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EDITORIAL

Surgical treatment for rectal cancer: An international perspective on what the medical gastroenterologist needs to know

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Abstract

Rectal cancer accounts for one third of all colorectal cancers. The age adjusted death rates from colorectal cancer have declined over recent decades due to a combination of colorectal cancer screening, improved diagnostic tests, improved standardized surgical technique, improved medical support, neoadjuvant chemotherapies and radiation treatment or combinations of these. Because of complex treatment algorithms, use of multidisciplinary teams in the management of rectal cancer patients has also been popularized. Medical gastroenterologists performing colonoscopies are frequently the first health care provider to raise the suspicion of a rectal cancer. Although the diagnosis depends on histological confirmation, the endoscopic presentation is almost diagnostic in many cases. In order to meet the patient's immediate needs for information, it is important that the endoscopist has knowledge about the investigations and treatment options that will be required for their patient. The aim of this paper is to describe the modern preoperative investigations and operative procedures commonly offered to rectal cancer patients taking into account perspectives of three colorectal surgeons, practicing in the USA, Europe and Asia.

Key words: Rectal cancer management; Evaluation; Staging; Neoadjuvant; Adjuvant; Surgical treatment; Surveillance

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INTRODUCTION

Rectal cancer accounts for one third of all colorectal cancers and in the USA 41 420 new rectal cancer cases were estimated in 2007[1]. The age adjusted death rate from colorectal cancer has declined over recent decades due to a combination of colorectal cancer screening, improved diagnostic tests, improved standardized surgical technique, improved medical support, neoadjuvant chemotherapies and radiation treatment or combinations of these[2]. Because of complex treatment algorithms, use of multidisciplinary teams in the management of rectal cancer patients has also been popularized[3].

Medical gastroenterologists performing colonoscopies are frequently the first health care provider to raise the suspicion of a rectal cancer. Although the diagnosis depends on histological confirmation, the endoscopic presentation is almost diagnostic in many cases. In order to meet the patient's immediate needs for information, it is important that the endoscopist has knowledge about the investigations and treatment options that will be required for their patient.

The aim of this paper is to describe the modern preoperative investigations and operative procedures commonly offered to rectal cancer patients taking into account perspectives of three colorectal surgeons, practicing in the USA, Europe and Asia.

PREOPERATIVE INVESTIGATION AND STAGING

Perhaps the most basic and informative test in patients with low rectal cancer is a digital examination. For many tumors, this will immediately give the experienced surgeon enough information to determine what treatment will be required. As well as a general health evaluation, such as appropriate cardiopulmonary investigations, the preoperative evaluation includes rigid proctoscopy, endoscopic rectal ultrasound, total colonoscopy, pelvic MRI, CT-scans of the abdomen, liver and lungs. These investigations will help the surgeon and his multidisciplinary team to determine: (1) The patient's health condition and comorbidities; (2) The stage of the rectal cancer; and (3) Which treatment option is best suited to meet the patient's preferences and at the same time be oncologically appropriate.

MEDICAL HISTORY AND PHYSICAL EXAMINATION

The three decisions that should be made initially include: whether the tumor is suitable for local therapy; whether preoperative therapy is required; and whether a permanent stoma is necessary. Severe comorbidities and poor health status can be a relative contraindication to abdominal surgery, whether open or even laparoscopic. A local or palliative approach may then be more reasonable. Accurate preoperative tumor staging is of extreme importance as it determines the indications for neoadjuvant therapy and the possibilities for a local resection versus a radical abdominal procedure. This must be balanced against the patient's preferences while at the same time giving the patients and their family the option to choose an individualized treatment plan with optimal chance for cure.

A patient history of previous pelvic or abdominal surgery will increase the difficulty of a laparoscopic approach, and thereby increases the likelihood of a decision for open rectal surgery. Abdominal wall scars should be noted as they might preclude the optimal stoma placement. Morbid obesity, especially in males, because of more intra-abdominal fat and narrow pelvis compared to females, will also favor open rectal surgery compared to laparoscopic surgery.

If the patient has a low rectal cancer, careful palpation of the groin lymph nodes is mandatory. Finding of one or several enlarged, hard and painless lymph nodes in the groin will ultimately lead to focus on palliative treatment once the finding is verified by MRI or biopsy. Excision (removing whole lymph nodes) should be considered after preoperative irradiation therapy including the affected groin.

Information about the benefits and limitations of the various surgical methods available, including the laparoscopic approach compared to the open operation should be given by the operating surgeon. However, most patients would also expect the medical endoscopist to have a brief overview and knowledge of the most common preoperative investigations and operative procedures performed in the treatment of rectal cancer. Frequently, the endoscopist receives questions about chemoradiation therapy or is involved in the diagnosis and treatment of its side-effects.

DIGITAL RECTAL EXAMINATION (DRE)

Despite the limited sensitivity and specificity of DRE, until recently the whole treatment plan was based upon its performance. Important information can still be gained from a correctly performed digital examination. What is the condition of the anal sphincters? Can the tumor be reached? If yes, is it occlusive? How much of the circumference is involved? Is it fixed to the surrounding tissue or can it be freely moved? What is the distance from the dentate line to the lower border of the tumor? Can the upper edge be reached?

By this simple examination the size, mobility and location of the cancer can be assessed. Before any decisions about treatment are made, the information gathered from DRE has to be confirmed by more objective means.

ENDOSCOPIC INVESTIGATIONS

A colonoscopy is used to rule out the presence of synchronous polyps and cancers in the rest of the colon with a reasonably high accuracy[4]. The findings of multiple polyps in a patient under the age of 50 should alarm the endoscopist of a hereditary colorectal cancer. A detailed family history of cancer is warranted and referral to genetic consultation should be considered. In patients with familial cancer syndromes, the planned operation is a total colectomy. A colectomy with ileorectal anastomosis is used for patients with hereditary non-polyposis colon cancer (HNPCC), and those with familial adenomatous polyposis (FAP) and fewer than 20 rectal polyps. Patients with FAP and more than 20 rectal polyps should undergo proctocolectomy and ileoanal anastomosis.

When the patient with rectal cancer meets the surgeon at the outpatient clinic, both transanal endoscopic rectal ultrasound (TRUS) and rigid proctoscopy will be performed. The diagnostic accuracy for TRUS is dependant on the experience of the operator, and the stage and location of the tumor. Because of limited reach, large tumors in the upper rectum are not suitable for rectal ultrasound. Occluding tumors that cannot be passed with the transducer are also not amenable for this examination. TRUS is most accurate for early rectal cancers in the distal half of the rectum, and is particularly valuable in assessing the T-stage. The limited penetration depth of 7 MHz ultrasound waves makes it difficult to access the N-stage with high precision, with most studies showing accuracy of 70%-75%[5]. Three dimensional rectal ultrasound imaging seems to improve the staging properties[5]. Thus, for making a decision about whether local resection is possible the results of TRUS are of significant importance.

MRI OF PELVIS

Because standard protocols can be used, and because it is less operator dependent, MRI has become the standard for preoperative stage assessment of rectal tumors[5]. With its high resolution and accuracy MRI can give information about T-stage and N-stage as well as distance to planned resection margins, especially lateral or circumferential margins within the pelvic cavity. MRI may also be used for the assessment of response to preoperative neoadjuvant chemoradiation treatment (CRT).

CT SCAN OF LIVER AND LUNGS

CT scans of liver and lungs are performed to rule out the presence of metastatic disease. Resectable liver metastasis can be removed in a one stage operation or as a second operation 3 mo after the primary rectal cancer surgery. Multiple metastases in both liver lobes or hilar lymph node involvement are signs of incurable disease. However, some of the new forms of chemotherapy have such excellent response rates that these patients may become surgical candidates after reassessment.

BLOOD TESTS

After the diagnosis of colorectal cancer, the carcinoembryonic antigen (CEA) level is measured in a simple blood test. The result of the CEA does not have any implications for the treatment, but increased levels are associated with poorer prognosis[6]. After resection of the cancer, elevated CEA levels should return to normal or metastatic disease should be suspected. CEA levels > 50 are very suggestive of liver metastases. In the surveillance program a three-fold increase in CEA level should alert the surgeon to search for local recurrence or metastatic disease[7].

Other blood tests such as electrolytes, hemoglobin, and albumin are frequently taken to assess the patient's general condition. A low serum albumin indicates poor nutritional status or deranged liver function and is associated with increased frequency of postoperative complications including anastomotic leaks.

MULTIDISCIPLINARY TEAMS

The complexity of individualized and highly specialized preoperative investigations and neoadjuvant treatment plans has evolved into the need for multidisciplinary teams. These teams are now being used in many institutions to ensure patients are appropriately placed on multidisciplinary care pathways. The results of the preoperative investigations and the clinical information about the patient are reviewed in the presence of dedicated specialists in medical oncology, gastrointestinal radiology and colorectal surgery. In the same meeting the pathology report of previous cases can be presented by a pathologist. The accuracy of the preoperative investigations, critical reevaluation of indications for adjuvant treatment, adjuvant treatment response as well as a judgment of the quality of the surgery performed can be discussed in relation to the pathological TMN stage and resection margins presented in the pathology report.

NEOADJUVANT TREATMENT

There is an important debate going on among surgical and oncological experts in rectal cancer treatment regarding the use of pre- or post operative radiation with or without chemotherapy in order to reduce rates of local recurrence and improve survival. Best evidence seems to support preoperative radiation in order to reduce local recurrence and at the same time reduce the side effects of radiation[8-10]. Adding chemotherapeutic agents to increase tumor radiosensitivity has been shown to be beneficial in improving local control, but was reported to have no effect upon survival[11]. Most centers nowadays have included preoperative chemoradiation therapy in their multimodality treatment options. However, there are still discussions about what gives best oncological results: short term radiation with 25Gy given in daily fractions of 5Gy and surgery the following week, or long term radiation treatment with chemotherapy in daily fractions of 1.8Gy five days per week, 50.4Gy in total, followed by surgery 4 to 6 wk later[12]. The latter treatment option probably has the advantage of down staging of the tumor and thereby increases the possibilities of a sphincter saving procedure, particularly in advanced low rectal cancers[13]. The connection between preoperative chemoradiation and achievement of uninvolved circumferential resection margin (CRM) is uncertain[14,15].

The long term follow up of the European Organisation for Research and Treatment of Cancer (EORTC) trial 22921 that compared adjuvant fluorouracil-based chemotherapy to no adjuvant treatment in patients with resectable T3-4 rectal cancer, reported no beneficial effects of adjuvant chemotherapy if the cancer did not respond to the preoperative radiation or chemoradiation therapy[16].

The role of postoperative radiation has recently been limited to inadvertent tumor perforations intraoperatively or involved resection margins if irradiation treatment was not given preoperatively. Intraoperative radiation therapy (IORT) can be given in cancers locally invading the pelvic side walls[17]. The definite role of postoperative chemotherapy for rectal cancer remains unclear[10].

However, the situation is even more complicated. Current discussion is not just about which is the best treatment, but also which patients should receive such treatment. Generally accepted international treatment guidelines are yet to be developed. Some countries recommend preoperative radiation or chemoradiation to almost all rectal cancer patients[11,18], whereas others recommend neoadjuvant chemotherapy to all patients with stage Ⅱ and Ⅲ rectal cancer[19]. Finally, others argue for a more selective neoadjuvant treatment policy offering it only to patients with preoperative MRI showing threatened CRM (nearest tumor tissue < 3 mm from predicted CRM) or for tumors in the lower half of the rectum[3,20-22].

SURGICAL TREATMENT OF RECTAL CANCER

Surgery is the only method to offer cure for rectal cancer. Rectal cancer surgery performed either as a minimally invasive or as an open procedure has four goals[23]: (1) To cure the patients and give long term survival; (2) To give local control and avoid local recurrence; (3) To preserve normal defecation-, bladder- and sexual functions when possible; (4) To maintain or improve the patients quality of life.

The best way to achieve goal number 1-cure and long term survival; and goal number 2-local control and avoidance of local recurrence, is by means of major surgery. However, this has its price and considerable efforts have been made to reduce the negative impact of rectal resections upon goals number 3-to preserve normal defecation-, bladder- and sexual functions and goal number 4-to maintain or improve the patients quality of life.

Functional disturbances such as impotency, retrograde ejaculation, urinary retention or disturbed urinary bladder function as well as defecational problems or formation of a stoma have negative impact on quality of life after surgical treatment. One of the main steps during the dissection of the mesorectum is to identify and preserve the hypogastric and parasympatic pelvic nerves and thereby preserve functions. Functional disturbances are still a problem after rectal cancer surgery in about 20% of the patients[24]. Table 1 shows a summary of abbreviations that are commonly used in the surgical treatment of rectal cancer.

Table 1 Vocabulary for rectal cancer treatment

	Treatment
Anterior resection	Resection of rectum with an anastomosis above the pelvic peritoneal reflection.
Low anterior resection	Resection of rectum with an anastomosis below the pelvic peritoneal reflection.
TME	Total mesorectal excision. The fatty tissue which contains the draining lymph nodes surrounding the lateral and posterior part of the rectal tube, are dissected down to the pelvic floor and resected. The hypogastric nerves are preserved.
PME	Partial mesorectal excision. The mesorectum is divided 5 cm below the cancer and rectum transected. PME is performed for cancers located in the upper rectum and rectosigmoid junction.
TEM	Transanal endoscopic microsurgery. A specially constructed proctoscope with an attached microscope permits local resection of premalignant lesions and selected cases of early rectal cancer up to 20 cm from the anal verge.
TAE	Transanal excision. Lesions in the lower third of rectum can be resected transanally.
APR	Abdominoperineal resection. Low rectal cancers that cannot be resected with a sphincter-saving procedure are resected with perianal tissue and the anal channel en block with the whole rectum and mesorectum.
Adjuvant	Additional treatment (chemotherapy, radiation therapy or chemoradiation) given after surgical resection.
Neoadjuvant	Preoperative treatment.
CRT	Chemoradiation treatment. Chemotherapeutic drugs, typically 5′-fluorouracil and/or leucovorin are given in order to increase cancer cells sensitivity to the radiation. CRT is frequently offered to patients preoperatively (neoadjuvant) in order to reduce the chances for local recurrence and improve survival.
Intersphincteric resection	The upper part of the internal anal sphincter muscle is resected continuously with the lower rectum in order to preserve anal function and avoid colostomy in cases of ultralow rectal cancer.
CRM	Circumferential resection margin is the distance in mm from the mesorectal fascia (the resection plane) to the nearest tumor growth.
DRM	Distal resection margin.

Local resections

Local resections are performed transanally using both specially developed instruments and sutures to expose the rectal mucosa (transanal excision, TAE), or the operation might be performed endoscopically using a microscope to improve visualization through a specially designed proctoscope to secure access and instrumentation of the tumor (transanal endoscopic microsurgery, TEM). Local resections would be the operation of choice if only goals 3 and 4 were to be considered. Early rectal cancers treated with local resections have been reported to be associated with unacceptably high local recurrence rates of up to 40%[25], and should only be offered to carefully selected patients, or to those who otherwise would need a permanent end stoma[26]. For patients with severe comorbidities or with extremely high risk from anesthesia and abdominal surgery, a local resection procedure can be the optimal solution despite its limitations regarding local recurrences. Studies are underway in which the results of combining chemoradiation therapy and TEM will be determined[27].

Studies of the mesorectum in rectal cancer have shown that 10% of early rectal cancer (T1) has micrometastasis in mesorectal lymph nodes, and close to 20% have local lymph node metastasis in T2 cases[28]. Performing local resections that leave metastatic lymph nodes is undoubtedly likely to increase local recurrence rates, although the exact risk has yet to be evaluated and the risk is likely dependent on the exact individual tumor stage biology.

Total mesorectal excision (TME)

Heald and coworkers standardized the approach to rectal cancer by performing a TME with sharp dissection in the avascular plane surrounding the mesorectum with preservation of the hypogastric and parasympathetic pelvic nerves[29]. They reported a 5-year recurrence rate of 5%-7% or lower, depending on the cancer stage, without the use of neoadjuvant treatment, showing the importance of adequate surgical quality upon local recurrence. By contrast, traditional rectal cancer surgery with blunt dissection and ignoring the importance of an intact mesorectum with adequate tumor resection margins,

has yielded local recurrence rates of 30% or higher[30]. The benefits of the mesorectal dissection technique have been confirmed in several European countries after introduction of training programs and national consensuses of TME as the standard operation method for rectal cancer[11,18,31]. It has been documented that cancers located in the upper rectum do not need to be removed along with all the fatty tissue surrounding the rectum (mesorectum) down to the pelvic floor[32]. They do need a TME-like radial margin, but can be resected with a 5-cm distal margin to the cancer, ie a partially mesorectal excision (PME), without compromising the oncological result. This helps minimize some of the functional disturbances seen after a coloanal anastomosis.

The development of suturing devices with stapled circular anastomosis has also made the formation of anastomoses in the lower pelvis feasible, reducing the need for permanent stomas. However, the reported rates of anastomotic complications still vary considerably between surgeons[33]. It is common practice to protect the lowest anastomosis, especially after radiation treatment, with a temporary diverting loop ileostomy. The ileostomy is normally closed after 8 to 12 wk.

The low anterior syndrome describes the functional disturbances that may be seen after rectal cancer surgery. Improved defecation function can be achieved by anastomosing a colon J pouch to the top of the anal channel or to the top of a short rectal remnant[34].

Laparoscopic mesorectal excision

Laparoscopic resection of the rectum has not gained the same international acceptance as laparoscopic colon surgery. However, it has proven to be technically feasible and safe with no more or perhaps fewer complications than after open rectal surgery[35,36]. Low anterior resection (LAR) technically performed as laparoscopic TME or PME has the same oncological outcome when compared to traditional open rectal surgery[37-40]. For patients, laparoscopic surgery gives benefits regarding reduced postoperative pain, shortened postoperative ileus with faster bowel recovery after surgery, improved abdominal cosmesis, fewer wound infections, less postoperative small bowel obstruction and ventral hernias[41,42]. For the health care providers the benefits are shorter hospital stay and reduced overall costs[43] and thereby more effective use of health care recourses.

Because of the technical challenges of laparoscopic pelvic surgery a standardization of the technique is important to reduce the rate of conversion and improve the operating team performance. The learning curve for laparoscopic mesorectal resection is higher than commonly stated for other laparoscopic procedures[44]. This has probably contributed to the centralization of laparoscopic rectal resections to high volume hospitals with trained and experienced surgeons.

Abdominoperineal resection (APR)

About one third of rectal cancers are located in the distal third of the rectum. Traditionally this tumor location has led to an APR and a permanent colostomy. A frequency of 30% or more of APR has therefore been reported in many series[45]. However, improved surgical technique and neoadjuvant CRT have made it possible to perform low resections and stapled or handsewn coloanal anastomosis[46]. For the ultralow rectal cancers, intersphincteric resection and a handsewn colonic J-pouch anastomosis can be performed with good oncological results[47]. Increased focus on sphincter saving surgery has reduced the frequency of APR to around 10% or less in some hands. Some authors even regard the frequency of APR as a surrogate marker of the surgical quality in rectal cancer treatment[48].

Hartmann's procedure

The Hartmann's procedure is a rectosigmoid resection where the bowel continuity is not restored by an anastomosis. Instead the proximal colon is diverted as an end colostomy and the distal rectum, or sometimes just the anal canal, is left behind as a pouch (Hartman's pouch). This procedure is performed in selected rectal cancer patients, such as those with preexisting fecal incontinence, or unacceptably high risk after an anastomotic complication.

Loop ileostomy

A loop ileostomy can be performed to divert the flow of stool until the anastomosis has healed. The ileostomy does not reduce the rate of anastomotic leakage but it will limit the infectious consequences and mortality of the leakage[49]. In cases with obstructive symptoms from the cancer, a loop ileostomy can relieve symptoms before preoperative chemoradiation therapy is initiated, as well as reducing the risk of complications associated with emergency surgery by converting emergency cases into later elective surgery.

ENHANCED RECOVERY PROGRAMS

The development of fast track surgery or enhanced recovery programs has dramatically reduced the recovery time and length of hospital stay after colorectal surgery[50,51]. By combining laparoscopic rectal surgery and enhanced recovery programs, hospital stay of 4 d or less can be expected for 90% of the patients[52]. Fast track pathways may include avoidance of preoperative mechanical bowel preparation, drinking of a carbohydrate enriched solution 2 h prior to surgery, use of total intravenous anaesthesia, early postoperative mobilization, avoidance of nasogastric tubes and abdominal drains, early postoperative intake of liquids and solid food, minimizing opiates for pain control and use of bowel stimulating drugs. Effective pain control can be achieved by patient controlled analgesia (PCA) pumps in most cases. Intravenous and urinary catheters are removed on postoperative day one. Using these strategies as a combined pathway leads to early recovery, with low risk for readmission within 30 d[53].

SURGICAL QUALITY

The aim to cure and improve survival as well as number

of lymph nodes in the specimen the surgical quality can be evaluated within a few days. Similarly, many of the important outcomes of early recovery after surgery can only be achieved in patients having high quality surgery. Tumor biology and stage are important prognostic factors, but so is the performance of the surgeon. The importance of the surgical quality can easily be obscured by focusing on short term and long term over all survival, cancer specific survival, long term and short term local recurrence rates, different radiation regimens with or without pre- or post operative chemotherapy, local versus major resections, or laparoscopic versus open technique. Overall local recurrence rates > 10% should lead to concerns about the surgical quality. However, it is rather late to change the technique, when the rates of local recurrence are commonly calculated 3-5 years after surgery.

By using the recommended pathological description[54], TME grading, CRM, distal mesorectal and mural margins as well as number of lymph nodes in the specimen can be evaluated within a few days of surgery. If preoperative MRI showed more than 2 mm distance from tumor to the lateral resection margin, the CRM measured in the specimen should be at least 2 mm. Because of distal spread of tumor cells in mesorectum, a 5-cm distal resection margin is advocated in cases of PME. When performing a TME all the mesorectal fatty tissue is removed, and the surgeon can focus on achieving a safe distal rectal wall resection margin which is shown to be 1 cm or even less in cases with preoperative chemoradiation[55].

If the surgeon repeatedly has tumor involvement in the CRM, too short distal mesorectal resection margins, or involved distal rectal wall resection margin, then his patients will suffer unnecessary local recurrence and shortened survival. Few, if any national colorectal associations have considered the consequences of this and started a certification program for colorectal surgeons who operate on rectal cancer. Development of centers of excellence could also help improve the quality of all aspects of rectal cancer treatment.

The complexity of individualized multimodal treatment plans and the challenges and technical difficulties of open or laparoscopic pelvic surgery, have centralized rectal cancer treatment to high volume institutions, hopefully to the benefit of the patients.

Additionally, there has been no broad discussion in the literature of possible overtreatment by giving neoadjuvant chemoradiation to all rectal cancer patients, since less than 10% of all rectal cancer patients will have local recurrence after optimal surgery alone.

COMPLICATIONS AFTER SURGICAL TREATMENT

The narrow pelvic cavity and the close relations of the rectum to functionally important organs and structures as the hypogastric and parasympathetic nerves, the urinary tract including ureters, bladder and urethra, the seminal vesicles and prostate gland in males, uterus and posterior vaginal wall in females, pelvic and sacral vessels, make rectal surgery technical challenging and risky. Impotency and sexual dysfunctions, bladder dysfunctions, defecational problems including evacuation difficulties, fecal incontinence and urgency significantly add to the mental stress of a recent cancer diagnosis. Stoma problems with fear of malodorous leakage can be socially crippling. An increased focus on quality of life has included preservation of normal defecation-, bladder- and sexual functions and maintaining or improvement of the patient's quality of life as main goals of the surgical therapy for rectal cancer. Still, up to 20% of the patients will experience one or more of the above-mentioned side effects of the surgical treatment[56].

POSTOPERATIVE SURVEILLANCE

The medical endoscopist frequently meets rectal cancer patients when they are coming in for colonoscopy, commonly at 6 mo and at 4 years in their postoperative surveillance program. The clinical benefit of a postoperative surveillance program is disputed[57], but there are several considerations. One is to discover signs of cancer local recurrence or metastatic disease. Another is to educate the patient to recognize signs and symptoms of recurrent disease as well as to encourage the patients to cope with the sequelae of treatment. Thirdly, it is an important way to monitor the results and quality of the rectal cancer treatment.

Details of recent development of weight loss despite normal appetite, increased fatigue, changes in bowel habits and vague abdominal discomfort should be questioned at every postoperative consultation. Physical examination, including palpation of the abdomen for any possible mass, surgical scars, the lower edge of the liver and palpation around stomas will be performed. The presence of ventral or parastomal hernias should be recorded, but any suggestions about surgical treatment should be balanced against symptoms, impact on quality of life or other possible benefits and risks. The left supraclavicular fossa (Virchows lymph node) and the groins should be palpated for enlarged lymph nodes. The perineal region should be inspected and palpated and a DRE performed in all cases with a residual anal canal. During DRE the anastomosis should be palpated if within reach and any pelvic mass recognized.

As mentioned earlier, postoperative CEA level should return to normal if elevated preoperatively. In these patients elevated CEA levels can be indicative of local recurrence or metastatic disease.

Unsuspected findings should be verified by CT or MRI scans. PET scan is the most accurate method to rule out presence and the extent of local or metastatic disease[58].

LOCAL RECURRENCE

If a local recurrence is verified, surgical resection must be considered either with curative intent or as a palliative effort. However, the side effects and complications of

any surgery for recurrent disease must not be underestimated. The plan for the investigation is to determine resectability and to assess risks to the patient. Second line chemotherapy is an option, however it is non-curative and with considerable side effects. Most cases of recurrent disease will be discovered between the surveillance controls, and two thirds within two years after surgery.

LIVER METASTASIS

The attitude towards liver metastasis from colorectal cancer has also changed during recent decades. An aggressive approach has been shown to prolong survival and increase chances for cure[59]. Even patients with multiple liver metastases should be considered for liver surgery because combination of surgical resection and ablation (radiofrequency ablation or cryo ablation) after downstaging chemotherapy can be a valuable option for the patient unless there is evidence of systemic cancer disease. Selective hepatic intraarterial chemotherapy and segmental liver embolization are also treatment options in selected cases.

LUNG METASTASIS

Rectal cancer does also spread to the lungs. In an otherwise fit patient with no other signs of metastatic disease, an aggressive surgical approach will prolong survival. Segmental pulmonary resection or lobectomy is advocated for selected patients[60]. Multilobular and bilateral location is a sign of systemic disease and is a contraindication for surgical treatment.

PALLIATIVE SURGERY FOR ADVANCED AND INOPERABLE RECTAL CANCER

Preoperative chemoradiation therapy might downstage a fixed and inoperable cancer to become resectable and even curable. All efforts should be made to resect a rectal cancer in order to avoid the painful and devastating conditions associated with an uncontrollable cancer growth inside the pelvic cavity. Stoma, intestinal bypass, stent, fulguration (burning down the cancer with diathermy) or laser evaporation can give temporary relief from an obstructing rectal cancer or its metastasis.

Large procedures as hemipelvectomy or anterior or total pelvic exenteration with or without combination with intraoperative radiation (IORT) have been performed in order to achieve a R0 resection (all cancer tissue removed) and thereby reduce the chances of local recurrence. Obviously, this has side effects for the patients.

CONCLUSION

The medical endoscopist is not commonly involved in the multidisciplinary teams deciding the treatment plans for patients with rectal cancer. However, the endoscopist frequently is the first health care provider to meet the patient with suspected rectal cancer in the setting of endoscopy for colorectal symptoms or screening, and is frequently the person that performs the postoperative endoscopic surveillance. By having knowledge about the complex investigation plans and treatment options available, the endoscopist can provide important information in order to help the patient to prepare for the coming meeting with the surgeon.

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EDITORIAL

Autoimmune liver diseases

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Abstract

The liver was one of the earliest recognized sites among autoimmune diseases yet autoimmune hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis, and their overlap forms, are still problematic in diagnosis and causation. The contributions herein comprise 'pairs of articles' on clinical characteristics, and concepts of etiopathogenesis, for each of the above diseases, together with childhood autoimmune liver disease, overlaps, interpretations of diagnostic serology, and liver transplantation. This issue is timely, since we are witnessing an ever increasing applicability of immunology to a wide variety of chronic diseases, hepatic and non-hepatic, in both developed and developing countries. The 11 invited expert review articles capture the changing features over recent years of the autoimmune liver diseases, the underlying immunomolecular mechanisms of development, the potent albeit still unexplained genetic influences, the expanding repertoire of immunoserological diagnostic markers, and the increasingly effective therapeutic possibilities.

Key words: Autoimmunity; Autoimmune liver diseases; Immunopathogenic processes; Clinical immunology

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The liver is one of the earliest recognized sites among those affected by autoimmune diseases. Such diseases became recognized during the 1950s as novel pathogenetic entities in humans and, later, in laboratory animals. Today there are 80 different disorders attributable to autoimmunity. Over the past five decades, clinical awareness of autoimmune liver disease has been greatly enhanced, knowledge of pathogenesis has become more refined, laboratory diagnosis far more precise, and therapy more effective. These advances are authoritatively described by the expert contributors to this dedicated issue of the *World Journal of Gastroenterology*.

The health burden of autoimmune liver diseases, numerically not of the same magnitude resulting from liver diseases due to alcohol abuse, hepatitis virus infection or steatosis-related pathology, is still very substantial. Thus autoimmune liver diseases can affect individuals in childhood, at highly productive stages of adult life, as well as in later years; and these diseases are life long, and have degrading effects on *joie de vivre* due to distressing symptoms and complications, or side-effects of therapy. Moreover, there is an impression sometimes given by doctors that medical knowledge has not yet fully explained the exact nature or cause of autoimmune disorders. This might not be surprising because autoimmune diseases, including those affecting the liver, result from intricate derangements of immunological functions, and the idea that "immunity" can work against the well-being of the individual is counter-intuitive. Furthermore, immunology as a discipline of science is still not well accommodated in the curricula for students of medicine; it is a fast-evolving and strongly laboratory-based discipline with its own arcane terminology; and it may be disadvantaged by a still incomplete severance from microbiology in many university departments. Expectedly, clinicians may not readily engage with the theoretical pillars of modern immunology, nor fully appreciate the intimate applicability of immunology to a wide variety of chronic diseases, whether in developed or developing countries.

The autoimmune liver diseases considered among these reviews are autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC), and primary (autoimmune) sclerosing cholangitis (PSC). AIH and PBC are very well proved in terms of an autoimmune background whereas PSC, as readers will discern, fits less readily into this category. Nevertheless, the evidence in PSC for some forms of immune derangement is quite impressive, and we can reasonably attribute "guilt by association" with, for example, ulcerative colitis, as pointed out by our authors

on this topic.

We have made special provisions in this series of articles on the etiopathogenesis for each of the three autoimmune liver diseases. We often read that "such-and-such" a disease is "an autoimmune disorder of unknown etiology". We would contend that autoimmunity, synonymous with tolerance deficit, should be regarded as aetiology in its own right, meaning that failure of natural immune tolerance itself can be pathogenic even in the absence of any overt environmental provocation. The regular and predictable occurrence of autoimmunity in certain tolerance-deficient inbred and genetically tilted strains of mice (NOD, NZB) is an ample witness to this. Among the autoimmune liver diseases, an environmental provocation is seldom discernible for AIH except for some "transient" examples after exposure to particular sensitizing medications, such as minocycline, whereas for PBC, there are a great variety of agents or processes claimed to act as "initiators". As for PSC, the co-morbidity with ulcerative colitis leads to an indictment of pathogenetic immune hyper-responsiveness to the normally tolerated microbial flora of the colon. Tolerance deficits in humans, as in animals, will be largely genetically based, and deciphering the nature of these errors is an urgent and exciting challenge for the future studies.

Each of the three reviews dealing with etiopathogenesis of an autoimmune liver disease has a partner article on clinical features and management, and readers will note that clinical presentations have changed compared with earlier days. For example, AIH is currently presenting more as an indolent disease with an onset in later life, in contrast to its major impact on young women in the past years. PBC occurs more often at minimally symptomatic earlier stages, with cases frequently ascertained as a result of automated laboratory screening, sometimes for unrelated purposes. It is encouraging for clinicians that both AIH and PBC are gratifyingly responsive to the current standardized treatment regimens, even though, for ursodeoxycholic acid in PBC, the undoubted therapeutic benefits are not readily explicable on theoretical grounds. Finally, some patients will still require liver transplantation for eventual end-stage disease, but readers will be glad to learn that AIH and PBC provide liver transplantation with more satisfying results in terms of post-transplantation morbidity and mortality. And, speaking of transplantation, the inescapable complications of allograft rejection or graft-versus-host disease do exist, but are now eminently manageable. Of much interest, there are credible examples of recurrence of autoimmune liver disease, or even *de novo* autoimmune hepatitis, in an allografted liver. This puzzling immunological scenario is engaged in one of our articles. Finally, a review article focuses on the particular aspects of autoimmune liver diseases occurring during childhood.

We have included an authoritative review on diagnostic serology in autoimmune liver disease. Previously diagnostic serological tests were usually provided by "academic" laboratories in either universities or major teaching hospitals. Currently commercial sectors are providing (increasingly more efficiently) the source materials and/or assay kits for private laboratories to perform autoimmune serologic assays. However, there is still a pressing need for the standardization of the assay procedures worldwide, and for a ready availability of calibrated anti-sera with which the laboratories can evaluate and quantitate their results. Clinicians should also be fully informed about the interpretation of the assay data rather than entirely rely on the printed results from the computer. For example, the hepatologist will be confronted from time to time with what is called an "overlap syndrome". This is a topic that has attracted the attention of authors of several recent review articles, given that the partner diseases, AIH and PBC, or AIH and PSC require different regimens of therapy. The theme is reviewed in these articles. The diagnostic serological laboratory can often help the clinicians to identify the dominant partner to which therapy should primarily be directed.

We recommend to readers this series of reviews as a timely and "state-of-the-art" outline of autoimmunity and liver, by research centres esteemed for their contributions to the science and practice of hepatoimmunology. Finally, we would express our deep appreciation to the invited authors for their painstaking preparation of the highly informative articles in this issue.

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TOPIC HIGHLIGHT

Pietro Invernizzi, MD; Ian R Mackay, MD, *Series Editors*

Historical reflections on autoimmune hepatitis

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Abstract

Autoimmune hepatitis (AIH), initially known as chronic active or active chronic hepatitis (and by various other names), first came under clinical notice in the late 1940s. However, quite likely, chronic active hepatitis (CAH) had been observed prior to this and was attributed to a persistently destructive virus infection of the liver. An earlier (and controversial) designation in 1956 as lupoid hepatitis was derived from associated L.E. cell test positivity and emphasized accompanying multisystem features and immunological aberrations. Young women featured prominently in early descriptions of CAH. AIH was first applied in 1965 as a descriptive term. Disease-characteristic autoantibodies were defined from the early 1960s, notably antinuclear antibody (ANA), smooth muscle antibody (SMA) and liver-kidney microsomal (LKM) antibody. These are still widely used diagnostically but their relationship to pathogenesis is still not evident. A liver and disease specific autoantigen has long been searched for but unsuccessfully. Prolonged immunosuppressive therapy with prednisolone and azathioprine in the 1960s proved beneficial and remains standard therapy today. AIH like many other autoimmune diseases is associated with particular HLA alleles especially with the "ancestral" B8, DR3 haplotype, and also with DR4. Looking forwards, AIH is one of the several enigmatic autoimmune diseases that, despite being (relatively) organ specific, are marked by autoimmune reactivities with non-organ-specific autoantigens. New paradigms are needed to explain the occurrence, expressions and pathogenesis of such diseases.

Key words: Medical history; Autoimmune hepatitis; Lupoid hepatitis; Liver disease autoantibodies; Immunosuppressive therapy; HLA-disease associations

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EARLY DAYS: 1940s-1950s

The recognition in the 1950s of the disease now known as autoimmune hepatitis (AIH) with its various guises and appellations is a richly interesting story, as hitherto recounted[1,2]. The history was recently embellished by Reuben in one of his memorable "Landmarks" published in Hepatology[3] (Figure 1). AIH, known in earlier days as chronic active hepatitis (CAH), was generally regarded as a "new" disease when reported in 1950 by Waldenstrom[4] in an out-of-the-way Conference Proceedings on nutrition. However, since it is hardly believable that any disease of autoimmune nature would have arisen then *de novo,* it is likely that it did exist but was unrecognized, for two reasons: first, autoimmunity in the 1940s had scarcely entered the medical mind and, second, most of the necessary diagnostic laboratory procedures (liver biopsy, serum aminotransferases, serum autoantibodies) were not then routinely available. The exception was the hallmark feature of hyperglobulinaemia, recognized by Waldenstrom[4], and also by others writing at around the same time (see below). Indeed increased levels of serum gamma globulin, first ascertained by moving boundary electrophoresis, are well illustrated in cases of cirrhosis of the liver reported in the early 1940s[5]. In fact, early descriptions that would fit AIH are discernible under the non-committal names of subacute hepatitis or subacute hepatic necrosis: such cases were ascribed to non-healing infectious hepatitis. As one example, Himsworth[6] in his 1947 monograph alluded to:

"the form of subacute hepatitis which appears to arise as such, without the patient ever having had acute illness suggestive of liver disease… jaundice being absent or so faint as to unnoticed… the conditions affects women more often than men… the patient may date her present illness from an acute infection, such as cystitis or bronchitis, some 1 or 2 years previously… since then she has never felt really well … rheumatic pains, without evidence of articular damage are often noticed… or she may delay attending until the condition has passed into

the next stage of post necrotic scarring… a particular problem is why do nearly all cases of sub-acute massive necrosis inevitably progress…?"

Similarly, Zimmerman *et al*[7] in 1951 described a case of subacute hepatic necrosis in a 36-year-old man with slowly developing jaundice and extreme hyperglobulinaemia (87 g/L) and in fact reached for an immunological explanation, in commenting that:

"the initial injury causes an alteration in liver protein, which stimulates the formation of anti-liver antibodies. These newly formed antibodies produce more liver injury, thus releasing more altered liver protein, which again contributes to the vicious circle of continuing necrosis."

The general interpretation in the 1940s of subacute or chronic hepatitis was that it was a sequel to, and a consequence of, an unresolved acute infectious hepatitis. For example Neefe[8], writing from a long experience of viral hepatitis, comments in his review in 1946 on the occurrence of chronic hepatitis as a sequel of viral hepatitis. The stage was actually set for the later nomenclature of CAH in 1945 by Barker, Capps and Allen[9] who followed the course of viral hepatitis in military personnel in the Mediterranean theatre. They defined "non-recovery" as persistence of symptoms after 4 mo and applied the term chronic hepatitis "without any implications regarding the nature of the pathologic process or the eventual prognosis". Further, their cases of "chronic hepatitis" were divided into active if symptoms were present, or inactive if there were only laboratory defined abnormalities[10]. Thereafter CAH became the standard descriptor of later years, albeit for a disease quite different from that exhibited by the soldiers. Even so, there still remained the strong impression that the precursor of CAH was hepatitis virus infection, according to articles by Wood and colleagues[11] in Melbourne in 1948, Kunkel and Labby[12] in New York in 1950, and Liebowitz[13] in New York in 1950 who followed up 68 patients with presumed acute viral hepatitis and reported that seven (11%) developed CAH. Waldenstrom[4] in his 1950 report had been more circumspect in specifying that the cause of the disease was unknown, *wie wir alle wissen*, although he also speculated on persisting viral infection (see below).

In the event, the disease seen during 1948-1950 that fulfils the modern description of AIH is illustrated by key phrases reproduced from the 1950 report of Waldenstrom[4], and by a 1951 Meeting Abstract of Kunkel and colleagues[14]. According to Waldenstrom:

"Dazu kommt bei fast allen Fällen das Auftreten von sog. Sternchen (Naevi aranei) und bei den Mädchen auch eine besondere Tendenz zur schweren Akneeruption. Eine langdauernde Amenorrhöe ist charakeristisch, die wahrscheinlich anovulatorisch ist… Es ist damit möglich, dass in der Zukunft eine Anzahl von diesen Fällen mit ACTH verbessert werden können… Die zweite Frage, ob die nachgewiesene Erhöhung des Gammaglobulins als Zeichen eines chronischen Immunisierungsprozesses aufzufassen ist, verdient meines Erachtens grösste Aufmerksamkeit… Die Atiologie dieser chronischen Leberleiden ist-wie wir alle wissen-immer noch unbekannt. Es werden toxische, infektiöse und Nahrungsfaktoren als Ursache angenommen… Es scheint sehr wohl möglich, dass die Gammaglobulinvermehrung als Symptom einer Immunisierung gegen das im Körper verbleibende Virus aufzufassen ist". Kunkel and colleagues[14] used the following phrases:

Figure 1 Montage and legend as prepared by Dr. Adrian Reuben for "Landmarks in Hepatology"[3]. **A**: Jan Gösta Waldenström (right) with Dr Göran Bauer, President of the Swedish College of Physicians, Stockholm, c.1989 (courtesy of Dr. Frank Wollheim); **B**: Henry George Kunkel, Paris, 1979, being informed of his naming for the Lita Annenberg Hazxen Award (courtesy of Dr. Eng M Tan); **C**: Ian Reay Mackay, on the occasion of his Retirement Symposium in 1987 organized by the Walter and Eliza Hall Institute (courtesy of the subject). Reproduced by courtesy of Dr. Reuben and Wiley and Sons Inc., publishers of Hepatology).

"Total proteins ranged from 9-13 per cent…this rise was due entirely to gamma globulin increase… eleven of these twelve patients were females… the maximum age was 32…onset of disease was insidious… course was prolonged and either stationary or downhill… frequently marked by periods of high fever, arthralgia, and arthritis … remarkable degree of plasma cell infiltration in the liver… which diminished during the course of disease… the etiology of the syndrome remains unknown."

This apparently new liver disease came under particular scrutiny in Melbourne following the report of Wood *et al*[11] referred to above, and particularly from the standpoint of clinical and histological correlations, described by Saint *et al*[15] in 1953, who noted:

"fairly well defined clinical features, a highly characteristic pattern of biochemical tests including hypergammaglobulinaemia, and a histological picture which seems to indicate active chronic inflammatory changes… in many cases an initial history of contact or

even a history of typical acute infectious hepatitis was lacking… but little doubt existed that the liver disease was due to infection with a virus".

This latter article followed the earlier nomenclature of Capps[10] in 1948 of active and inactive chronic hepatitis but used histological rather than clinical criteria to draw the distinction. Accordingly Saint *et al* introduced the term active chronic hepatitis[15] that for a while coexisted with CAH, although the latter eventually prevailed. The dire outcome was recorded as follows:

"the course was progressively downhill… jaundice became a permanent feature… bleeding episodes became more frequent… alternatively these patients became permanently bedridden owing to their dropsical enfeebled state, and finally lapsed into coma… the time or presentation until death has varied between six months and two years".

Among the cases assembled by Saint *et al*[15], one stands out. This was a 36-year-old woman, described further in 1955 in a case study by Joske and King[16] as active chronic viral hepatitis with positivity for the lupus erythematosus (L.E.) cell test. Their report includes the following:

"liver biopsy showed the typical picture of an active chronic viral hepatitis… cellular infiltration with lymphocytes and plasma cells and fibroblastic activity… fairly numerous L.E. cells present… cortisone produced a dramatic improvement in her arthralgia… we may recall that Leonie (1954) found L. E. cells in ascitic fluid from a patient with hepatic cirrhosis… we suggest that the L.E. cell and related phenomena might be based either: (1) on an abnormality of the antibody-producing mechanism… or (2) on changes in red or white cells or their constituents… which modify their characteristic "self markers".

The latter 3-4 lines can be reasonably attributed to the pen of FM Burnet who, at this time in 1955 had already turned his mind to immunological aberrations in disease states. Also noteworthy in the report are comments on the plasma cellular content of the inflammatory infiltration into the liver, and the improvement conferred by treatment with cortisone.

The L.E. (lupus erythematosus) cell effect requires a few lines here. L.E. cells had been discovered incidentally in the 1940s by Hargraves[17] in bone marrow preparations from patients with "collagen diseases" of the lupus erythematosus type, and were modestly reported after a delay of some years in 1948 in the Mayo Clinic Proceedings. Hargraves himself was quite surprised by the interest that his report aroused[18]. This interest intensified further with the discovery that the L.E. cell effect depended on a serum factor, of gamma globulin nature[19], which only a few years thereafter became recognized by various groups as an antinuclear autoantibody. An item of interest, unreported and transmitted as a personal comment from Hargraves to this author, was that among the initial patients in whom L.E. cells were detected was a young girl (PC) suffering from chronic hepatitis! The L.E. cell test soon became a surrogate marker for an autoimmune basis for a given disease and, as such, was deployed in Melbourne in the early 1950s as the single available routine laboratory indicator for multisystem autoimmunity-hence its application to cases of CAH.

LUPOID TO AIH

After my return to Melbourne from abroad in 1955, it took only a little time to identify several instances of CAH in young women with hypergammaglobulinaemia and a lymphoplasmacytic infiltration in the liver and, in each, a positive test for the presence of L.E. cells in blood. Then the case for an immunological derangement as the cause of the disease became even stronger because at the time, DC Gajdusek who was a visiting scientist to the Hall Institute was attempting to develop a diagnostic serological test for viral hepatitis based on a complement fixation (CF) reaction, using as antigen liver tissue obtained at autopsy from a patient with fatal acute viral hepatitis. However sera from cases of acute hepatitis were at most only weakly positive using this CF reaction, whereas sera from cases of CAH (inserted as disease controls) tested positive, not only using as antigen the virally infected liver tissue but also using normal liver as well[20]. We can recall that during the development of the Wassermann serological test for syphilis infection, *Spirochaeta*-infected tissue was used initially, but normal tissue was found to serve equally well to elicit a positive reaction.

The several patients with CAH and a positive L.E. cell test appeared to represent a unique disease entity since most had, additionally, extrahepatic disease expressions including arthralgia, rash, haemolytic anaemia and others, typically seen in cases of systemic lupus erythematosus (SLE) wherein sera also gave a positive autoimmune CF test. In a report to Lancet in 1957[21], the concept was developed thus:

"linking certain types of active chronic hepatitis and lupus erythematosus… possibly through the common factor of disturbed immunological response… this group of cases has been provisionally designated as 'lupoid hepatitis' since 'lupus' has now acquired a far broader significance than the original term suggests… we consider that immunological destruction of the host's liver cells best explains the perpetuation of the hepatitis and progression to cirrhosis. If this is so, it would be rational to use therapeutic measures (e.g. cortisone therapy) designed to modify this process, and our experience suggests that cortisone is of benefit in this autoimmune hepatitis."

The global response to this report was remarkable. There were journal reports of patients with lupoid hepatitis from far and wide and, as well, detailed case studies, by Bearn *et al*[22], Reynolds *et al*[23] and others[1,2]. Thereafter there arose controversy, still not completely resolved[3,24], as to whether "lupoid hepatitis" was intended to specify a particular form of CAH and thus a disease in the realm of hepatology, or a component of a multisystem syndrome in the realm of rheumatology.

Admittedly, this dilemma was not helped by our earlier writings which attempted to distinguish cases of "lupoid hepatitis" from those of "CAH" in which L.E. cell positivity was not demonstrable[25]. The development of the immunofluorescence test for antinuclear antibody (ANA), and its application to patients with CAH[26] soon revealed that cases of "lupoid" and "ordinary" CAH were indistinguishable on important criteria such as biochemical indices of liver dysfunction, histological abnormalities in the liver, and responsiveness to immunosuppressive therapy. Also, by the early 1960s, it was clearly evident that the disease in all its guises was best accounted for by an autoimmune reaction in the liver and thus, in 1965, we suggested that the disease be named "autoimmune hepatitis[27], although this appellation did not become formally endorsed until 1993[28]. Meanwhile, lupoid hepatitis lived on for quite some years, having heuristic appeal in some jurisdictions and causing semantic grievance in others, to the extent that one publication was directed to establishing that lupoid hepatitis was a "non-entity" within the disease group known as CAH[29]. Actually our Clinic had already discarded a possible association between lupoid hepatitis and SLE by showing in 1959 that liver lesions in classic instances of SLE were relatively trivial and nondescript[30], and a serological distinction was forthcoming a few years later (see below).

THERAPIES FOR CHRONIC ACTIVE/AIH

In our 1957 publication on lupoid hepatitis[21], the notion of immunological destruction of host liver cells was seen to provide a rationale for the use of "anti-immune" therapies to modify the process. Corticosteroid therapy in fact had been used in cases of CAH in Melbourne from as early as 1953, and, although initial results were equivocal[15], later experience proved more encouraging[31]. Also, Bearn *et al*[21] in their 1956 article that formalised observations made in the 1951 Meeting Abstract[14], reported that cortisone induced improvement in symptoms, and in physical and biochemical expressions of disease, and that withdrawal of cortisone from two patients resulted in prompt relapse. However they did retain the proviso that "there was no conclusive evidence that cortisone modified the disease process or that it will alter the eventual outcome". A telling observation in Melbourne, reported in 1958[32], was that by serial daily monitoring of levels of serum aspartate transaminase after treatment was started, prompt falls in highly raised levels occurred, often within hours, and other indices of impaired liver function improved in turn. Moreover, since relapse tended to occur when cortisone was withdrawn, the need for long term maintenance therapy became evident. Hence co-therapy with a "steroid-sparing" agent was sought and, on theoretical grounds, there was chosen azathioprine, a derivative of the immunosuppressive drug 6-mercaptopurine, and this conferred added benefit[33]. However there was scepticism whether our routine prednisolone-azathioprine regimen, albeit symptomatically beneficial, actually altered the natural history of the disease. The view in Melbourne was that a trial that included randomly allocated placebo control patients was ethically problematic. Our decision was that all trial patients would receive active therapy after a 4-8 wk non-treatment run-up with close monitoring of multiple liver functional indices, and comparison would be made of mean levels for such indices for a group of 15 patients pre- and post -therapy with prednisolone, or prednisolone plus azathioprine. The outcome, published in 1968[34], led to the recommendation for long term (2-3 years) maintenance therapy with either prednisolone monotherapy, or combined prednisolone-azathioprine. However, conventional trials with randomly allocated non-treated controls were still called for, and these convincingly indicated survival benefits for the treated patients. The study of Cook *et al*[35] is exemplary, albeit with a substantial burden of mortality among the control group (Figure 2). Detailed studies on immunosuppressive therapy by the Mayo Clinic through the 1970s-1980s, with substantial case numbers, have shown sustained efficacy according to various criteria, survival, biochemical and histological[36].

PATHOLOGICAL FEATURES OF CHRONIC ACTIVE/AIH

The increasing use of percutaneous biopsy of the liver in the 1950s greatly contributed to better knowledge of the nature of chronic hepatitis, well illustrated by the description in 1958 by Klatskin[37] of nine cases bearing the pre-1950s diagnoses of subacute hepatic necrosis/post-necrotic cirrhosis, and attributed to "anicteric infections with the hepatitis virus" because histopathological appearances met existing criteria for subacute hepatic necrosis of viral origin. However scrutiny of the clinical, biochemical and histological features strongly suggests that the illness in some, perhaps most, of the nine cases was actually AIH: there was female preponderance, hyperglobulinaemia, and corticosteroid responsiveness. However the particular feature of Klatskin's study was the introduction of, or emphasis on, detailed histological features characteristic of those associated with autoimmune (as well as with viral) hepatitis. These included extensive "bridging" hepatocellular necrosis, ballooning of hepatocytes, regenerating hepatocytes with arrangement of cells in the form of "rosettes", intense mononuclear inflammatory reaction; regenerative nodules, and acidophilic bodies (Councilman bodies) which are rounded intensely eosinophilic homogeneous cytoplasmic masses derived from hepatic cells undergoing coagulation necrosis. These latter appearances of course represent apoptosis of hepatocytes, recognized by Kerr[38] some 30-40 years before but not widely appreciated until interest developed in the 1980s in the apoptosis process. A detailed analysis of apoptosis in the context of chronic hepatitis and liver cell degeneration is given by Searle *et al*[39].

There were two morphological features additional

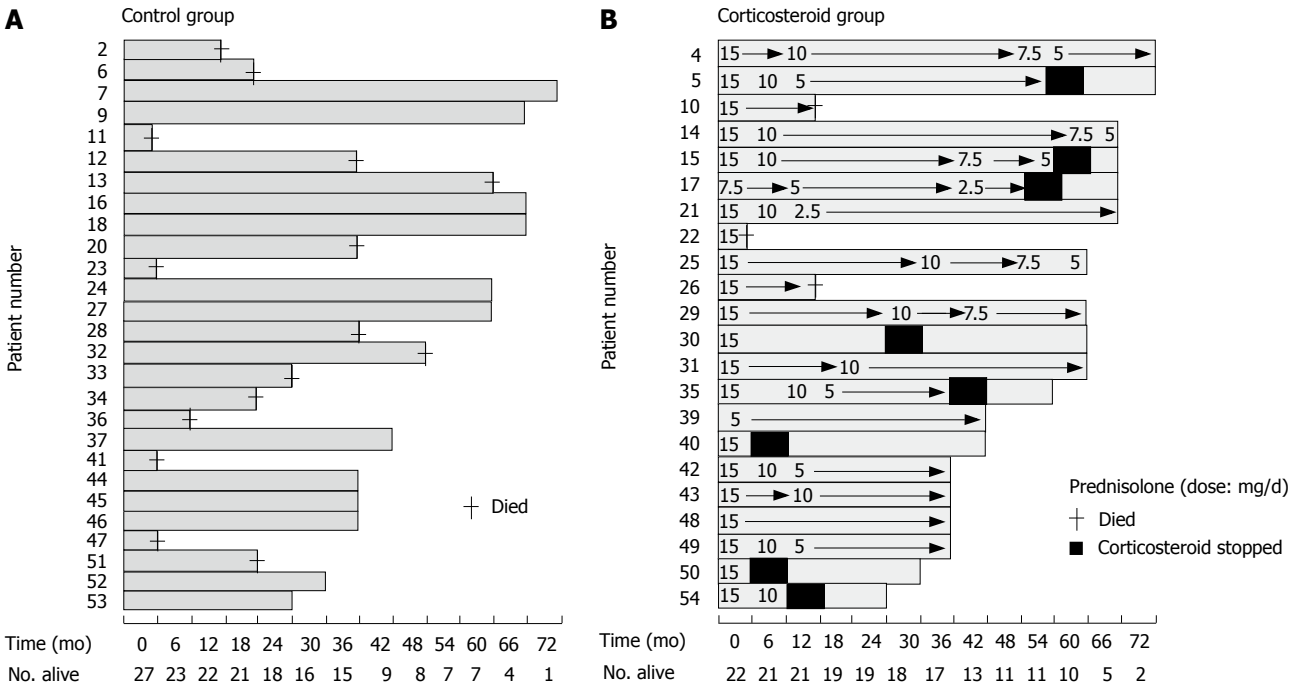

Figure 2 Combined Figures 1 and 2 from a publication[35] on long-term prospective controlled trial of corticosteroid treatment of patients with active chronic (autoimmune) hepatitis. The graphs show duration of treatment and survival for (**A**), left, non-treated control group and (**B**), right, corticosteroid treated group, and length of time (months) that the individual patients in either group had been in the investigation at the time of assessment. Numbers indicate the sequence of entry to the trial; +, time of death. Survival was 19/22 for the treated group and 12/27 for the control group. Reproduced with permission of Oxford University Press, publishers of Quarterly Journal of Medicine.

to those described by Klatskin that deserve comment. One was the concentration of inflammatory activity and necrosis in the junctional region between the portal tract and liver lobule, initially described by Popper *et al*[40] as piecemeal necrosis, and currently referred to as interface hepatitis. The other feature was the striking accumulation among the inflammatory infiltrates of plasma cells, noted in most pathological descriptions, although not all[41], and earlier on leading to the disease being designated as "plasma cell hepatitis"[42]. This feature of CAH, i.e. prominence of plasma cells in liver, and bone marrow as well, aligns with the characteristic hypergammaglobulinaemia, although the antigenic specificity of these plasma cells and their secreted immunoglobulins has not yet been ascertained.

Finally we can note the protracted debate on whether histological features, irrespective of clinical diagnosis, would distinguish cases of chronic hepatitis with a progressive course and cirrhogenic potential-CAH-from those without such potential-chronic persistent hepatitis[43,44]. After much elaboration in the 1970s these terms slowly disappeared from the lexicon.

HETEROGENEITY OF CAH

The term CAH used in 1950s and 1960s was generic, for what tended to be regarded as a single disease entity, with the prototypic cases being those that would fulfil criteria that presently define the autoimmune type of disease. However, impressions developed from the 1960s that CAH was not a homogeneous disease entity. Thus a collaborative study with colleagues in Singapore ascertained that CAH seen among Caucasians in Melbourne and Chinese in Singapore differed substantially, clinically, histologically and serologically[45]. Similarly, in unpublished observations in Melbourne, features of cases of chronic hepatitis and cirrhosis among recent Southern European immigrants differed from those among Australian-born individuals, such that we spoke of "Mediterranean cirrhosis". An explanation was soon forthcoming. This depended on the fortuitous discovery by Blumberg and colleagues of an antigenic particle in serum initially called "Australia antigen" (Au), because it was first detected in serum from an Australian aboriginal serum donor[46]. Au was later found to be a surface protein of a virus identified with as the transmissible agent responsible for infectious (serum) hepatitis type B. The particle became known as hepatitis B surface antigen-HBsAg[47].

It became evident, as reported from several centres that cases of "CAH" segregated into those that were autoantibody positive and HBsAg negative, or vice versa and, in fact, prototypic (autoimmune) CAH became referred to as HBsAg-negative CAH[36]. Moreover, certain histological features of CAH, chiefly interface hepatitis, could be recognized in various other causally different cases chronic liver disease, whether due to viral infection, ethanol abuse, Wilson's disease or other causes. An invitation was extended to me in 1972 to write an editorial on the "Prognosis (I changed this to Prognoses) of CAH" which led to a proposal that CAH was indeed heterogeneous in terms of aetiology, histological appearances, immunogenetic background, therapy requirements and outcome[48]. Moreover it seemed

reasonable to assume that aetiological proportions among all cases of CAH would differ according to geographic region, and this proved to be the case, as judged by differences ascertained for cases of CAH in Australia and Singapore (see above) or Yugoslavia wherein proportions of cases due to chronic hepatitis B were substantially higher[49]. Finally, there were cases regarded as cryptogenic CAH, with some perhaps resulting from infection with non-A, non-B hepatitis virus; these later became attributable to the subsequently discovered hepatitis C virus (HCV). Currently, world-wide, the proportion of cases of chronic hepatitis B, followed by that of hepatitis C, far outnumber that of all other types.

All this led to the perception of a need for a clearer definition of CAH. This was met by the convening of a widely representative expert panel of hepatologists, the International AIH Group whose consensus deliberations led to reports in 1993 and 1999 on generally acceptable diagnostic criteria[28,50].

GENETIC FEATURES OF CHRONIC ACTIVE/AIH

The wide regional differences in prevalence of different types of chronic hepatitis depend on differing environmental exposures and genetic composition of the particular population groups. Among the former, the endemicity of hepatitis virus infections is one important element.

The first genetic factor of interest in AIH was female predisposition, common to most autoimmune diseases, and for which the basis is still not well understood. Then, recognized in 1972, were genes that encoded for human leucocyte antigens, HLA. Cases of "classical" CAH, selected for typing for the then testable HLA alleles encoded at the HLA A and B loci, showed a significantly increased frequency of HLA-B8[51], an allele already implicated among Caucasian subjects with certain other autoimmune diseases. This association was soon confirmed in other centres, and was followed by the finding of an increased frequency in CAH of the D-related (DR) locus allele, DR3; a family study showed the combined inheritance of HLA A1, B8 and DR3 *en bloc* (a haplotype) from one or other parent, or both[52]. Possession of HLA DR3, particularly in those homozygous for these alleles, was predictive of a severe course and lesser responsiveness to immunosuppressive therapy[53]. The culprit allele is now styled as *HLA-DRB1*0301*. Later an additional HLA type, DR4 (*HLA-DRB1*0401*), not evident in our earlier studies, was identified[54]. The 6-7 fold risk for disease conferred by HLA DR3/4 is substantial but not highly potent meaning that, like all other "complex" autoimmune diseases, there must exist multiple other polymorphisms in "tolerance/ autoimmunity" genes that contribute to susceptibility: these are mostly undiscerned pending application of population genetics by genome wide screening.

IMMUNOSEROLOGICAL AND T-CELL STUDIES IN CHRONIC ACTIVE/AIH

The reactivities that initially (in the 1950s) were indicative of autoimmunity in CAH, the L.E. cell test and the AICF reaction, were soon superseded by more discriminatory and simpler laboratory assays. These are described in detail in other articles in this issue and in contemporary reviews[55-57].

Nuclear antigen(s)

Detection of ANA by indirect immunofluorescence (IIF) was introduced in the early 1960s[26] and remains the standard diagnostic screening procedure[57]. Superficially at least, the nuclear reactant(s) is the same as that responsible for the ANA reactivity observed in SLE i.e. the nucleosome (chromatin), although anti-DNA is much less frequent[56]. The idea that patients with AIH and SLE share one or more of the gene loci that determine ANA reactivity may be revealed by future population genome studies.

Smooth muscle antigen(s)

In 1963 there was observed a novel reactivity with smooth muscle of rodent gastric mucosa[58]. Detection of this smooth muscle antibody (SMA) to high titre proved to have high specificity for the diagnosis of CAH and notably, in "conventional" cases of SLE in which inflammatory destruction of liver cells is not evident, the test proved negative[59]. Further observations showed that some SMA+ve sera reacted by IIF with the mesangium of renal glomeruli, indicative of a wider distribution of the antigenic reactant than merely gastric smooth muscle tissue[60]. A subsequent observation was that some positive sera gave reactivity only with blood vessel walls (SMAv), and others reacted as well with renal glomeruli and renal tubular cells (SMAgt)[61]. The recognition that SMAv pointed to non-specific reactivity, and SMAvgt to reactivity specifically associated with AIH has led laboratory serologists to retain the designations SMAv and SMAgt in their diagnostic reporting.

The first indication of the identity of a reactant for SMA+ve sera was that reactivity could absorbed from serum by exposure to the cytoskeletal protein F-actin[62]. Further studies using IIF on cultured tissue cells revealed that SMA+ve sera stained cytoskeletal microfilaments (actin "cables"), representing polymeric F-actin, whereas SMA+ve sera from cases other than CAH stained intermediate filaments representing vimentin, desmin or others[63]. After much developmental work, there are now commercially available ELISA formats based on highly purified F-actin that have good specificity and sensitivity for the diagnosis of AIH[56]. The need at present is for better knowledge on the basis of anti-F-actin reactivity, including the significance (if any) for the pathogenesis of AIH, the epitope specificity of the antibodies, the relationship of epitopes to binding sites for the numerous F-actin binding proteins in the cell, and functional effects of anti-F-actin on cell motility[64].

LKM-1 antigen

In 1973, yet another serum reactant in AIH was discovered by IIF, to an antigen that was enriched in cytoplasm of liver and kidney proximal tubular cells[65]. This so-called liver-kidney "microsomal" (LKM) antigen, later designated LKM-1 because other LKM antigens became demonstrable[56], is actually located in the endoplasmic reticulum of liver and kidney proximal tubular cells[66]. A notable feature of anti-LKM-1-positive-AIH, versus ANA/SMA positive AIH, is that the serologically defined reactivities appear mutually exclusive, providing grounds for distinguishing these serological variants of AIH as type 2 and type 1[67]; this distinction has been retained by hepatologists even though few other differences exist. The point of interest heuristically on the serological distinction is that the respective autoantibody responses (ANA/SMA, anti-LKM-1) cannot be ascribed simply to (hepato) cellular injury, an explanation often levelled for the appearance of at least for some types of autoantibody. Although cases of anti-LKM-1-positive AIH are numerically far less than the traditional type, the ratios being about 1:10 in adults and 1:4 in children, type 2 AIH has proven far more amenable to investigation, since the LKM-1 antigen has been molecularly identified by screening a gene expression library as the cytochrome P450 isoform 2D6, enabling epitopes to be mapped[68]; there is demonstrated a CD4+ T-cell responsiveness to peptide antigens of CYP450 2D6[69]; and an experimentally credible model in mice has been developed[70].

Soluble liver/pancreas antigen

A soluble cytoplasmic antigen was independently discovered by CF[71], and ELISA[72], using pancreas or liver cell extracts respectively, and the reactant was found to be identical; it is generally known as "soluble liver/pancreas antigen (SLA). Sero-positivity, initially thought to identify a type 3 AIH, occurs in cases of AIH that are negative for other reactivities, but also in sero-positive cases of type 1 AIH. SLA has been cloned, identified and purified[56], and commercially available ELISA kits are diagnostically reliable. The pathogenetic significance of SLA is uncertain.

A liver-specific and disease specific antigen

This has been long searched for, since AIH behaves very much like an organ-specific autoimmune disease. In earlier times much effort was put into the characterisation of a preparation called liver-specific lipoprotein (LSP)[73], and assessment of the autoantigenic potential of this claimed liver-specific molecule[74]. Stemming from this was the recognition of the liver-specific membrane antigen, the asialoglycoprotein receptor[75], but this has not quite fulfilled the earlier hopes[76]. The liver cell membrane has been repeatedly studied, mostly by immunoblotting using AIH sera, for a molecular signal corresponding to a specific autoantigenic moiety, with the consistent finding being that of multiple reactive components of mw ranging from 20 to > 100 kDa[77,78], but with none of these reaching candidate status as a liver-specific or disease-specific autoantigen.

CONCLUSION

Despite weighty circumstantial evidence for autoimmunity as one proximal cause of chronic hepatitis, and the existence of diagnostic serological reactants of high sensitivity and specificity, there is a disconcerting lack of mechanistic immunological explanation for AIH, and particularly the more prevalent type 1. There are high expectations for "the way we hope to be"[79] but, for these to be realised, new paradigms will be needed to explain AIH as well as other examples of organ-specific autoimmunity with non-organ-specific immune-dependent accompaniments.

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TOPIC HIGHLIGHT

Pietro Invernizzi, MD; Ian R Mackay, MD, *Series Editors*

Clinical features and management of autoimmune hepatitis

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Abstract

Autoimmune hepatitis (AIH) is a chronic hepatitis of unknown etiology which can progress to cirrhosis. Its clinical manifestations are highly variable and sometimes follow a fluctuating course. Diagnosis is based on characteristic histologic, clinical, biochemical and serological findings. Anti-inflammatory/immunosuppressive treatment frequently induces remission but long-term maintenance therapy is often required. Liver transplantation is generally successful in patients with decompensated cirrhosis unresponsive to or intolerant of medical therapy.

Key words: Autoimmunity; Autoimmune hepatitis; Chronic hepatitis; Cirrhosis; Liver disease

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INTRODUCTION

Autoimmune hepatitis (AIH) is chronic hepatitis of unknown etiology, which is thought to occur as a result of escape from normal suppression of self-reactivity. It occurs worldwide in children and adults. Clinical manifestations are highly variable and sometimes follow a fluctuating course. Diagnosis is based on characteristic histologic, clinical, biochemical and serological findings. Anti-inflammatory/immunosuppressive treatment induces remission but long-term maintenance therapy is often required. Liver transplantation is generally successful in patients with decompensated cirrhosis unresponsive to or intolerant of medical therapy.

HISTOLOGY

The histologic appearance of AIH is that of chronic hepatitis, and, although certain changes are characteristic, there are no findings specific to the disease. The histologic differential diagnosis of chronic hepatitis is shown in Table 1. Based on the advances in virologic studies and refinements of cholangiographic methods, exclusion of other entities has become easier, although co-existence of chronic viral hepatitis and AIH may make the diagnosis difficult.

The inflammatory component is characterized by a mononuclear cell infiltrate, which invades the sharply demarcated hepatocyte boundary (limiting plate) surrounding the portal triad and permeates the surrounding parenchyma (periportal infiltrate; piecemeal necrosis; interface hepatitis) and beyond (lobular hepatitis). It may include an abundance of plasma cells and/or eosinophils, but the portal lesion generally spares the biliary tree. In all but the mildest forms of AIH, fibrosis is present. In advanced disease, fibrosis is extensive (bridging fibrosis) and, with distortion of the hepatic lobule and appearance of regenerative nodules, cirrhosis occurs. On occasion, centrizonal disease may be present.

The histologic findings differ somewhat comparing patients with acute onset AIH to those with an insidious presentation. Patients presenting with fulminant hepatic failure have more interface and lobular hepatitis, lobular disarray, hepatocyte necrosis, central necrosis and submassive necrosis, but less fibrosis and cirrhosis compared to patients presenting with a more chronic course[1,2]. Steatosis occurs in a minority of patients, although, given the increasing prevalence of diabetes, dyslipidemia and obesity in many parts of the world, non-alcoholic fatty liver disease may be seen more often accompanying AIH. Whether the co-morbidity of steatosis and/or steatohepatitis accelerate progression of disease in AIH is unknown. The prevalence of cirrhosis in patients $\geqslant$ 60 years at presentation was found to be higher than that in patients $\leqslant$ 30 years; when comparing groups of patients $\geqslant$ 60 years with those < 60 years, however, no differences were found[2,3]. In patients with a spontaneous or pharmacologically-induced remission, histologic findings may revert to normal; inflammation

Table 1 Histologic differential diagnosis of chronic hepatitis

Histologic differential diagnosis
Autoimmune liver disease
Autoimmune hepatitis
Primary biliary cirrhosis
Primary sclerosing cholangitis
Variant syndromes
Chronic viral hepatitis
Chronic hepatitis B
Chronic hepatitis C
Chronic hepatitis delta
Chronic hepatitis due to other viruses
Chronic drug-induced hepatitis
Alpha$_1$-antitrypsin deficiency
Wilson's disease
Granulomatous hepatitis
Systemic lupus erythematosus
Graft-versus-host disease
Alcoholic steatohepatitis
Nonalcoholic steatohepatitis

may be confined to portal areas; cirrhosis may become inactive; and fibrosis may regress or disappear[4].

CLASSIFICATION

The most commonly accepted classification of AIH is based on patterns of circulating antibodies, although there is little evidence to support a role for these antibodies in pathogenesis (Table 2).

Type 1 AIH is most frequently characterized by antinuclear antibody (ANA), smooth muscle antibody (SMA) and anti-actin antibody (AAA). Titers of significance vary depending on the autoantibody in question and assays employed[5]. Anti-actin (IgG anti F actin) antibodies measured by ELISA appear to be more sensitive than SMA measured by immunofluorescence[6,7].

The identification of other circulating autoantibodies, in particular anti-soluble liver antigen/liver-pancreas antigen (anti-SLA/LP) and atypical perinuclear anticytoplasmic antibody (pANCA) are sometimes helpful in diagnosing type 1 disease. Anti-SLA/LP is the most specific autoantibody detected in type 1 AIH but is found in only 10%-30% of type 1 AIH. Atypical pANCA is non-specific, but commonly present. Antimitochondrial antibodies (AMA) occur infrequently in type 1 AIH. At times AMA may be the sole antibody present and identify an entity sometimes referred to as AMA-positive AIH or the overlap syndrome[8].

Anti-liver/kidney microsome -1 (ALKM-1) and anti-liver cytosol-1 (ALC-1) antibodies occurring alone or together characterize type 2 AIH. Anti-liver cytosol-1 generally occurs in conjunction with anti-liver/kidney microsome-1, but may be the sole autoantibody[9].

Type 1 AIH in Caucasians is associated with the *HLA-DR3* serotype, which is found in linkage disequilibrium with *HLA-B8* and *HLA-A1* and in *HLA-DR3*-negative patients with *HLA-DR4*. *HLA-DR3*-associated disease is more commonly found in patients $\leqslant$ 40 years at presentation[2]. In Japan, where HLA-DR3 is rare, the primary association is with *HLA-DR4*. Polymerase chain reaction studies genotyping for *HLA-DRB*, *DQA* and *DQB* have confirmed the serologic findings. In children, type 1 AIH is commonly associated with the *HLA-DRB1*03* and *HLA-DRB1*13* alleles. Type 2 AIH has been associated with *HLA-DRB1* as well as *HLA-DQB1* alleles[10].

Table 2 Classification of autoimmune hepatitis

Disorder	Characteristic autoantibodies
Type 1	ANA (antinuclear antibody)
	SMA (smooth muscle antibody)
	AAA (anti-actin antibody)
	Anti SLA/LP (anti-soluble liver antigen/liver-pancreas antigen)
	pANCA (atypical perinuclear antineutrophil cytoplasmic antibody)
	AMA (antimitochondrial antibody)[1]
Type 2	ALKM-1 (anti-liver/kidney microsome-1)
	ALC-1 (anti-liver cytosol-1)

[1]Occurs infrequently in association with other characteristic autoantibodies. It may be the sole antibody present in AMA-negative autoimmune hepatitis, also referred to as the overlap syndrome.

CLINICAL FEATURES

Although there is a female predominance, AIH occurs in children and adults of both sexes in diverse ethnic groups worldwide. Type 2 disease, which is seen predominantly in children and young women, is rare in North America[9,10]. Although AIH was thought previously to be primarily a disease of the young or middle aged, it is now clear that it also occurs in the elderly (generally defined as $\geqslant$ 60 years of age)[2,3,11].

The heterogeneous, sometimes fluctuating nature of AIH, leads to marked variability in clinical manifestations. Presentation may be asymptomatic or insidious, with mild non-specific symptoms only or may mimic acute viral hepatitis. Rarely, AIH presents as fulminant hepatic failure[1,12]. Patients with occult disease may have undetected cirrhosis and present only when decompensation occurs. The group of patients now labeled as cryptogenic cirrhosis, includes some patients with seronegative AIH, underscoring the possibility of the absence of circulating autoantibodies in AIH[13].

Many patients with an acute presentation have histological evidence of chronic disease in the liver biopsy, indicating that they have had antecedent subclinical disease, although the duration of the subclinical anicteric course is generally difficult to ascertain. In retrospect, a fluctuating course, which had been thought to reflect some other diseases, can be identified occasionally. Long periods of subclinical disease may also ensue after presentation. Recent surveys of pregnancy in AIH have indicated that the initial presentations of AIH may occur not only during pregnancy but in the early post-partum period[14,15]. AIH may occur in conjunction with a variety of autoimmune disorders, including celiac disease[16,17]. Arthralgia involving small joints is common, and inflammatory arthritis may be particularly troublesome.

One presentation of AIH is in the setting of medications, or herbal agents, used for other diseases. It is not

clear if they unmask and/or induce AIH or simply result in a drug-induced hepatitis with histological findings that mimic AIH. Minocycline and, more recently, statins[18], both of which induce other autoimmune syndromes, have been considered as drugs capable of "triggering" AIH.

Complications of AIH are those seen in any progressive liver disease and primary hepatocellular carcinoma is an expected, although uncommon, consequence[2,19,20]. There are no established guidelines for hepatocellular carcinoma screening in cirrhosis associated with AIH. A reasonable approach would be surveillance with an ultrasound and alpha feta-protein every 6-12 mo.

DIAGNOSIS

In the presence of a compatible histologic picture, the diagnosis of AIH is based on characteristic clinical and biochemical findings, circulating autoantibodies and abnormalities of serum globulins. A scoring system, proposed and subsequently revised by the International AIH Group for experimental purposes to standardize diagnosis for clinical trials and population studies, has been adopted by clinicians, but found to be problematic when applied to individual patients, especially children. Thus attempts were undertaken by the International AIH Group to devise a less complicated and more accurate system for wider application in clinical practice. A scoring system, using autoantibodies, gamma globulins, absence of viral hepatitis and histologic findings from patients form a wide geographic distribution, has been proposed as a sufficiently sensitive and specific scoring system[21].

TREATMENT OF ADULTS WITH AIH

Appropriate management of AIH can mitigate inflammation, slow progression of disease, prolong survival, improve quality of life and delay or avoid liver transplantation. However, depending on a variety of definitions of response, success rates only range from 65% to 80%, which leaves a significant number of patients in need of other than standard treatment. Considerable challenges still exist in the areas of initial and maintenance regimens, management of relapse, non-response, drug toxicity and intolerance, and non-compliance[8,22].

Standard medications for initial and maintenance regimens are still considered to be prednisone (or prednisolone) alone or in combination with azathioprine (or 6-mercaptopurine) (Table 3). A recent retrospective analysis of corticosteroid treatment in AIH patients with severe and fulminant AIH, suggested that steroids did not obviate the need for transplantation and may have promoted septic complications[23].

One issue of treatment of particular concern is toxicity and/or intolerance to 6-mercaptopurine and its prodrug azathioprine. The methylation of 6-mercaptopurine and 6-thioguanosine 5'-monophosphate is catalyzed by thiopurine methyltransferase (TPMT). The genes encoding thiopurine methyltransferase are highly polymorphic.

Table 3 Drugs used in standard treatment of autoimmune hepatitis in adults

Regimen	Single-drug therapy	Combination therapy
Initial	Prednisone 20-60 mg/d	Prednisone 15-30 mg/d and azathioprine 50-100 mg/d
Maintenance	Prednisone 5-15 mg/d or azathioprine 50-200 mg/d	Prednisone 5-10 mg/d and azathioprine 50-150 mg/d

Homozygosity and heterozygosity for mutations in TPMT genes occur in Caucasian and other populations, and these patients may accumulate high levels of thioguanine nucleotides in bone marrow cells. Patients who are homozygous for a mutation of TPMT are at high risk for severe toxicity, including death. Patients, who are heterozygous for the TMPT mutation, probably have an intermediate risk of toxicity. These findings have led to the suggestion that prior to placing patients on azathioprine or 6-mercaptopurine, TPMT genotyping may be appropriate. Despite reliable methods for TMPT genotyping and measurement of levels of 6-mercaptopurine metabolites, their assessment in the clinical management of AIH is not established, and must be evaluated in the context of severity of disease, as well, as advanced fibrosis has been shown to predict azathioprine toxicity[24-26].

Although some patients will remain in remission when drug treatment is withdrawn, the majority requires long-term maintenance therapy. In general, the response is better with milder disease. Adults with cirrhosis at the time of initial biopsy and children, particularly those with type 2 disease, rarely stay in remission when treatment is withdrawn and will almost require life-long maintenance therapy.

No firm guidelines exist for decisions regarding withdrawal of medications because histologic changes may lag biochemical responses and a quiescent histologic appearance and normal biochemical findings while patients are still receiving therapy, are not necessarily predictive of continued remission once therapy is withdrawn. Although, in the past, aminotransferase levels $\leqslant$ 2 x normal were proposed as a guideline to reducing medications, relapse has been shown to be less likely in patients who achieve normal transaminase and gamma globulin (or IgG) levels[27].

Progress in non-standard treatment for patients with inadequate responses or intolerance to therapy with glucocorticosteroids alone or in combination with azathioprine or 6-mercaptopurine (including mycophenolate mofetil, methotrexate, cyclophosphamide, tacrolimus, budesonide and ursodeoxycholic acid) has been slow. In view of the paucity of trials with non-standard forms of therapy most decisions must be based on data obtained from case reports and series of small numbers of patients. Cyclosporine, which has been used successfully in children to induce remission[28], and tacrolimus are used occasionally to treat adults[29,30]. Off-label use of mycophenolate mofetil has become more frequently employed in intolerant or non-responsive patients[31,32]. The roles of cyclosporine, tacrolimus, mycophenolate mofetil,

methotrexate, cyclophosphamide, ursodeoxycholic acid, budesonide[33,34] and other immunosuppressive medications have not been established.

AIH patients who develop decompensated cirrhosis may require liver transplantation. Five-year patient and graft survivals range from 80% to 90%. As in other autoimmune liver diseases, recurrence and cirrhosis may occur after transplantation[35,36] and mandate modifications of the post-transplantion therapeutic regimens. So-called *de novo* AIH, also referred to as post-transplantion immune hepatitis or graft dysfunction mimicking AIH, occurs after liver transplantation for diseases other than AIH in adults and children, and may require changes in post-transplantion therapy as well[37,38].

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TOPIC HIGHLIGHT

Pietro Invernizzi, MD; Ian R Mackay, MD, *Series Editors*

Aetiopathogenesis of autoimmune hepatitis

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Abstract

The histological hallmark of autoimmune hepatitis (AIH) is a dense portal mononuclear cell infiltrate that invades the surrounding parenchyma and comprises T and B lymphocytes, macrophages, and plasma cells. An unknown but powerful stimulus must be promoting the formation of this massive inflammatory cellular reaction that is likely to initiate and perpetuate liver damage. An autoimmune attack can follow different pathways to inflict damage on hepatocytes. Liver damage is likely to be orchestrated by $CD4^+$ T lymphocytes recognizing an autoantigenic liver peptide. To trigger an autoimmune response, the peptide must be embraced by an HLA class Ⅱ molecule and presented to naïve $CD4^+$ T helper (Th0) cells by professional antigen presenting cells, with the co-stimulation of ligand-ligand fostering interaction between the two cells. Th0 cells become activated, differentiate into functional phenotypes according to the cytokines prevailing in the microenvironment and the nature of the antigen, and initiate a cascade of immune reactions determined by the cytokines produced by the activated T cells. Th1 cells, arising in the presence of the macrophage-derived interleukin (IL) -12, secrete mainly IL-2 and interferon-gamma (IFN-γ), which activate macrophages, enhance expression of HLA class Ⅰ (increasing liver cell vulnerability to a $CD8^+$ T cell cytotoxic attack), and induce expression of HLA class Ⅱ molecules on hepatocytes. Th2 cells, which differentiate from Th0 if the microenvironment is rich in IL-4, produce mainly IL-4, IL-10, and IL-13 which favour autoantibody production by B lymphocytes. Physiologically, Th1 and Th2 antagonize each other. Th17 cells, a recently described population, arise in the presence of transforming growth factor beta (TGF-β) and IL-6 and appear to have an important effector role in inflammation and autoimmunity. The process of autoantigen recognition is strictly controlled by regulatory mechanisms, such as those exerted by $CD4^+CD25^+$ regulatory T cells, which derive from Th0 in the presence of TGF-β, but in the absence of IL-6. If regulatory mechanisms fail, the autoimmune attack is perpetuated. Over the past three decades different aspects of the above pathogenic scenario have been investigated. In particular, a defect in immunoregulation affecting $CD4^+CD25^+$ regulatory T cells (T-regs) has been demonstrated in AIH, particularly at diagnosis or during relapse. Advances in the study of autoreactive T cells have occurred mostly in AIH type 2, since the knowledge that CYP2D6 is the main autoantigen has enabled the characterization of both CD4 and CD8 T cells targeting this cytochrome. CD4 T cells from patients with type 2 AIH positive for the predisposing HLA allele *DRB1*0701* recognize seven regions of CYP2D6, five of which are also recognized by CD8 T cells. High numbers of IFN-γ producing CD4 T cells and CD8 T cells are associated with biochemical evidence of liver damage, suggesting a combined cellular immune attack.

Key words: Autoimmune hepatitis; Aetiopathogenesis; Lymphocyte; Cellular immune attack; Histocompatibility lymphocyte antigen

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INTRODUCTION

Autoimmune hepatitis (AIH) is an inflammatory liver disease, affecting mainly females, characterized by elevated serum transaminase activity, positive organ and non-organ specific autoantibodies, elevated IgG, and a histological picture of interface hepatitis. There are two types of AIH according to their serology: type 1 is characterized by anti-nuclear (ANA) and/or anti-smooth muscle (SMA) antibodies; type 2 by anti-liver kidney microsomal type 1 (anti-LKM-1) antibody. The aetiology of AIH is unknown, though both genetic and environmental factors are involved in its expression.

Immune reactions against host liver antigens are believed to be the major pathogenic mechanism.

GENETICS

AIH is a "complex trait" disease, i.e. a condition not inherited in a Mendelian autosomal dominant, autosomal recessive, or sex-linked fashion. The mode of inheritance of a complex trait disorder is unknown and involves one or more genes, operating alone or in concert, to increase or reduce the risk of the trait, and interacting with environmental factors.

Susceptibility to AIH is imparted by genes within the major histocompatibility complex (MHC) - the human leukocyte antigen (HLA) region - on the short arm of chromosome 6, especially genes encoding HLA *DRB1* alleles. Since the role of class Ⅱ MHC molecules is to present peptide antigens to CD4 T cells, HLA class Ⅱ antigen presentation with ensuing T cell activation is likely to be involved in the pathogenesis of AIH.

In Europe and North America, susceptibility to AIH type1 is conferred by the presence of HLA *DR3* (*DRB1**0301) and *DR4* (*DRB1**0401), both heterodimers containing a lysine residue at position 71 of the *DRB1* polypeptide and the hexameric amino acid sequence LLEQKR at positions 67-72[1,2]. In Japan, Argentina, and Mexico, susceptibility is linked to *DRB1**0405 and *DRB1**0404, alleles encoding arginine rather than lysine at position 71, but sharing the motif LLEQ-R with *DRB1**0401 and *DRB1**0301[3]. Thus, K or R at position 71 in the context of LLEQ-R may be critical for susceptibility to AIH, favouring the binding of autoantigenic peptides, complementary to this hexameric sequence. However, an alternative model based on valine/glycine dimorphism at position 86 of the DR-β polypeptide has been proposed, better representing the key HLA associations in patients from Argentina and Brazil[1,2]. In a study from Japan, patients with AIH type 1 were found to have *DRB1* alleles which encode histidine at position 13[1,2]. There appears therefore to be at least three different models, suggesting that different genetic associations are present in different populations and that the peptides presented by HLA class Ⅱ molecules to the T cell receptors are different and may be derived from different antigens. Thus, these HLA associations may be the molecular footprints of the prevailing environmental triggers that precipitate AIH type 1 in different environments, though at the effector level the same autoantigenic target would be recognized. In this context, it is of interest that in South America presence of the HLA *DRB1**1301 allele, which predisposes to paediatric AIH type 1 in that population, is also associated with persistent infection with the endemic hepatitis A virus.

The lysine-71 and other models for AIH type 1 cannot explain completely the disease, since for example in European and North American patients presence of lysine-71 is associated with a severe, mainly juvenile, disease in those *DRB1**0301 positive, but to a mild, late onset, disease in those *DRB1**0401 positive. Other genes within or/and without the MHC are, therefore, likely to be involved in determining the phenotype. Possible candidates are the MHC encoded complement and tumour necrosis factor α genes, that are located in the class Ⅲ MHC region, and the MHC class I chain-related (MICA) A and B genes.

Susceptibility to AIH type 2 is conferred by HLA DR7 (*DRB1*0701*) and DR3 (*DRB1*0301*); patients positive for *DRB1*0701* have a more aggressive disease and worse outcome[4].

In an attempt to define additional susceptibility genes, a genome-wide approach was applied to a Japanese cohort of patients with AIH type 1[5]. This study found that 2 microsatellite markers (on chromosomes 11 and 18) out of 400 studied are associated with AIH type 1, though no protein of clear relevance to the disease is encoded in proximity of these two markers. The use of a larger number of microsatellites may prove more informative.

A form of AIH resembling AIH type 2 affects some 20% of patients with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), a condition also known as autoimmune polyendocrine syndrome 1. APECED is a monogenic autosomal recessive disorder caused by homozygous mutations in the *AIRE1* gene and characterized by a variety of organ-specific autoimmune diseases, the most common of which are hypoparathyroidism and primary adrenocortical failure, accompanied by chronic mucocutaneous candidiasis[6,7]. The *AIRE1* gene sequence consists of 14 exons containing 45 different mutations, with a 13 bp deletion at nucleotide 964 in exon 8 accounting for more than 70% of APECED alleles in the UK[6]. The protein predicted to be encoded by *AIRE1* is a transcription factor. *AIRE1* is highly expressed in thymic medullary epithelial cells and thymic stromal cells involved in clonal deletion of self-reactive T cells. Studies in a murine model indicate that the gene inhibits organ specific autoimmunity by inducing thymic expression of peripheral antigens in the medulla leading to central deletion of autoreactive T cells. Interestingly, APECED has a high level of variability in symptoms, especially between populations. Since various gene mutations have the same effect on thymic transcription of ectopic genes in animal models, it is likely that the clinical variability across human populations relates to environmental or genetic modifiers. Of the various genetic modifiers, perhaps the most likely to synergize with *AIRE* mutations are polymorphisms in the HLA region. HLA molecules are not only highly variable and strongly associated with multiple autoimmune diseases, but are also able to affect thymic repertoire selection of autoreactive T cell clones. Carriers of a single *AIRE* mutation do not develop APECED. However, although the inheritance pattern of APECED indicates a strictly recessive disorder, there are anecdotal data of mutations in a single copy of *AIRE* being associated with human autoimmunity of a less severe form than classically defined APECED[6,7]. A role of the heterozygote state for mutant *AIRE1* in the development of AIH remains

to be established. *AIRE1* mutations have been reported in 3 children with severe AIH type 2 and extrahepatic autoimmune manifestations[8].

IMMUNE MECHANISMS

The liver is regarded as a lymphoid organ with unique immunological properties[9]. Because of its location and function, the liver is continuously exposed to a large antigenic load that includes pathogens, toxins, tumour cells, dietary, and self-antigens. The liver contains large numbers of phagocytic cells, antigen presenting cells (APC) and lymphocytes and is a site for the abundant production of cytokines, complement components and acute phase proteins. The intrinsic lymphocyte population mainly resides in the portal tracts but is also scattered throughout the parenchyma, consisting of both cells of the innate (natural killer T cells, natural killer cells, and macrophages) and the adaptive (T and B cells) arms of the immune system. The blood entering the liver from the gut is rich in bacterial and dietary antigens that intermingle with lymphocytes. Immunoregulatory mechanisms are required to determine whether an antigen encounter will result in immunological unresponsiveness (tolerance) or reactivity. Liver autoimmunity implies loss of self-tolerance. Programmed cell death - apoptosis - which is responsible for the normal turnover of hepatocytes and the elimination of liver cells and unwanted lymphocytes in inflammatory pathologies is also relevant to the breakdown and/or maintenance of liver tolerance. First, death by apoptosis allows for non-inflammatory elimination of cell components in contrast to necrosis, which is pro-inflammatory and potentially autoantigenic. Second, apoptosis is the mechanism whereby the immune system is "cleansed" of autoreactive T and B lymphocytes as illustrated by the process of "activation induced cell death".

Various mechanisms have been proposed to account for the onset of an autoimmune liver response with no single initiating event being able to explain all instances of autoimmunity. Two general conditions, however, should prevail: self reactive B and T lymphocytes must exist in the immunological repertoire and autoantigens must be presented in conjunction with MHC class Ⅱ molecules by APC.

Humoral autoimmunity

Titres of antibodies to liver specific protein (LSP), a macromolecular complex present on the hepatocyte membrane[10], and to its well characterized components asialoglycoprotein receptor (ASGPR)[11] and alcohol dehydrogenase (ADH)[12] correlate with the biochemical and histological severity of AIH. Immunofluorescence studies on monodispersed suspensions of liver cells obtained from patients with AIH show that these cells are coated *in vivo* with antibodies reacting with antigens on the liver cell membrane[13]. A pathogenic role for these autoantibodies has been indicated by cytotoxicity assays demonstrating that autoantibody-coated hepatocytes from patients with AIH are killed when incubated with autologous or allogeneic lymphocytes[13]. The effector cell was identified as an Fc receptor positive mononuclear cell, presumably a natural killer (NK) cell.

In AIH type 2 the target of the disease-defining antibody, anti-LKM-1, is CYP2D6, a member of the hepatic P450 cytochrome family. Since CYP2D6 is expressed on the membrane of the hepatocytes and readily "accessible"[14], anti-LKM-1 antibodies might well have a pathogenic effect. In AIH type 2 anti-LKM-1 antibodies recognize linear regions (autoepitopes) of CYP2D6 in a hierarchical manner. Thus, the principal linear B-cell epitope, $CYP2D6_{193-212}$ is recognized by 93% of patients, $CYP2D6_{257-269}$ by 85%, $CYP2D6_{321-351}$ by 53%, and two additional minor epitopes $CYP2D6_{373-389}$ and $CYP2D6_{410-429}$ are recognized by 7% and 13% respectively[15]. Intriguingly, anti-LKM-1 antibodies are also found in some 5% of patients with hepatitis C virus (HCV) infection, among whom they appear to correlate with increased disease severity and adverse reactions to interferon α treatment[16]. The major CYP2D6 epitope recognized by patients with AIH type 2, $CYP2D6_{193-212}$, is also recognized by 50% of patients with anti-LKM-1 positive HCV infection[15]. Interestingly, these patients have antibodies that cross-react with homologous regions of HCV ($NS5B\ HCV_{2985-2990}$) and CYP2D6 ($CYP2D6_{204-209}$), and also of cytomegalovirus (exon $CMV_{130-135}$)[15]. Cross-reactive mechanisms to explain the emergence of CYP2D6 specific autoimmunity have also been suggested for other sequences of CYP2D6 which share homologies with HCV and herpes simplex virus (HSV), such as the sequence spanning aa 310-324 of E1 HCV and aa 156-170 of IE175 HSV1, which share homology with the CYP2D6 region comprising aa 254-271. As anti-LKM-1 antibodies cross-react with homologous regions of CYP2D6, HCV, HSV, and CMV, a "multi-hit" mechanism for the generation of these antibodies and possibly of AIH type 2 may be envisaged. In this model, multiple exposures to CMV or HSV, common viral pathogens, may establish permissive immunological conditions, by priming a cross-reactive subset of T cells, in a genetically predisposed host. Depending on the degree of immunological priming, i.e. level of exposure and the degree of genetic susceptibility (particularly at the HLA locus and coding regions for "innate" components of immunity), a minority of recurrently infected individuals may progress to autoimmune disease. It is therefore conceivable that an as yet unsuspected virus infection may be part of the origin of the autoimmune attack in AIH; this is to some degree in agreement with the concept expressed by Rolf Zinkernagel that an autoimmune disease is a viral disease in which the virus is unknown[17].

Molecular mimicry

The central function of the adaptive immune system is to generate T and B lymphocytes that can specifically recognize a potentially infinite number of non-self antigens without any prior information as to their structure. This is achieved by randomly generating a

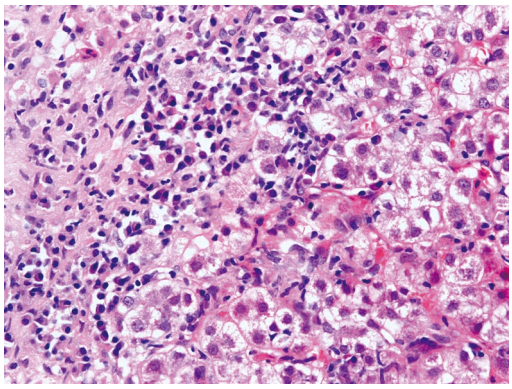

Figure 1 The portal and periportal inflammatory infiltrate characteristic of autoimmune hepatitis is composed by lymphocytes and plasma cells (interface hepatitis) (HE, × 40, provided by Dr. Alberto Quaglia).

large number of T and B cell specificities (*via* their respective antigen receptors-the T cell receptor and the antibody receptor) that are then able to clonally expand and recruit effector mechanisms on recognition of their cognate antigen or epitope. It is however, becoming clear that even this versatile system cannot cope with the extent of non-self antigenic diversity, and in the past decade convincing evidence has emerged for cross reactivity as an inherent property of immune ontogeny[18]. This has been studied primarily in the context of T lymphocytes, where it is clear that altered peptide ligands (APLs) - peptides similar in structure to the peptide antigen which are initially encountered - are able to induce both stimulatory and inhibitory T cell responses and, indeed, endogenous APLs operate in selecting the T cell repertoire in the thymus. This implies that a single T cell, rather than responding to a single antigen specificity, is able to cross-reactively respond to a number of antigens, thus expanding the antigenic specificities of the immune system to a level that reflects the antigenic diversity of the external environment[19].

This inherent potential for cross-reactivity, whilst allowing efficient responses to a vast array of pathogens also provides the immune system with the potential to cross-react with self, leading to autoimmunity. This process has been termed "molecular mimicry" as described above, whereby immune responses to external pathogens become directed towards structurally similar self components. Molecular mimicry has been shown to participate to the pathogenesis of autoimmune disease both in experimental models and in the human setting at the level of both T and B cells[18].

Cellular autoimmunity

The histological picture of interface hepatitis (Figure 1), with its striking infiltrate of lymphocytes, plasma cells, and macrophages was the first to suggest an autoaggressive cellular immune attack in the pathogenesis of AIH. Whatever is the initial trigger, this massive recruitment of activated inflammatory cells is likely to cause damage. Immunohistochemical studies have identified a predominance of T lymphocytes mounting the α/β T cell receptor[20]. Amongst the T cells, a majority are positive for the CD4 helper/inducer phenotype, and a sizeable minority for the CD8 cytotoxic phenotype. Lymphocytes of non-T cell lineage are fewer and include (in decreasing order of frequency) natural killer cells (CD16/CD56 positive), macrophages, B cells, and plasma cells. The involvement of natural killer T cells is the focus of ongoing studies.

There are different possible pathways that an immune attack can follow to inflict damage on hepatocytes (Figure 2) as discussed below.

Impairment of T regulatory cells

An impairment of immunoregulatory mechanisms, which would enable the autoimmune response to develop, has been repeatedly reported in the setting of both human and experimental autoimmunity. Thus, in early studies it was shown that patients with AIH have low levels of circulating T cells expressing the CD8 marker[21], and impaired suppressor cell function which segregates with the possession of the disease-predisposing HLA haplotype B8/DR3[22] and is correctable by therapeutic doses of corticosteroids[23]. Furthermore, patients with AIH have been reported to have a defect specifically in a subpopulation of T cells that control the immune response to an as yet unidentified liver-specific membrane antigen(s)[24]. Recent experimental evidence confirms an impairment of the immunoregulatory function in AIH. Thus, among recently defined T cell subsets with potential immunosuppressive function, $CD4^+$ T cells constitutively expressing the interleukin 2 receptor α chain (CD25) (T-regulatory cells, T-regs) have emerged as the dominant immunoregulatory lymphocytes[25]. These cells, which in health represent 5%-10% of the total population of peripheral $CD4^+$ T cells, control the innate and the adaptive immune responses by preventing the proliferation and effector function of autoreactive T cells. Their mechanism of action involves mainly a direct contact with the target cells, and to a lesser extent the release of immunoregulatory cytokines, such as interleukin 10 and transforming growth factor β 1 (TGF-β). In addition to CD25, which is also present on T cells undergoing activation, T-regs express a number of additional markers such as the glucocorticoid induced tumour necrosis factor receptor, CD62L, the cytotoxic T lymphocyte associated protein-4 (CTLA-4) and the forkhead/winged helix transcription factor FOXP3, the expression of which is closely associated with the acquisition of regulatory properties. In patients with AIH, T-regs are defective both in number and function compared to normal controls and these abnormalities relate to the stage of disease, being more evident at diagnosis than during drug-induced remission[26-28]. The percentage of T-regs inversely correlates with markers of disease severity, such as levels of antibodies to anti-soluble liver antigen[29] and anti-LKM-1 autoantibody titres, suggesting that a reduction T-regs favours the serological manifestations of autoimmune liver disease. If loss of immunoregulation was central to the pathogenesis of autoimmune liver disease, treatment should concentrate on restoring T-regs ability to expand, with consequent

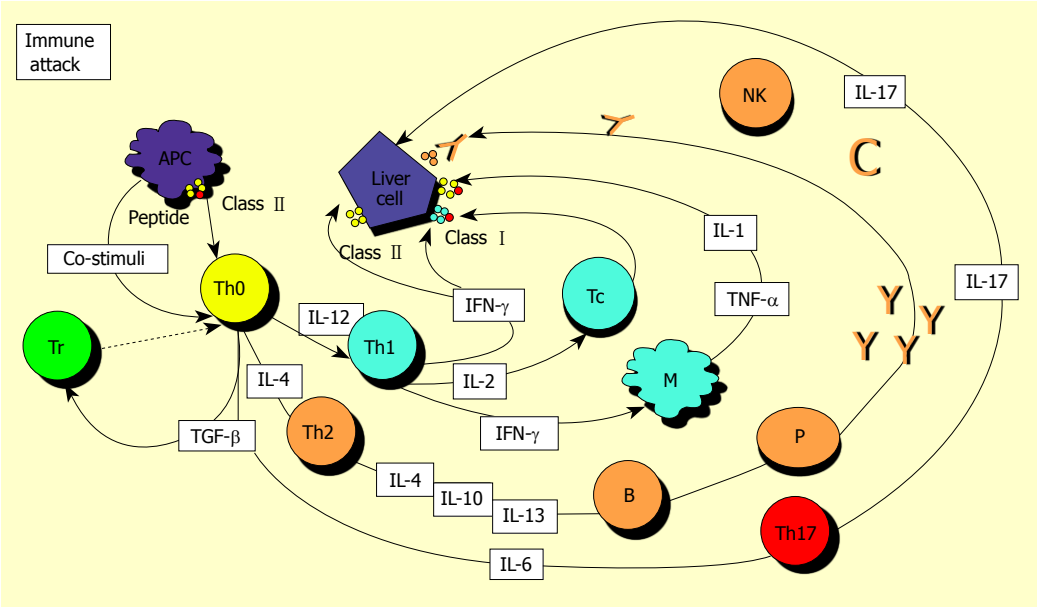

Figure 2 Autoimmune attack to the liver cell. A specific autoantigenic peptide is presented to an uncommitted T helper (Th0) lymphocyte within the HLA class II molecule of an antigen-presenting cell (APC). Th0 cells become activated and, according to the presence in the microenvironment of IL-12 or IL-4 and the nature of the antigen, differentiate into Th1 or Th2 and initiate a series of immune reactions determined by the cytokines they produce: Th2 secrete mainly IL-4, IL-10 and IL-13, and direct autoantibody production by B lymphocytes; Th1 secrete IL-2 and IFN-γ, which stimulate T cytotoxic (Tc) lymphocytes, enhance expression of class I and induce expression of class II HLA molecules on hepatocytes and activate macrophages; activated macrophages release IL-1 and tumour necrosis factor alpha (TNF-α). If regulatory T cells do not oppose, a variety of effector mechanisms are triggered: liver cell destruction could derive from the action of Tc lymphocytes; cytokines released by Th1 and recruited macrophages; complement activation or engagement of Fc receptor-bearing cells such as natural killer (NK) lymphocytes by the autoantibody bound to the hepatocyte surface. The role of the recently described Th17 cells, which arise in the presence of transforming growth factor beta (TGF-β) and IL-6, is under investigation.

increase in their number and function. This is at least partially achieved by standard immunosuppression, since T-regs numbers do increase during remission[26,28].

CD4 autoreactive T cells

To trigger an autoimmune response, a peptide must be embraced by an HLA class Ⅱ molecule and presented to uncommitted T helper (Th0) cells by professional APC, with the co-stimulation of ligand-ligand (CD28 on Th0, CD80 on APC) interaction between the cells (Figure 2). Once the autoimmune response has been initiated and in the absence of effective immunosuppressive treatment, tissue damage ensues and persists. In an inflammatory milieu, hepatocytes from patients with AIH, in contrast to normal hepatocytes, express HLA class Ⅱ molecules[20], as well as class Ⅰ. Although lacking the antigen processing machinery typical of APC, MHC-class Ⅱ -bearing hepatocytes may present peptides through a bystander mechanism[30]. In the presence of impaired immunoregulation and inappropriate expression of HLA class Ⅱ antigens on the hepatocytes, an intracellular autoantigenic peptide from intact hepatocytes could be presented to the CD4 helper/inducer T cells leading to their activation. Although no direct evidence exists as yet that an autoantigenic peptide is in fact presented by MHC-class Ⅱ-bearing hepatocytes and recognized by CD4 T helper cells, activation of such cells has been documented in AIH[21]. Circulating T cells specific for liver autoantigens are found also in normal subjects, but in AIH their frequency is at least 10-fold higher[31]. This finding suggests that the pool of liver-autoreactive T cells undergoes a significant expansion in patients with AIH and hence may be involved in the initiation and perpetuation of the immune attack to the liver.

Given that T cells recognize antigens in a precise fashion, studies in the early 1990s were conducted at a single T cell level in order to characterize antigen-specific T cell recognition. T cell clones generated from the peripheral blood were mainly $CD4^+$ α/β T cells[32], while a large proportion of liver-derived clones were either $CD4/CD8^-$ γ/δ or $CD8^+$ α/β T cells[31,33,34]. Both α/β and γ/δ T cell clones proliferated in the presence of a crude liver membrane preparation, liver specific protein and asialoglycoprotein receptor, α/β being more reactive than γ/δ clones. Some of the liver membrane reactive clones also proliferated in the presence of LSP and/or ASGPR, responded in an HLA class Ⅱ restricted fashion and helped autologous B cells to produce immunoglobulins, and in particular autoantibodies to LSP and ASGPR[32].

T cell ligands are best studied in AIH type 2, since the target of anti-LKM-1 has been characterized as *CYP2D6*. *CYP2D6*$_{262\text{-}285}$ specific T cell clones generated from liver tissue and peripheral blood express a Th1 $CD4^+$ phenotype[33,34]. In contrast to the latter study that focused on a short antigenic sequence of CYP2D6, a systematic approach based on the construction of overlapping peptides covering the whole CYP2D6 molecule was recently adopted to define the specificity of *ex vivo* CYP2D6 reactive T cells in patients with AIH type

2[4]. This study showed that T cells from patients positive for the predisposing HLA allele *DRB1**0701 recognize in a proliferation assay seven regions of CYP2D6, four of which are also partially recognized by T cells of *DRB1**0701 negative patients. While distinct peptides induce production of IFN-γ, IL-4 or IL-10, peptides that induced IFN-γ and proliferative responses overlap. There was also an overlap between sequences inducing T and B cell responses. The number of epitopes recognized and the quantity of cytokine produced by T cells are directly correlated to biochemical and histological markers of disease activity. These results indicate that the T cell response to CYP2D6 in AIH type 2 is polyclonal, involves multiple effector types targeting different epitopes, and is associated with hepatocyte damage[4].

CD8 autoreactive T cells

In addition to the unfolding role of CYP2D6 specific CD4 T cells in AIH type 2, there is growing evidence implicating an HLA class I restricted CD8 response in the pathogenesis of autoimmune liver damage. In the early 1990s CD8 T cell clones specific for ASGPR were described in patients with AIH[32]. Recent studies have identified CYP2D6 specific CD8 T cells capable of secreting IFN-γ and of exerting cytotoxicity after recognition of CYP2D6 epitopic sequences in an HLA class I restricted fashion[35].

Taken together, the data presented above suggest that a failure of immune homeostatic processes, normally keeping the response against self-antigens under control, is involved in the pathogenesis of AIH. The prime mechanism for tolerance breakdown remains to be elucidated. There is some evidence that molecular mimicry mechanisms involving viral self-mimicking and autologous sequences may be involved[36,37] and such mechanisms are the focus of ongoing studies.

ANIMAL MODELS

Research on the pathogenesis of AIH has been hampered by the lack of animal models reproducing faithfully the human condition. The ideal model for AIH should have a well-defined initiating event followed by chronic inflammation leading to fibrosis. Recently, researchers have focused on animal models of AIH type 2, since in this condition the autoantigen is well defined. The model produced by the group of Alvarez[38] is based on immunizing every two weeks for three times C57BL/6 female mice with a plasmid containing cDNA for the antigenic region of human CYP2D6, which is the target of anti-LKM-1, and formimino-transferase cyclodeaminase, which is the target of anti-liver cytosol-1 and an additional marker for AIH type 2[39], together with the end of the terminal region of murine CTLA-4. The latter was added to facilitate antigen uptake by antigen presenting cells. In a parallel set of experiments a plasmid containing the cDNA encoding IL-12, a Th1 skewing pro-inflammatory cytokine, was also used. When autoantigens and IL-12 were used to break tolerance, antigen specific autoantibodies were produced, a relatively modest elevation of transaminase levels at 4 and 7 mo was observed, and a portal and periportal inflammatory infiltrate composed of CD4 and CD8 T cells and, to a lesser extent, B cells was demonstrated 8-10 mo after the third immunization. When the same immunization protocol was used in different mouse strains, either a mild hepatitis or no inflammatory changes were observed indicating the importance of a specific genetic background. Another model of AIH type 2 uses CYP2D6 transgenic mice and aims at breaking tolerance with an adenovirus-CYP2D6 vector[40]. While focal hepatocyte necrosis was seen in both mice treated with the adenovirus-CYP2D6 vector and control mice treated with adenovirus alone, only the former developed chronic histological changes, including fibrosis, reminiscent of AIH. The hepatic lesion was associated to a specific immune response to an immunodominant region of CYP2D6 and a cytotoxic T cell response to adenovirus-CYP2D6 vector infected target cells. Though these two experimental approaches provide useful information on the possible pathogenic mechanisms leading to human AIH type 2, a model that closely reproduces human AIH type 1 is still lacking, hampering the elucidation of pathogenic mechanisms in this form of AIH.

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TOPIC HIGHLIGHT

Pietro Invernizzi, MD; Ian R Mackay, MD, *Series Editors*

Clinical features and management of primary biliary cirrhosis

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Abstract

Primary biliary cirrhosis (PBC), which is characterized by progressive destruction of intrahepatic bile ducts, is not a rare disease since both prevalence and incidence are increasing during the last years mainly due to the improvement of case finding strategies. The prognosis of the disease has improved due to both the recognition of earlier and indolent cases, and to the wide use of ursodeoxycholic acid (UDCA). New indicators of prognosis are available that will be useful especially for the growing number of patients with less severe disease. Most patients are asymptomatic at presentation. Pruritus may represent the most distressing symptom and, when UDCA is ineffective, cholestyramine represents the mainstay of treatment. Complications of long-standing cholestasis may be clinically relevant only in very advanced stages. Available data on the effects of UDCA on clinically relevant end points clearly indicate that the drug is able to slow but not to halt the progression of the disease while, in advanced stages, the only therapeutic option remains liver transplantation.

Key words: Primary biliary cirrhosis; Epidemiology; Clinical course; Natural history; Treatment

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INTRODUCTION

Primary biliary cirrhosis (PBC) is an autoimmune liver disease characterized by progressive destruction of intrahepatic bile ducts with cholestasis, portal inflammation, and fibrosis which may lead to cirrhosis, to its complications, and eventually to liver transplantation or death. Thus, primary biliary cirrhosis is indeed a historically-based misnomer, since currently a substantial proportion of patients may not develop cirrhosis as the final event. The disease predominantly affects women who are usually diagnosed in their fifties mainly in an asymptomatic stage. The loss of bile ducts leads to the retention within the liver of detergent bile acids which contribute to parenchymal damage through interaction with cell membranes and cellular organelles. The derangement of the entero-hepatic circulation of bile acids may also induce important pathophysiological changes which may determine, if untreated, some of the extra-hepatic alterations characteristic of established disease. It is well known that both clinical features and natural history vary greatly among individual patients ranging from asymptomatic and stable or only slowly progressive to symptomatic and rapidly progressive disease. The clinical presentation has progressively changed from one characterized by a serious outcome to that of a slowly evolving disease since natural history and outcome have improved, during the last few decades, due to the recognition of earlier more indolent cases and, likely, to widespread use of ursodeoxycholic acid (UDCA).

Since aetiology and immunological aspects are reviewed separately in this series, the aim here is to review the evidence on epidemiology, diagnosis, clinical features, and treatment. Both management of the consequences of long-standing cholestasis and specific therapy for PBC will be discussed.

EPIDEMIOLOGY

Descriptive epidemiology of a particular disease is important in order to establish the magnitude of the problem and to find clues for aetiopathogenesis. There are a number of epidemiologic studies reported among patients affected by PBC[1]. The key issue involving all these studies is that they rely upon the number of diagnoses recorded in a defined location rather than on the screening of the entire population at risk. Obviously, this latter approach

Table 1 Epidemiology of primary biliary cirrhosis: Results from the most relevant studies[2-9]

Area	Patients (No.)	Prevalence (per million)	Incidence (per million/yr)	Age (yr)	Gender (M:F)
Europe (1984)	569	23	54	54	1:10
Northern Sweden (1990)	111	151[1]	13.3	55	1:6
North East England (1990)	347	129[1]	19	58	1:9
Ontario, Canada (1990)	225	22	3.3	59	1:13
Victoria, Australia (1995)	84	19	-	-	1:11
Newcastle, England (1997)	160	240[1]	22	66	1:10
Olmsted County, MN (2000)	46	402[1]	27	-	1:8
Victoria, Australia (2004)	249	51[1]	-	61	1:9

[1]Data include survey of laboratories for antimitochondrial antibodies.

would be particularly expensive in view of the relatively low prevalence of the disease thus requiring large populations to be screened. At present, we must consider the prevalence indicated by case-finding studies as underestimates, to a degree inversely related to the accuracy of the methodology employed to identify the potential diagnoses made in the area under consideration. In Table 1, relevant data from the available epidemiologic studies are reported in chronological order[2-9].

Several difficulties however exist when attempting to compare results of these studies among each other, and over time. Heterogeneity in the methodology of case finding and, to a lesser extent, the criteria used for the diagnosis represent the most problematic issue. In particular, only a few studies used multiple strategies to reduce selection bias by capturing the entire spectrum of illness associated with PBC, especially cases at the preclinical stage[10]. Ascertainment from laboratory determination of anti-mitochondrial antibodies (AMA), which are highly sensitive and specific markers of the disease, has been a valuable approach. Differences in estimates of incidence and prevalence of PBC among populations, coming from the earlier studies[2,11-15], may be due to differences in diagnostic criteria and study design, as well as to the different disease awareness among physicians, and to the differing degrees of access to health care systems. Similarly the same limits may explain the lack of confirmation of preliminary observations of associations between the occurrence of PBC and environmental factors[12,16].

The methodological quality of reported investigations has improved over time which allows some capacity to compare incidence and prevalence rates by geographic areas. Initial studies published between 1974 and 1986 described annual incidence rate of PBC ranging from 0.6 to 13.7 cases per million[2,11,13-15]. Prevalence rates from these studies varied between 23 and 128 cases per million[2,11,13-15]. The majority of data originated from the United Kingdom and Sweden. Since 1989 a larger number of studies have been reported, mainly performed in Europe but also coming from Asia, North America, and Australia[3-10,16-31]. From these more recent studies, both the annual incidence rates and prevalence of PBC have increased[3-10,16-31]. In particular, from the United Kingdom the annual incidence rates increased from 5.8 to 20.5 cases per million between 1980 and 1999 among residents of Sheffield[12,28] and from 11 to 32 cases in Newcastle-upon-Tyne between 1976 and 1994[4,7,27]. A parallel increase of the prevalence rate occurred reaching the number of more than 200 cases per million in the middle-late nineties[4,7,27]. A similar picture has been reported by very recent studies coming from Europe[30,31]. These data may be explained by the progressively higher proportion of asymptomatic cases with early-stage disease, resulting in growing prevalence rates, and the increased use of biochemical and serologic testing leading to the increasing diagnosis of new cases per year. Interestingly the mean age at diagnosis did not change from initial to more recent studies (Table 1), thus indicating that the increasing prevalence and incidence reported by the literature is more related to wider rather than to earlier diagnoses.

Only recently, several epidemiological data are available also from the USA in full indicating an annual incidence rate of 27 cases per million with prevalence rates ranging between 160 and 402 cases per million, thus leading to an estimate of 3500 new cases each year with 47 000 prevalent cases among the white population[8]. However, these data come from specific regions and difficulties in obtaining more complete epidemiological evaluations are mainly due to two reasons: (1) the lack of an universal health care system; and (2) the large number of patients followed in secondary and tertiary centres. Lower prevalence and incidence have been reported in Canada and Australia[5,6,9,18,22].

For PBC there is a well known high prevalence of female gender (F/M 9 to 1), and based on this observation several studies provided greater insight into the aetiopathogenesis of the disease[32]. Little information is available regarding the influence of race or ethnicity on the descriptive epidemiology of PBC[1] indicating that host susceptibility plays a significant role in the development of the disease. PBC occurs more commonly among individuals with a family history of either PBC itself or other autoimmune disorders[33-35] and there is a high concordance rate (63%) *versus* that in other autoimmune diseases in monozygotic twins[36]. Taken altogether, these observations point towards the relevance of genetic factors in the occurrence of PBC. On the other hand, the recent finding of several clusters of PBC within defined spatial boundaries suggests that also environmental factors, such as pollution, may contribute to the development of the disease[37,38]. These associations are statistically extremely weak and may be flawed by quite a high number of biases of different types[39]. The role of a previous infection as the triggering factor for the development of PBC by the mechanism of molecular mimicry has been repeatedly suggested, in analogy with other autoimmune diseases, but data are inconsistent[40-42].

In conclusion, data coming from more recent surveys of diagnoses performed in different geographical areas

indicate that PBC is not a rare disease and its prevalence and incidence are apparently increasing in recent years mainly due to easier recognition of the disease and improved case finding strategies. No firm suggestion on the aetiologic role of any specific environmental factors has come from epidemiology, whereas familial clustering indicates a major role for genetic background.

DIAGNOSIS

The diagnosis of PBC is currently based on three criteria: the presence of AMA in serum which is highly specific for the disease, elevation of biochemical indices of cholestasis for more than 6 mo, and histological features in the liver that are indicative of the diagnosis. The presence of two of these criteria allows a probable diagnosis but for a definite diagnosis the occurrence of all criteria is needed[43]. However, alternative diagnoses of liver disease should be ruled out and particularly in the absence of detectable AMA, a nuclear magnetic resonance cholangiography is necessary to exclude a primary sclerosing cholangitis.

Determination of AMA using routine methods, however may lead to underestimation of their presence[44]. Up to 5% to 10% of patients have no detectable antimitochondrial antibodies, but their disease appears to be identical to that in AMA positive patients[45].

Serum liver enzymes are the earliest biochemical indices to increase in serum: gamma glutamyl transpeptidase, alkaline phosphatase, and aminotransferases in descending order of sensitivity, but each lacks specificity, except, to some extent, alkaline phosphatase, if bone disease can be ruled out. On the other hand, serum bilirubin concentrations increase only in advanced stages of the disease, and accurate measure of serum bile acid concentrations requires state of the art methods, like gas chromatography-mass spectrometry (GC-MS), which are not available routinely[46]. In addition, serum bile acids are extremely sensitive but poorly specific and their detection by GC-MS is more useful to study derangement of the bile acid circulation or the effects of therapeutic bile acids[47].

The utility of liver biopsy in the diagnosis of PBC has been questioned by several hepatologists[43] and even for staging purposes it is scarcely justified in patients who have obvious features of cirrhosis by clinical evaluation including imaging techniques.

HISTOLOGICAL FEATURES

The pathological lesion typical for PBC is a chronic non-suppurative destructive cholangitis involving interlobular bile ducts of 40-80 μm in diameter[48]. Overall, coexistence of portal inflammatory infiltrate with bile duct paucity is needed for diagnosis. PBC is divided into four histological stages but the liver is not affected uniformly and even a single biopsy sample may demonstrate the presence of different stages of the disease. If this is the case, the most advanced stage of those present is assigned, according to convention[43]. Stage 1 is characterized by localization of inflammation to portal triads. Stage 2 entails extension of inflammation beyond the portal triads into the lobular parenchyma and reduction in number of normal bile ducts. Stage 3 entails fibrous septa linking adjacent portal tracts. Stage 4 is the most advanced histological stage in which liver cirrhosis has occurred[49].

Table 2 Modifications during time of the clinical spectrum of primary biliary cirrhosis at presentation[50-52]

	Sherlock 1973 (*n* = 100)	James 1981 (*n* = 93)	Nyberg 1989 (*n* = 80)
Jaundice (%)	28	16	3
Pruritus (%)	57	14	26
Complications (%)	4	9	1
Asymptomatics (%)	11	61	70
Mean age (yr)	50	57	58

CLINICAL FEATURES

Symptoms

Asymptomatic disease: PBC is now diagnosed earlier in its clinical course and most cases are only slowly progressive in comparison with the past, and the large majority of patients are asymptomatic at diagnosis (Table 2)[50-52]. It has been suggested that symptoms develop within five years in most asymptomatic patients, although one third of patients may remain symptom-free for many years[53,54]. Pruritus and fatigue are early symptoms and occur in about 20% of the patients[53,55].

Fatigue: This is reported in up to 78% of PBC patients overall and is suggested to be a significant cause of disability from numerous studies[56-59]. However, a well-preserved quality of life has been recently reported in a very large cohort of patients with PBC in the USA thus arguing against the clinical relevance of fatigue in such a population[60]. Several studies have explored the pathogenesis of this symptom and indicated heterogeneous mechanisms ranging from autonomic dysfunction[59,61,62], to excessive daytime somnolence[63], and to altered manganese homeostasis within the central nervous system[64], while concomitant depression could not be ruled out[65-67]. In addition, studies aimed at demonstrating the clinical relevance of fatigue in PBC are affected by significant flaws, since the correlation of inaccurate quantification of the symptom with both scores related to quality of life and clinically relevant events appears to be inappropriate, and a possible role of concomitant diseases could not be excluded[56-59]. Therefore fatigue seems a poorly specific symptom and a predominant psychogenic component is likely, as usually occurs in carriers of a chronic progressive illness who are aware of the potential impact on their future life.

Pruritus: This appears to be the most typical symptom of PBC. It was reported to occur in 20% to 70% of patients and occasionally is quite distressing[68]. In latter years its frequency in PBC has been decreasing because

the disease is increasingly recognized in its asymptomatic stage. The availability of therapeutic options such as UDCA which has been widely administered during the last two decades, seems to have also modified the occurrence and intensity of this symptom. The onset of pruritus generally precedes the onset of jaundice by months to years. The cause of pruritus remains unknown. However there is consensus that in the course of cholestasis biliary excretion of several compounds is impaired, thus leading to increased systemic concentrations of a putative "pruritogenic" compound. The occurrence of pruritus would result from the interaction between these substances and nervous terminations at the skin level. The extreme variability of the degree of pruritus between patients, or even in the same patient, may have two explanations: (1) inter-individual or time variability of the systemic concentrations of the "pruritogenic" compounds, which are generally confined within the enterohepatic circulation; and (2) subjective variability of the perception of pruritus, mainly due to psycho-emotional factors. Increased serum concentrations of bile acids are associated with cholestasis by definition, and a direct causative relationship between increased bile acid concentrations and the occurrence of pruritus has been suggested[69]. Several observations support this hypothesis, including: (1) the presence of bile acids in the skin in cholestatic patients[70]; (2) the capability of bile acids to produce pruritus when injected subcutaneously[71,72]; (3) the relief of pruritus by external biliary drainage, and by cholestyramine which can bind bile acids and thus favours their fecal elimination[73-75]. However, this hypothesis has never been proven since no relationship was found between degree of pruritus and bile acid levels measured in cutaneous interstitial fluid[76-78]. In addition, it is possible that many other substances are eliminated during both biliary drainage and cholestyramine administration.

The hypothesis that pruritus in cholestatic liver disease may have a central origin has been suggested by the observation of an increased opioidergic activity in both experimental models of cholestasis[79-82] and in cholestatic patients[79-81,83], and by the observation that opioid receptor ligands with agonist properties (morphine for example) mediate pruritus[84-86]. Therefore, there have been studies using opioid antagonists for the treatment of pruritus in cholestatic conditions with positive results[87-89], thus confirming the hypothesis that an increased opioidergic activity plays a role in the occurrence of pruritus associated with cholestasis. In cholestatic conditions high concentrations of bile acids in the systemic circulation may alter several central regulatory systems such as the opioid-mediated system.

Portal hypertension: This may occur even before cirrhosis develops. However, usually, ascites, variceal bleeding, and hepatic encephalopathy complicate the course of PBC only in advanced stages. Similarly, the incidence of hepatocellular carcinoma is elevated among patients with long-standing histologically advanced PBC[90].

Consequences of long-standing cholestasis

Other common findings in advanced PBC include the consequences of long-standing cholestasis that can lead to hyperlipidemia, fat malabsorption, renal tubular acidosis, and osteopenia. However, the clinical relevance of hyperlipidemia in patients with PBC remains questionable since neither cardiovascular risk[91] nor more precocious signs of atherosclerosis[92] are associated with alterations of lipid metabolism in PBC. In addition, the wide use of therapeutic bile acids in the last decade may have modified the metabolic pattern of plasma lipids in PBC[93,94].

Metabolic bone disease described in patients with PBC is the result of two different pathological processes: osteomalacia and osteoporosis. Osteomalacia which is a consequence of lipid malabsorption may be easily corrected by supplementation with calcium and vitamin D[95-97]. The changing spectrum of bone disease associated with cholestasis with a progressive disappearance of osteomalacic features over time may be due to the increasingly wide use of vitamin D and calcium supplementation in clinical practice[97,98]. Therefore, at present, osteoporosis is the predominant component of metabolic bone disease[98]. During end-stage liver disease, which is characterized by reduced physical activity, malnutrition, and, possibly, infectious complications, bone loss is a major clinical issue[99]. On the other hand there is no consensus on the clinical relevance of cholestasis in inducing bone loss at less advanced stages of liver disease[100]. In a recent longitudinal controlled study, we demonstrated that cholestasis was not an additional risk factor for bone demineralization in women with well-compensated PBC if adequate calcium and vitamin D supplementation had been provided[101]. These data are in accordance with several studies[102-104] but in contrast with others[105-107]. Different results may be due to: (1) the cross-sectional nature of many studies; (2) the lack of an adequate control group in the majority of the published studies so precluding the protection against confounding factors such as menopausal status, which is important in a population wherein perimenopausal women are largely represented; (3) the lack of adequate vitamin D and calcium supplementation in most of the published studies; and (4) the confounding effects of other concomitant medications.

Malabsorption, deficiencies of fat-soluble vitamins, and steatorrhea are uncommon except in the late stages of the disease[108]. Finally, the occurrence of renal tubular acidosis which was once thought to be quite frequent[109] was not found in a large population of PBC in the absence of complication of liver cirrhosis[110], thus indicating that such a complication, if present at all, may be restricted to very late stages of the disease in association with multiorgan dysfunction.

Associated diseases of autoimmune type

Symptoms of coexisting autoimmune diseases including Sjogren syndrome, scleroderma, rheumatoid arthritis autoimmune thrombocytopenia, and haemolytic anaemia may be present. Interestingly, liver disease was recently shown to have a slower progression when systemic

sclerosis is associated with PBC compared with matched patients with PBC alone[111]. Overlap syndromes with autoimmune hepatitis are described in another article in this issue.

NATURAL HISTORY AND PROGNOSTIC MODELS

The natural history and prognosis of PBC have become more difficult to characterize given the rising number of asymptomatic cases which require long-term follow-up[1,43,109,112]. Furthermore, patients are more likely than in the past to be asymptomatic at diagnosis[1,43,112] and to receive medical treatment as soon as diagnosis is made. Hence, estimated survival has significantly improved compared to the past. Earlier data on survival suggesting a poor outcome were obtained from patients in whom the disease had been diagnosed many years ago when no effective treatment existed[1,43,112]. In addition, most of these patients were symptomatic[1,43,112].

A different outcome of the disease has been reported for symptomatic *versus* asymptomatic patients. In 1983, the reported survival of asymptomatic PBC patients was similar to that of a normal U.S. population matched for age and sex[113], but, when their survival data were extended for a longer duration, the asymptomatic patients had a shortened survival compared with controls[114]. In this latter study, 279 patients from the USA were observed for up to 24 years, and the median survival of asymptomatic PBC patients was significantly longer than symptomatic patients at presentation[114]. Additional studies confirmed that initially asymptomatic patients had a longer survival than symptomatic ones[109,115]. In one of these studies from Canada, asymptomatic PBC patients had a shortened survival compared with a healthy population[115]. The results described in a community-based study from the UK are at variance with all of the other reports[54]. Here 770 patients (61% asymptomatic) living in England were diagnosed between 1987 and 1994 and observed until death, transplantation, or until data were censored in January 2000. The median survival was similar in asymptomatic and symptomatic patients, and symptom development was not associated with shorter survival. However, the design of this study, in which patients were followed by regular interview and by examination of their medical records may be not as informative as a single centre cohort study to assess the natural history of PBC, even though it is sufficient for epidemiological purposes. In fact, these UK results are confounded by the fact that 45% of the deaths in asymptomatic patients occurred while these patients were remained asymptomatic, suggesting that many of these patients would have been died of non-hepatic causes and that age at diagnosis was a major determinant of survival. Since the prognostic relevance of the presence of symptoms is well documented, the higher proportions of asymptomatic patients enrolled in the more recent cohort studies explain, partly at least, the observed improvement in the natural history of PBC since 1980s.

Table 3 Parameters independently associated with bad prognosis in different prognostic models based on a single point observation[16,113,119,121-123]

Parameters	Yale	European	Mayo	Glasgow	Oslo	London
Increase in serum bilirubin	+	+	+	+	+	+
Decrease in serum albumin		+	+			+
Increase in PT (INR)			+			
Advanced age	+	+	+	+		+
Hepatomegaly	+					+
Ascites, fluid retention			+	+		+
Esophageal varices						+
Gastrointestinal bleeding				+	+	
Cirrhosis	+	+		+		+
Cholestatic picture at histology		+		+		
Mallory bodies				+		

Most patients with PBC are now treated with UDCA[43] and the widely used administration of this drug has greatly changed the natural history of the disease[43,112]. At least 20% of patients treated with UDCA will have no histologic progression over four years, and some will have no progression over a decade or longer[116]. In a recent study, the survival rate of patients with stage 1 or 2 disease given UDCA long-term was similar to that of a healthy control population[117]. In the above-mentioned community-based study from the UK no improvement in survival was found in UDCA-treated patients[54]. We reiterate that such a study design albeit excellent for epidemiological purposes, is not adequate for the evaluation of the effects of medical treatment. In addition, there is sufficient evidence that UDCA treatment does prevent the development of esophageal varices[118]. Therefore, sufficient information is now available to indicate that, among the reasons for the improving prognosis of PBC, is the wide use of bile acid therapy. Detailed information on the effects of UDCA therapy on survival is described below.

Cox proportional hazards regression analysis has been used to develop prognostic models. There are different prognostic models for predicting survival for PBC patients. Of these models, the Mayo survival model is the most popular. The Mayo model was based on combined data from more than 400 patients who were observed at the Mayo Clinic and was then externally cross-validated using PBC patients from other medical centers[119,120]. The Mayo model uses five independent prognostic variables: age, total serum bilirubin, serum albumin, prothrombin time, and the severity of fluid retention. Serum bilirubin is the most heavily weighted among these variables, consistent with the presence of this index in all the proposed prognostic models[16,113,119,121-123] (Table 3). All these models are based on a single assessment but several have been modified to include repeated measures of prognostic indices[121,124,125]. The Mayo model has been widely used to assess the efficacy of medical treatment in clinical trials, but also serum bilirubin concentrations

have been similarly used as surrogate markers of disease improvement, due to the prognostic value of this index in PBC patients with more advanced disease[126].

Recently, also an immune marker was shown to be of prognostic value since a particular specificity of antinuclear antibodies that directed against nuclear pore complex, identified patients destined to experience more rapid disease progression[127].

TREATMENT OF SYMPTOMS AND COMPLICATIONS

Fatigue

No therapy that has been evaluated for the treatment of PBC has proven able to ameliorate fatigue[128-130]. However, this symptom is not specific, only indirect quantitative measurement is available, and there are no convincing data to support any organic pathophysiological mechanism with even a psychological basis possible in some cases[65-67].

Pruritus

Pruritus in several, albeit very rare, cases may severely affect the quality of life, leading to sleep disturbance and major depression. This is the reason why intractable pruritus has been considered an indication for orthotopic liver transplantation (OLT). A large number of pharmacological approaches have been tested on the basis of both pathophysiologic considerations and serendipitous observations. The heterogeneity of the treatments suggested reflects the difficulties in treating this symptom which is extremely variable in severity and type, influenced by subjective factors and not easily quantifiable. The administration of UDCA, the only approved treatment for PBC, was not associated with a consistent improvement of pruritus in most controlled clinical trials; however, since the majority of them were not designed specifically to test the effects of this drug on pruritus, no definite conclusion can be drawn. In addition, as reported above, epidemiological data indicate that the disease expression has changed during the last two decades towards less symptomatic disease[1,43,112], and a possible effect of the widely administered UDCA in decreasing pruritus certainly cannot be ruled out.

The oral anion exchange resin cholestyramine has been the mainstay of therapy for pruritus associated with cholestasis[73-75]. The mechanism of action is related to binding of bile acids and other biliary molecules, with their subsequent fecal excretion. Dose of cholestyramine should start from 4 g daily and should be increased, in case of therapeutic failure, until a maximum of 16 g. The timing of administration is before meals. The drug is more effective in those patients with an intact gallbladder when taken before and after breakfast, because the greatest amount of bile is likely to be available for binding at this time. Since cholestyramine binds also other medications, notably UDCA, oral contraceptive hormones, digoxin and thyroxin, it is advisable that at least 4 h should elapse between the administration of cholestyramine and other medications. In the majority of cases this drug is effective within a few days from starting treatment, but in about 10% to 20% of the patients it is ineffective. In addition, many patients find cholestyramine unpleasant to take and complain of dyspeptic symptoms or diarrhea or, alternatively, constipation so leading to poor compliance with treatment.

Table 4 Pharmacological characteristics of the opiate antagonists investigated in clinical studies

	Pharmacological characteristics
Naloxone	Very short half life Intravenous continuous infusion Dose: 0.2-0.4 μg/kg per minute
Nalmefene	Longer half life Oral administration 2 mg twice/d with a gradual increase until 20 mg twice/d
Naltrexone	Longer half life Oral administration 50 mg/d (in two divided doses the first day and subsequently in a unique dose)

Rifampicin is an enzyme-inducing antibiotic which was serendipitously identified as an agent that improves pruritus in cholestasis[131]. A subsequent crossover trial indicated that the drug provided good control of pruritus in PBC at doses of 150 mg twice per day or three times per day[132]. In subsequent studies higher doses were used up to 600 mg/d[133] and 10 mg/kg per day[134]. Its mechanism of action remains unknown but it may alter bile acid composition[135,136] and stimulate the hepatobiliary transport systems[137,138]. When given long-term, rifampicin was shown to improve also the biochemical expression of PBC[139]. However, it is not effective in all patients and may cause side effects[140]. Two cases of acute hepatitis were reported (12.5% of treated patients) during long-term administration[139], but this spontaneously resolved after discontinuation of treatment. In any case, the potential hepatotoxicity of rifampicin precludes long-term administration of this drug to patients with PBC.

Many studies endorse the use of opioid antagonists, given intravenously or orally, for the treatment of cholestasis-related pruritus[87-89]. The main pharmacological characteristics of the three compounds investigated clinically are reported in Table 4. Each compound was shown to be highly effective in improving pruritus, but the main limit on their use was the occurrence of withdrawal-like symptoms in several patients. In addition, after initial enthusiasm following elegant studies supporting the intriguing hypothesis of an increased opioidergic activity in cholestatic patients[79-82], opioid antagonists have lapsed for the treatment of pruritus. Larger and longer studies are needed to fully assess the actual clinical value of opioid antagonists in controlling pruritus in PBC.

Since the serotoninergic system participates in the mediation of nociception, it appears rational to use drugs acting on this system. Several studies suggested that a possible beneficial effect may be exerted by ondansetron a type Ⅲ serotonin antagonist[141-143], but subsequent studies showed only limited or no effects on pruritus[144-146]. Surprisingly, the results of a recently published small randomized, double-blind, placebo-controlled trial based

on a heterogeneous group of patients with pruritus and liver disease suggested a beneficial effect of sertraline, a serotonin reuptake inhibitor[147]. Finally, since the cannabioidergic system plays a role in the mediation of nociception, uncontrolled observations on the effects of dronabinol, a cannabinoid B1 receptor, suggested relief of pruritus in course of cholestasis[148].

In conclusion, since UDCA is the only accepted therapy for PBC, this bile acid represents the treatment of choice for pruritus. If the symptom persists, cholestyramine be initiated. Only in the case of a lack of response to maximal doses of cholestyramine a therapeutic approach with rifampicin or opioid antagonists should be considered.

Metabolic bone disease

Osteomalacia may be easily corrected by parenteral supplementation of vitamin D (vitamin D_3 100 000 UI intramuscular monthly). Supplementation with calcium carbonate (1 g/d) has been largely recommended based on pathophysiological considerations and on data coming from experience in postmenopausal osteoporosis whereas only indirect evidence is available in PBC patients[97,149].

As reported above, it is highly questionable whether osteoporosis during cholestatic conditions represents a separate clinical entity[100,101]. Therefore the available data on treatment of metabolic bone disease in PBC are similar to those reported for postmenopausal osteoporosis noting that most patients with PBC are females at a menopausal age. Various data indicate that hormone replacement therapy is effective and safe, contrary to previous beliefs[150-154]. Etidronate was suggested to be effective[155,156], but not all studies reported positive results[157], while alendronate was shown to be superior[158,159]. Calcitonin failed to improve bone mineral density in female patients with PBC[149]. The negligible improvement observed in one study[160], is perhaps attributable to concomitant vitamin D and calcium supplementation. Several indications for the clinical management of metabolic bone disease associated with PBC are reported in Table 5. Finally it should be highlighted that UDCA, the specific treatment for PBC was shown to have no effects on the occurrence of bone loss[161].

Table 5 Clinical management of metabolic bone disease associated with primary biliary cirrhosis

Clinical management	Efficacy	
	Moderate efficacy	Mild efficacy, insufficient data
Prevention		
1 Parenteral vitamin D3 supplementation 2 Calcium carbonate supplementation	Indicated for all patients to prevent osteomalacic lesions	
Treatment		
1 Estrogen		Few data but effective and safe
2 Etidronate		Conflicting data Indicated in case of concomitant corticosteroid administration
3 Alendronate		Few data but effective and safe
4 Calcitonin		Probably ineffective

Hyperlipidemia

It is still questionable if hypercholesterolaemia associated with PBC should be treated, and which patients need pharmacological treatment. Since increased cholesterol concentrations associated with cholestasis do not increase the atherosclerotic risk, it seems reasonable to treat hypercholesterolaemia only when hyperlipidemia of familial and nutritional origin probably coexists[162]. The extent of cholesterol reduction by UDCA administration[93] may be insufficient to protect this group of patients from cardiovascular risk. These patients probably would benefit from dietary modifications, weight loss, and the administration of specific lipid-lowering drugs. Cholestyramine may be indicated for its cholesterol lowering capacity in hypercholesterolaemic patients, especially if there is associated pruritus, while HMGCoA-reductase inhibitors should be limited to hypercholesterolaemic patients in whom serum levels of HDL are below the protective range, or if additional risk factors for cardiovascular disease are present[162]. In pilot studies, both simvastatin and atorvastatin proved to be safe and effective in reducing serum cholesterol levels in patients with PBC[163-165].

Malnutrition

During severe cholestasis, which occurs only at very advanced stages of PBC when liver transplantation is precluded, lipid malabsorption occurs with steatorrhoea and weight loss. In such cases a reduction to 40 mg of the daily dietary fat intake is indicated and the same amount should be administered as medium chain triglycerides, which are digested and absorbed in the intestine even in the presence of low bile acid concentrations. In several cases administration of cholestyramine has to be discontinued.

Since malabsorption of lipophilic vitamins occurs even in the absence of clinically evident steatorrhoea, preventive supplementation with vitamin D may be advisable in case of significant alterations of biochemical markers of cholestasis. Parenteral vitamin K supplementation should be given if prothrombin time is increased.

SPECIFIC TREATMENT FOR PBC

Many therapeutic agents have been tested for PBC but difficulties have been encountered in establishing statistically significant long-term benefits for a disease with such a variable natural history. In addition, PBC surrogate markers of prognosis have several limitations: impairment of indices of liver synthetic function occurs only at very advanced phases of the disease, and the likelihood of sampling errors limits the value of liver histology. The only index which may be useful to assess prognosis is serum bilirubin, and this only in late phases of the disease. Randomized, controlled trials, recently

Table 6 Efficacy and toxicity of the principal drugs investigated for the medical treatment of primary biliary cirrhosis

	Efficacy	Toxicity
D-penicillamine	-	+
Chlorambucil	+/-	+
Cyclosporine	+/-	+
Azathioprine	+/-	+
Methotrexate	+/-	+
Colchicine	+/-	-
Glucocorticoids	+/-	+/-
UDCA	+	-

re-evaluated by a meta-analysis[166], have endorsed the failure of penicillamine. The only accepted treatment for PBC is UDCA that may delay but not halt the progression of the disease[167]. For several other agents, mainly immunosuppressive components, some interesting possibilities have been revealed but mainly in terms of combination treatment with UDCA. Data are summarised in Table 6. Regarding corticosteroid drugs, data are scanty mainly because bone demineralization represents a big concern in a population of female patients at postmenopausal age[168,169]. Corticosteroid monotherapy does not seem to offer a sufficient benefit *versus* side effects ratio for most PBC patients and its use should be limited to patients with other concomitant autoimmune diseases or with a PBC-autoimmune hepatitis overlap syndrome[170]. In such cases, co-administration of etidronate may prevent bone loss[156].

Azathioprine administration should not be recommended on the ground of a limited efficacy and the substantial risk of side effects[121,171,172]. For chlorambucil, the frequency and potential severity of side effects outweighs potential benefits of this immunosuppressive drug, thus contraindicating its use in PBC[173]. After preliminary encouraging data coming from a pilot study[174], Kaplan and colleagues have repeatedly reported biochemical and histological improvement after the administration of low dose of methotrexate (15 mg/wk), but no data on survival have been presented[175,176]. Aside from potentially serious complications[177], the beneficial effects of methotrexate in the treatment of PBC, alone or in combination with UDCA, could not be confirmed by randomized, controlled trials performed by other groups[178-180]. There is no indication for the clinical use of cyclosporine in PBC, given the limited efficacy and known side effects[181].

Available information indicates that colchicine with its anti-inflammatory and antifibrotic properties may exert limited beneficial effects on the natural history of PBC but without relevant side effects[182-185]. This is the reason why it has been largely tested in association with UDCA but showing no additional benefit in terms of clinically relevant end-points in comparison with UDCA monotherapy[186,187].

UDCA for the therapy of PBC

The rationale for the use of UDCA in the treatment of PBC depends on its ability in displacing and/or diluting detergent and hepatotoxic bile acids from the bile acid pool. It is well known that in cholestatic conditions, endogenous bile acids are retained within hepatocytes, thus leading to the progressive deterioration of liver function. The beneficial effects of UDCA on indices of liver dysfunction have been attributed to its physicochemical properties, since UDCA is very hydrophilic and therefore non-toxic to biological membranes[188,189]. However, experimental data failed to support this hypothesis since a substantial shift towards hydrophilicity of the bile acid pool was not observed during UDCA administration[47]. It has been suggested that UDCA has a direct cytoprotective effect, and different molecular mechanisms may be responsible, such as regulation of cellular signalling systems and protection against apoptosis[190]. Immunomodulatory effects of UDCA have been also described[190], although it is not conventionally used as an immunosuppressive drug in non-hepatic diseases.

A number of randomized controlled studies have been conducted to evaluate UDCA efficacy[43]. In all studies UDCA was well tolerated since no relevant side effects were reported. In all studies a significant improvement of serum liver enzymes markers of cholestasis and cytolysis occurred. Serum concentrations of bilirubin, the most important prognostic marker of the disease, were reduced by UDCA administration. A consistent reduction of IgM, which is an immunological marker of PBC was also reported.

Results of randomized placebo-controlled trials with a duration long enough to evaluate the effects on histology and on survival are summarized in Table 7[191-197]. Among the six studies that evaluated the effects of UDCA on pruritus[191-196], an improvement was described in only three[191,194,196], but these studies were not specifically designed to assess pruritus. In four studies a significant improvement of several histological indices was reported[191,192,194,196]. The Mayo Clinic group did not report any improvement of liver histology, but have suggested in a separate paper that UDCA delays the occurrence of esophageal varices[118], thus indicating a positive effect on the progression of the disease.

To evaluate the effectiveness of a specific therapy for a severe life-threatening disease, the effects on survival should be explored. However, since PBC is a relatively uncommon disease with a long and variable natural history, a very large sample size and a very long follow-up are needed to obtain reliable data. No effect on survival was observed in any of the single studies reported in Table 7, and only after an extension of follow up was a positive effect on survival without OLT reported by the French and the Mayo Clinic studies[198,199]. During the 2-year extension of the French study all patients administered placebo were switched to UDCA, while in the Mayo Clinic study, UDCA was offered to all patients but, for the analysis, follow-up was censored at the end of the randomized phase for patients initially assigned to the placebo group, thus avoiding the limits of a switch-over design.

A combined analysis of three studies[167] and two meta-

Table 7 Randomized, double-blind, placebo-controlled trials on ursodeoxycholic acid administration to patients with primary biliary cirrhosis

First author	No. of patients	Study design and duration of follow up	UDCA effects on		
			Pruritus	Histology	Survival
Poupon[191]	146	2 yr	Improved	Improved	No effect
Heathcote[192]	222	2 yr	No effect	Improved	No effect
Lindor[193]	180	Mean follow up: 2 yr	No effect	No effect	No effect
Combes[194]	151	2 yr	Improved	Improved (early stages)	No effect
Eriksson[195]	116	2 yr + 2 yr as open trial (UDCA)	No effect	No effect	No effect
Pares[196]	192	Mean follow up: 3.4 yr	Improved	Improved	No effect
Papatheodoritis[197]	86	Mean follow up 7.3 yr for UDCA 8.1 yr for controls	Not evaluated	No effect	No effect

analyses[200,201] have been performed, since the majority of the published studies had insufficient statistical power to explore the effects of UDCA on survival. The combined analysis was obtained by pooling of results from three trials with similar designs but dissimilar results. The analysis included 548 patients and a significant improvement of survival free from OLT was reported with the relative risk of death being 0.53 (0.36-0.77; 95% CI). A significant improvement of survival could be recorded only in patients with serum bilirubin higher than 1.4 mg/dL at baseline. The lack of an effect on survival in patients with less severe disease may well indicate that the time of observation was not sufficient to detect effects of UDCA in a population with a low probability of developing clinically relevant events. On the other hand, results of the two meta-analyses indicate no effects of UDCA on the natural history of the disease. Formal meta-analysis includes consideration of all relevant trials, justifies eventual exclusion of trials from the analysis, and explores heterogeneity between trials and the reason for variation in results. The main limit of a meta-analysis is that trials evaluated may be too different in their designs to be truly comparable. The reason for the opposite results reported by the combined analysis[167] and by the two meta-analyses[200,201] remains unclear. The main criticisms directed against the combined analysis were the limits of the switching over design, but the "intention to treat" basis of the analysis is protective against type I error, thus reducing the probability of demonstrating benefits of UDCA in the absence of a true beneficial effect. Conversely, the inclusion in the meta-analyses of studies using low doses of UDCA, and with a follow-up too short for assessment of effects on clinically relevant end-points, has been strongly criticized. The effects on surrogate markers of clinical outcome, such as serum bilirubin concentration, do indicate that UDCA may positively affect survival in PBC. In addition, the UDCA safety and its relatively low cost permit a wide scale use of this therapeutic bile acid.

So, in conclusion, our opinion is that UDCA does exert a favourable effect on the natural history of PBC, but since many studies had been characterized by an insufficient number of patients, insufficiently long follow-up periods, heterogeneity of evaluated indices, and inadequate study designs, an absolutely clear-cut demonstration of benefit was precluded. Indirect data on the beneficial effects of UDCA also in patients at the initial stages of the disease are now available[117,202]. An excellent long-term survival, comparable to that observed in a control population, has been recently reported in patients with PBC showing biochemical response to UDCA[202]. These data were obtained by studying a cohort of 192 patients, mainly with stage 1 and 2 of the disease, who had been treated for a mean period of more than 6 years. In addition, in a recent study of 262 patients with PBC who received UDCA for a mean of 8 years, the survival rate of patients with stage 1 or 2 disease was similar to that of a healthy control population[117]. However, not all patients have a response to treatment, since in the same study, the probability of death or undergoing OLT in patients with stage 3 or 4 of PBC was significantly increased compared with a healthy population, despite UDCA treatment. Therefore strategies aimed at improving therapeutic agents for PBC are still needed, mainly by the use of associated treatments.

Several drugs have been tested in association with UDCA. The results obtained with colchicine, and budesonide are the more promising but none of the drugs studied was shown to provide any additional benefit, in terms of clinically relevant events, compared to UDCA monotherapy[186,187,203-205].

OLT for the therapy of PBC

Finally, OLT has greatly improved survival in patients with PBC since this is the only effective treatment in patients with very advanced disease. 'The survival rates are 92% and 85% at 1 year and 5 years, respectively[206]. While the recurrence rate is 30% at 10 years[207]. Note that OLT is considered in detail in another article in this series.

CONCLUSION

Data coming from the more recent epidemiological studies indicate that PBC is not a rare disease and its prevalence and incidence are apparently increasing. In addition, the clinical presentation of PBC has progressively changed from a highly symptomatic disorder with a bad prognosis to a slowly evolving disease. The changing methods used for the diagnosis, with an increasingly wide assessment of laboratory indices related to both cholestasis and immunology, together with improved case finding strategies, may explain these observations.

As a result, the recognition of earlier more indolent

cases led to the presence of a substantial proportion of asymptomatic patients within PBC cohorts. Therefore development of early prognostic indices may be useful to predict which patients are destined to develop a progressive disease thus requiring a more intensive follow-up.

UDCA does not act on the aetiology of the disease but reverses the detrimental effects of the retention of endogenous bile acids within the liver. Although several flaws of the available studies prevented a clear-cut demonstration of its efficacy, many indirect observations suggest that a beneficial effect occurs and we cannot exclude that the wide use of UDCA may have significantly changed the clinical course of the disease. However, UDCA is able to slow but not to halt the progression of the disease and, in advanced stages, when the large majority of bile ducts have been destroyed, OLT remains the only therapeutic option.

In the future, reliable epidemiological data to be obtained by screening the entire population at risk, will provide both a correct measurement of the real prevalence and incidence of PBC and a greater insight into aetiology and pathogenesis, thus leading to the possibility of a specifically targeted therapy.

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TOPIC HIGHLIGHT

Pietro Invernizzi, MD; Ian R Mackay, MD, *Series Editors*

Etiopathogenesis of primary biliary cirrhosis

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Abstract

Primary biliary cirrhosis (PBC) is an autoimmune disease of the liver characterized by progressive bile duct destruction eventually leading to cirrhosis and liver failure. The serological hallmark of the disease is the presence of circulating antimitochondrial antibodies (AMA). These reflect the presence of autoreactive T and B cells to the culprit antigens, the E2 subunits of mitochondrial 2-oxo-acid dehydrogenase enzymes, chiefly pyruvate dehydrogenase (PDC-E2). The disease results from a combination of genetic and environmental risk factors. Genetic predisposition is indicated by the higher familial incidence of the disease particularly among siblings and the high concordance rate among monozygotic twins. Environmental triggering events appear crucial to disrupt a pre-existing unstable immune tolerance of genetic origin allowing, after a long latency, the emergence of clinical disease. Initiating mimotopes of the vulnerable epitope of the PDC-E2 autoantigen can be derived from microbes that utilize the PDC enzyme or, alternatively, environmental xenobiotics/chemical compounds that modify the structure of native proteins to make them immunogenic. A further alternative as a source of antigen is PDC-E2 derived from apoptotic cells. In the effector phase the biliary ductular cell, by reason of its proclivity to express the antigen PDC-E2 in the course of apoptosis, undergoes a multilineage immune attack comprised of $CD4^+$ and $CD8^+$ T cells and antibody. In this article, we critically review the available evidence on etiopathogenesis of PBC and present interpretations of complex data, new developments and theories, and nominate directions for future research.

Key words: Autoantibodies; Autoreactive T cells; 2-oxoacid dehydrogenase; Biliary epithelial cells; Primary biliary cirrhosis

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INTRODUCTION

Primary biliary cirrhosis (PBC) is a chronic cholestatic liver disease of autoimmune origin characterized by a striking female predominance, high titer serum anti-mitochondrial autoantibodies (AMA), disease-specific antinuclear autoantibodies (ANAs), and an autoimmune-mediated destruction of the small and medium size intrahepatic bile ducts[1]. PBC is a peculiar, yet representative, organ-specific autoimmune disease. The presence of serum AMA and autoreactive T and B cells, in conjunction with the co-occurrence of other autoimmune diseases, all point to an autoimmune pathogenesis for PBC. Although most patients with PBC have AMA against the E2 subunit of the mitochondrial pyruvate dehydrogenase complex (PDC), there is no direct correlation between the titer of AMAs and disease severity. However, certain disease-specific antinuclear antibodies (ANAs) are present in about one third of patients and these carry a risk for more severe and progressive disease[2].

A multifactorial genetic background is suggested by a higher incidence of the disease among first-degree relatives[3], by the high concordance rate among monozygotic twins[4], and by an apparent role for X chromosome defects in PBC, based on the observation that women with PBC have preferential loss of one X chromosome

in peripheral white blood cells[5,6]. A vital question in the pathogenesis of PBC is why biliary epithelial cells (BEC) in particular are the primary target of pathology despite the ubiquitous presence of the PDC autoantigen in all tissue cells. Recent studies suggest that enhanced apoptosis in BEC is a critical step in ductular destruction in PBC[7,8], and some clues exist on mechanisms by which apoptosis in BECs cause the tissue-specific autoimmune reactivity characteristic of PBC.

GENETICS IN PBC

It is currently accepted that PBC pathogenesis is multifactorial, with genetic and environmental factors interplaying to determine disease onset and progression. Although the etiology of PBC remains enigmatic, there are several items of data indicating that genetic predisposition contributes strongly to the overall pathogenesis of PBC. The lines of evidence are these: (1) Data from monozygotic twins indicate that the concordance rate of PBC in monozygotic twins is 63%[4], among the highest reported for autoimmunity; (2) Approximately 6% of patients with PBC have a first-degree relative that also suffers from PBC[3]; (3) There is a high female:male disease incidence ratio (8:1), with suggestions of a significant role for X chromosome defects in PBC, based on the observation that women with PBC have a significantly enhanced monosomy X frequency in peripheral white blood cells compared to age-matched healthy women[5] and that the X chromosome loss is preferential[6]. Interestingly, similar genetic defects were also found in women with systemic sclerosis and autoimmune thyroid disease[9], but not with systemic lupus erythematosus[10]. Future studies should assess whether haploinsufficiency for specific X-linked genes may lead to loss of tolerance; (4) PBC is exceptional among autoimmune diseases in having controversially variable associations with alleles of the major histocompatibility complex (MHC, HLA); only a weak and regional association with HLA *DRB1*08* has been widely confirmed[11], although there is growing evidence on a protective association with HLA *DRB1*11* and **13*[12,13].

Several association studies have attempted to identify gene loci associated with PBC but no family study of genetic linkage has been performed. Associations are often not applicable to all populations but available evidence suggests that a "multi-hit" genetic model might apply to PBC, with different genetic variants conferring initial susceptibility, and others influencing subsequent disease progression. Genetic influences operative in PBC may reflect mutations transmitted through germline genes, or conceivably, somatic mutations in hemopoietic precursor cells[14].

In summary, a susceptible genetic background is considered to be necessary, but is not sufficient to explain either PBC onset or the strong female predominance[15]. Thus several environmental factors have been invoked as additional elements in tolerance breakdown.

ENVIRONMENTAL FACTORS

Bacterial infection in various settings has been repeatedly invoked in the etiopathogenesis of PBC. This etiology is usually linked to the concept of molecular (epitope) mimicry. The cross-reactivity of AMA with prokaryotic antigens has been reported for a number of microbes. This cross-reactivity is not particularly surprising given the conserved sequence of PDC-E2 across all species, from eubacteria to mammals.

We provided experimental evidence suggesting that *Novosphingobium aromaticivorans*, a ubiquitous xenobiotic-metabolizing Gram-negative bacterium, is the best microbial candidate yet for the induction of PBC[16,17]. Briefly, we can extrapolate theories on microbial molecular mimicry in PBC as follows. The microbial motif CpG enhances IgM production in peripheral blood mononuclear cell cultures, with CD27+ memory B cells in PBC patients being responsible for this IgM production through Toll-like receptor (TLR) 9 signaling. Also, CpG can stimulate AMA production and expression of TLR9, CD86, and one of the potassium channels, KCa3.1, in B cells of PBC patients. Moreover upregulated expression of TLR9 and CD86, and AMA secretion induced by CpG, can be suppressed by a specific blocker of the KCa3.1 channel, namely TRAM. These data indicate that B-cell immunity of PBC patients depends on an enhanced innate immune response and imply that TRAM-34 can influence B-cell autoimmunity in PBC[18].

Another source of antigenic mimicry is xenobiotics. These are foreign compounds that may either alter or complex to defined self or non-self proteins, inducing a change in the molecular structure of the native protein sufficient to induce an immune response[19]. Such immune responses may then result in the cross-recognition of the self molecule, which could in turn perpetuate the immune response, thus leading to chronic autoimmunity. Interestingly, most xenobiotics are metabolized in the liver, thereby increasing the potential for liver-specific alteration of proteins. Recent data demonstrate that certain chemical/xenobiotic compounds can induce AMA and are in fact recognized by PBC sera with higher affinity compared to the analogous self protein and that such compounds are found in products in common use as food flavorings and cosmetics[20-23]. This implicit involvement of cosmetics could contribute to the female predisposition to PBC.

ROLE OF BEC

PBC is characterized by destruction of the small and medium size intrahepatic bile ducts, lined by BECs (cholangiocytes). BECs express cell surface adhesion molecules which permit adhesion and recognition of lymphocytes. Moreover, several studies have demonstrated that BECs of both healthy and diseased liver have the capacity to increase the expression of adhesion molecules, ICAM-1 and others, MHC class Ⅰ and Ⅱ, TNF-alpha, interferon (IFN) -gamma

Table 1 Immunopathological characteristics of biliary epithelium in PBC[90]

	Normal	PBC
Expression level of PDC-E2	+	+ + +
Adhesion molecules		
- ICAM-1	+	+ +
- VCAM-1	-/+	+
- LFA-1	-/+	+
- E-selectin		+ +
Biliary intra-epithelial lymphocytes	Large bile ducts, few $CD4^+$	Small bile ducts, increased $CD4^+CD28^-$
Apoptosis-related molecules		
- Fas (CD95)	-	+
- granzyme B	-	-/+
- perforin	-	-/+
- bcl-2	+ +	-
BEC phagocytosis of apoptotic BECs	-	+ +
Cytokines		
- INF-γ	-	+ +
- IL-2	-	+ +
- IL-6	-	+ +
- IL-6 receptor	-	-/+
- TNF-α	-/+	+ +
- TNF receptor	-/+	+ +

and IL-1[24-26] upon stimulation with proinflammatory cytokines[27]. Up-regulation of VCAM-1 and LFA-1 can also be identified[28]. Adhesion molecules expressed on the BEC surface, and the up -regulation by proinflammatory cytokines, which are abundant in the course of inflammatory reactions, allow BECs to modulate the intensity and localization of inflammatory reactions. The other immune feature attributed to the BECs is a capacity to act as APCs. Several studies demonstrate that BECs express HLA class Ⅱ[27,29], and such expression is increased after injury and after stimulation with IFN-gamma and IL-1. BECs also express accessory molecules responsible for the second (co-stimulatory) signal to T cells, CD80, 86 (B7-1, B7-2)[30]. These interactions with T cells might also be responsible for bile duct loss, one of the fundamental characteristics of progression of disease.

Data obtained in recent years point towards apoptosis as a leading mechanism for ductopenia. Years ago, Harada and colleagues demonstrated susceptibility to apoptosis via the perforin/granzyme B pathway, and this was enhanced by interaction of CD95 (Fas) with CD178 (FasL) in BECs of patients with PBC[31]. The hypothesis was further confirmed when apoptotic BECs were shown to express CD40, and Fas and FasL, with transcriptional up regulation of the latter molecules after stimulation with CD154 (CD40L), culminating in apoptosis[32]. Odin and colleagues discovered that glutathiolation of the lysine-lipoic acid moiety of PDC-E2 was dramatically reduced by serum AMA[33]. Recently it has been demonstrated that apoptotic cells are phagocytosed by BECs and consequently could be an endogenous source of autoantigens from BECs[34-36]. Importantly, these findings support the concept that tissue specific damage in PBC is due to cell type-specific differences in apoptosis, and phagocytosis of apoptotic cells.

Antigenicity of BEC self-molecules, or highly homologous epitopes, could also be related to their role in mucosal immunity. Like other epithelial cells, BECs actively transfer IgAs, and in PBC these IgAs have specificity for PDC-E2. These specific IgA-type AMA can be detected in almost all body fluids of patients with PBC, including saliva, urine and bile[37,38]. Further, Fukushima and colleagues[39] detected deposits representing co-localization of such antibodies with PDC-E2 (or a highly homologous molecule) at the apical surface and in the cytoplasm of BECs, and also detected their presence in liver allografts in patients with recurrent PBC after receiving a liver transplant. To assess the direct pathogenicity of the IgA antibody class, Matsumura and colleagues exposed canine kidney cells transfected with the human polymeric Ig receptor to highly purified AMA-IgAs, thereby inducing caspase up-regulation, and thus providing evidence for direct toxic effects[40]. The immunogenic characteristics of BECs in PBC are summarized in Table 1. Finally, the still unknown role of autophagy in autoimmunity could in the future provide interesting data for the pathogenesis of PBC[41].

B CELLS AND AUTOANTIBODIES

As mentioned, the presence of serum AMA and autoreactive B cells strongly endorses the concept of an autoimmune pathogenesis of PBC[42-44].

AMA is highly specific for PBC and can be detected in nearly 100% of patients, when sensitive diagnostic methodologies based on recombinant antigens are used[45]. They are directed against members of the 2-oxoacid dehydrogenase complexes (2-OADC) existing in the inner membrane of mitochondria. Among them, the major autoantigen is the E2 subunit of the pyruvate dehydrogenase complex (PDC-E2). The epitopes for this antibody to the E-2 subunit localize to three domains of PDC-E2 component: (1) the inner (and outer) lipoic acid (lipoyl) domains; (2) the E3 binding domain; and (3) the catalytic and E2-binding domain[46]. Reactivity at lower frequency is also found against other 2-oxoacid dehydrogenase complexes (2-OADC), the 2-oxo glutarate dehydrogenase (OGDC-E2) and the branched-chain 2-oxo acid dehydrogenase (BCOADC-E2), involved respectively in the citric acid cycle and in amino acid catabolism. The 2-OADC autoantigens in PBC are summarized in Table 2.

Although the E-2 subunits of the three E-2 subunits are structurally similar, immunochemical studies have shown that the reactivities are independent, and do not depend on cross-reactivities, at least at the antibody level[47]. Antibodies to PDC-E2 and to the other 2-OADC enzymes are capable of inhibiting PDC-E2 enzyme activity *in vitro*, but this has not been shown to occur *in vivo*. Targeting of enzymes is a common feature of autoantibodies detected in patients with autoimmune

Table 2 Mitochondrial and nuclear autoantigens in PBC

		Autoantigens
Mitochondrial antigens	E2 subunits of 2-OADC	PDC-E2
		OGDC-E2
		BCOADC-E2
	Pyruvate dehydrogenase complex	E3BP
		PDC E1α
Nuclear antigens	Nuclear pore complex	gp210
		nucleoporin 62
	Multiple nuclear dots	Sp100
		PML
	Anticentromere	

2-OADC: 2-oxo-acid dehydrogenase complex; PDC: Pyruvate dehydrogenase complex; OGDC: Oxoglutarate dehydrogenase complex; BCOADC: Branched chain 2-oxo-acid dehydrogenase complex; E3BP: Dihydrolipoamide dehydrogenase (E3)-binding protein.

diseases, as is the inhibition of their activity by these autoantibodies. A pathogenic role for AMA is uncertain, since no clinical correlations with levels of AMA can be found, and in certain experimental animal models there is occurrence of serum AMA but no overt PBC-like liver lesions[14]. The role of the lipoic acid co-factor attached to lysine$_{173}$ (K_{173}) in the composition of the epitope recognized by AMA is unclear. Both lipoylated and non-lipoylated PBC-E2 react with AMA and the question is to what degree does lipoic acid serve to enhance antigenicity.

In addition to AMA, PBC sera can present other disease-specific autoantibodies, particularly anti-nuclear (ANA) specificities[2]. PBC-specific ANA reactants include nuclear pore glycoproteins of the inner nuclear membrane, gp210[48] and p62[49], with a detection rate up to about 30% and with an apparently higher prevalence among AMA-negative PBC. This subtype of PBC-specific ANA has been shown to correlate with disease severity and progression[50,51]. Other PBC-specific nucleoprotein reactants include the Sp100-promyelocytic leukemia (PML) autoantigen antigen that gives the characteristic fine nuclear dot pattern by immunofluorescence[52]; both appear specific for PBC, but the prevalence differs, being from about 20% to 30%. Finally anti-centromere antibodies occur in PBC (-10% of cases) often in association with a limited scleroderma syndrome. Recently, it has been demonstrated that anti-centromere antibodies were a significant predictive factor in PBC for the development of portal hypertension[51,53]. Table 2 specifies the nuclear autoantigens in PBC.

There seems no way of incorporating the co-occurrence of AMA and ANA into a unifying theory of pathogenesis of PBC, other than specifying both reactivities as reflecting a systemic failure of maintenance of immune tolerance.

T CELLS

Autoreactive $CD4^+$ and $CD8^+$ T cells are demonstrably involved in the pathogenesis of PBC and, histologically, infiltration of presumably autoreactive T cells in the liver and periductular spaces is one of the major features of the disease[54,55]. Both $CD4^+$ and $CD8^+$ T lymphocytes can be purified from biopsy samples of PBC patients and both subsets recognize epitopes of PDC-E2[56]; moreover, using recombinant fragments of PDC-E2 it has been demonstrated that there is a sequence overlap in the PDC-E2 specific T and B cell epitopes[57]. The minimal T-cell epitope for $CD4^+$ T cells was identified as amino acid residues 163 to 176 (GDLLAEIETDKATI), within the inner lipoyl domain of PDC-E2[57]. Phenotypically, the PDC-E2$_{163\text{-}176}$ T-cell clones were positive for CD4, CD45RO, and T cell receptor (TCR) αβ. The MHC Class Ⅱ human leukocyte antigen (HLA) -restriction molecules for this epitope have been identified as HLA-DR53 (B4*0101)[58]. In addition, PDC-E2$_{163\text{-}176}$-specific $CD4^+$ T-cell clones recognize other functionally related mitochondrial autoantigens, including OGDC-E2 and BCOADC-E2, and also E3BP[59]. More specifically, these T-cell clones were cross-reactive with the amino acid residues 100 to 113 of OGDC-E2, residues 90 to 103 of BCOADC-E2, and residues 34 to 47 of E3BP, all located in the respective E2-lipoyl domain of these enzymes; thus the suggestion is that OGDC-E2$_{100\text{-}113}$, BCOADCE$_{290\text{-}103}$, and E3BP$_{34\text{-}47}$ all represent $CD4^+$ T-cell epitopes.

$CD8^+$ T cells (CTLs) from peripheral blood of patients with PBC have been studied in the context of MHC Class I HLA-A2.1 restriction, and have been found to identify amino-acid residues 159-167 and 165-174 of PDC-E2[57]. Specific MHC class I restricted CTLs can also be generated by *in vitro* stimulation with antigen pulsed dendritic cells[60] from blood of patients with PBC, but not from healthy controls, indicative of the presence in PBC of specific precursors of PDC-E2 -reactive T cell clones in peripheral blood. Interestingly, there was a greater increase in numbers of CTL precursors in blood in early *versus* advanced stages of PBC, and in the same study there was a 10 -fold increase in specific CTLs in the liver compared to the peripheral blood, supporting the role of these cells and their specific recruitment in the evolution of bile duct injury in PBC. Thus the two major subsets of T cells recognize the same or very close amino acid sequences within the same epitope regions in the lipoyl domain, thus supporting the hypothesis of a common etiological trigger mechanism, potentially molecular mimicry, associated with other particular immune modifications.

Coming now to $CD4^+CD25^{high}$ natural regulatory T cells (Tregs), a decreased reactivity appears to contribute to a number of human autoimmune diseases[61-65] including PBC. A relative reduction of Tregs compared with healthy controls was detected and, as well, the ratio of hepatic Tregs over hepatic $CD8^+$ cells in PBC patients was lower than that in patients with chronic hepatitis C or autoimmune hepatitis[66,67].

INNATE IMMUNITY IN PBC

Innate immunity is a first line of defense against

infections and neoplasms, but its importance for adaptive immunity has been appreciated only recently, and its role in the induction of autoimmunity is only partially known[68]. The cellular components of innate immunity, including dendritic cells (DC) and other professional APCs[69], and natural killer T cells (NKT), are known to have a regulatory function by modulating the quality and quantity of subsequent adaptive immune responses, including antigen-specific antibody and T cell responses. Innate immunity in PBC patients is characterized by an increased response to pathogen-associated stimuli, as indicated by higher levels of pro-inflammatory cytokines secreted *in vitro* by monocytes after exposure to micro-organisms[70].

NK/NKT cells have been linked to autoimmune diseases in murine models, including autoimmune diabetes in NOD mice and experimental autoimmune encephalomyelitis, a model of multiple sclerosis[71], and the role of such cells in autoimmunity in general is attracting increasing attention. In PBC, Chuang and colleagues recently demonstrated a marked increase in the frequency and absolute number in blood and liver of NK cells. Moreover, in the same study, the cytotoxic activity and perforin expression by isolated NK cells were significantly increased, associated with increased levels of plasma IL-8 and the expression of CD128a (IL-8 receptor) on such cells. In contrast, the levels of IFN-γ, IL-6 and IL-8 synthesized by NK cells were significantly decreased in PBC compared to controls[72].

Hyper-responsiveness of the innate immune system of itself would be insufficient to account for the breakdown of natural immune tolerance, but these alterations might come to influence the initiation and perpetuation of the subsequent adaptive autoimmune response.

CYTOKINES

In PBC, a Th1 cytokine predominance has been reported in serum and liver[73], and a high prevalence of INF-γ, a Th1 cytokine, has been detected as a transcriptional up-regulation[74]. Moreover, BECs of patients with PBC overexpress TNF-α and the corresponding receptor, thus favoring the idea of a paracrine activity of, and effect on these cells, leading to their proliferation and, potentially, to apoptosis[75]. Recent findings further suggest the involvement of cytokine-cytokine receptor interactions in the effector stages of the pathogenesis of PBC[72]. Whilst T cells and NKT cells are major sources of cytokines, B cells, endothelial cells, macrophages and other cell types also contribute to cytokine production. Furthermore, different types of APC, genetic background, availability of costimulator molecules, and types and amounts of antigenic stimuli may also influence the differentiation of Th0 cells into either the Th1 or Th2 cell pathways, each with their particular cytokine profiles. Of course, cytokines also come into play in the earlier inductive stages of PBC, in particular transforming growth factor-beta (TGF-β). Deficiency of TGF-β is prejudicial to immunoregulatory functions, as illustrated by recent mouse models of PBC (see below).

ANIMAL MODELS

The occurrence of a spontaneous animal model would be extremely helpful in elucidating causation and progression of PBC, but none has been identified, and there is some element of "artificiality" with induced models. However, recently, there have been developed three informative genetically manipulated mouse strains that simulate features of human PBC[76].

The first of these mouse models is a congenic variant of the non-obese diabetic (NOD) mouse designated NOD.c3c4 that presents as an autoimmune larger bile duct cholangiopathy and PBC-like serology, with AMA positivity of 50%-60% and ANA positivity of 80%-90%[77]. Histologically, there is lymphocytic infiltration within portal tracts with appearances of chronic nonsuppurative destructive cholangitis and epithelioid granuloma formation, although certain features of the bile duct lesions differ from those in human PBC, particularly the occurrence of cystic changes[77]. Detailed analysis of the introgressed genetic intervals that determine the autoimmune switch from pancreatic insulitis to cholangitis is awaited.

The second of the mouse models was derived by transgenic introduction of a dominant negative form of TGF-β receptor Ⅱ (dnTGF-βRⅡ)[78]. These mice have inflammatory cholangitis and show 100% AMA positivity against PDC-E2. TGF-β receptor Ⅱ is essential for signal transduction of TGF-β, which regulates activation of lymphocytes. This model suggests a specific dysfunction of T cells with impaired TGF-β signaling which, in the presence or absence of B cells, is implicated in the pathogenesis of a PBC-like disease, at least in mice[78]. Interestingly, it has been demonstrated that CD1d-restricted NKT cells in these mice are a critical factor in liver injury[79].

The third of the mouse models depends on knockout of the gene for the IL-2 receptor (IL-2Rα knockout mouse)[54]. These mice have inflammatory cholangitis with lymphocyte infiltration around the portal tracts accompanied by cholangiocyte injury, and show 100% AMA positivity against PDC-E2 and 80% ANA positivity. The IL-2Ra is the CD25 molecule which, when highly expressed on $CD4^+$ T cells, is a marker for cells with immunoregulatory activity. This model further implicates deficiency of TGF-β-dependent regulatory pathways in the pathogenesis of PBC.

Another useful model has been developed by experimental immunization with xenobiotically modified molecular variants of the PDC-E2 epitope region. Such immunization appears promising in that AMA have thus been elicited in different animal species, rabbits, guinea pigs and mice, as recently reviewed[14].

PATHOGENIC MECHANISMS

Several theories have been proposed for the

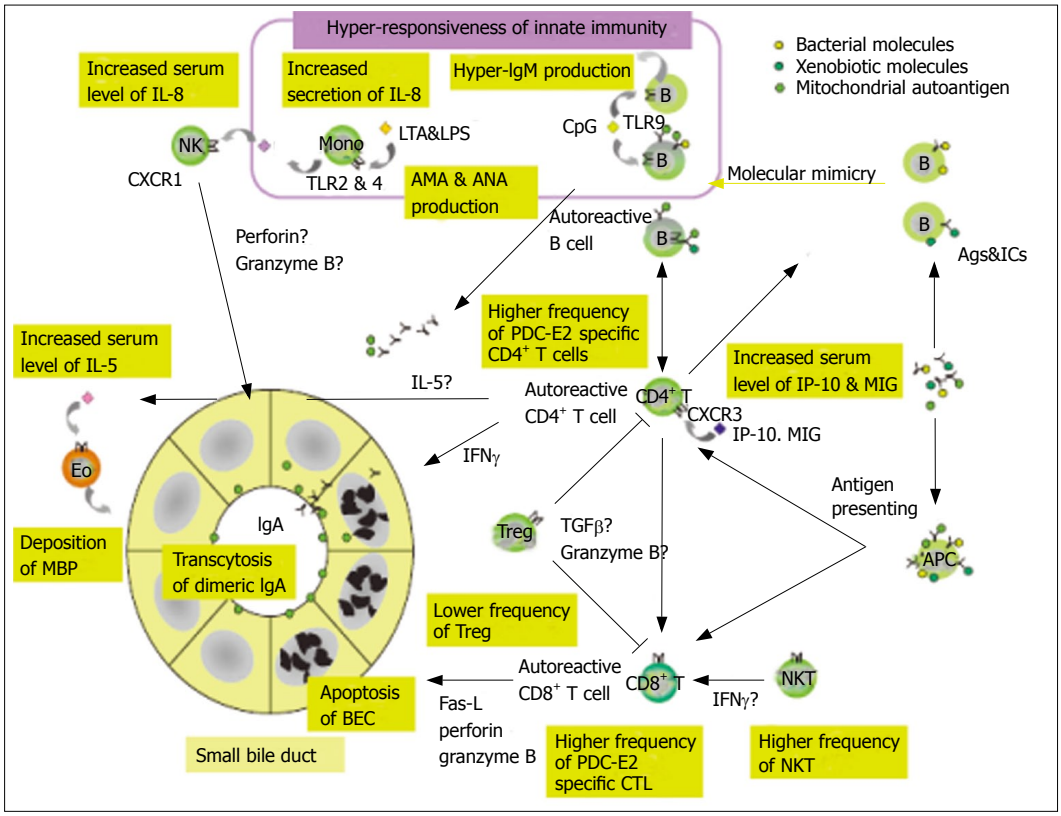

Figure 1 Model of pathogenic mechanisms in primary biliary cirrhosis (PBC). PBC is initiated by an autoantigenic stimulus (upper, right) provided either by a bacterial mimic of the autoepitope of PDC-E2, a xenobiotically modified PDC-E2, or "spillage" of native mitochondrial autoantigens derived perhaps from apoptotic cells. Hyper-responsiveness of innate immunity (top, centre) can facilitate autoantigenicity; bacterial Cpg enhances IgM production and cellular expression of TLR9. Genetic susceptibility is critical overall, and depends particularly on multiple inherited deficits in immune tolerance, mostly as yet undefined. APCs that become activated (lower, right) by stimulation through TLRs present immunogenic self peptides (or mimics) *via* MHC Class II molecules to autoreactive $CD4^+$ T lymphocytes (centre) which in turn activate $CD8^+$ cytotoxic T lymphocytes and B lymphocytes that produce AMA. Treg lymphocytes (lower, centre) that normally restrain activated autoreactive T cells are deficient in PBC, thus further impeding T cell tolerance. Effector mechanisms converge on the target cell in PBC, the BEC (lower left), which can be damaged by injurious cytokines (IFN-γ) from $CD4^+$ T cells, direct cytotoxicity (Fas-L, perforin, granzyme B) from $CD8^+$ T cells, or transcytosis of IgA-AMA. A toxic effect might even be supplied by activated eosinophils (centre, left) by release of eosinophil MBP. BECs thus undergo apoptosis and in doing so contribute immunogenic mitochondrial PDC-E2 autoantigen to sustain a self-perpetuating autoimmunization process and, by reason of a BEC-specific anomaly of apoptosis retain PBC-E2 intact in apoptotic blebs (see text), so conferring particular vulnerability on these cells. Ags: Antigens; AMA: Antimitochondrial antibodies; ANA: Antinuclear antibodies; APC: Antigen-presenting cell; BEC: Biliary epithelial cells; CTL: Cytotoxic T lymphocytes; ICs: Immune complexes; IL: Interleukin; IFN: Interferon; IP-10: Interferon-γ-inducible protein 10; LTA: Lipoteichoic acid; LPS: Lipopolysaccharide; MIG: Monokine induced by γ-interferon; MBP: Major basic protein (primary cytotoxic granule protein); NKT: Natural killer T cells; PDC-E2: Pyruvate dehydrogenase complex E2; TGF: Transforming growth factor; TLR: Toll-like receptor; Treg: Regulatory T cells.

etiopathogenesis of the immune-mediated tissue injury observed in PBC (Figure 1). Such theories are not necessarily independent, but rather each may be directed to different phases of etiopathogenesis. In other words, we need to consider processes particular to initiation; processes particular to perpetuation, notably deficiencies in immune tolerance; and processes particular to the selective destruction of BECs, with the assumption that these express the target of the disease in an accessible form, namely the AMA autoantigen PDC-E2.

Initiation

Initiation has been considered already and possibilities include microbial infection or chemical-xenobiotic modification of the PDC-E2 epitope sequence with tolerance-breaking effects due to molecular mimicry. Alternatively mere spillage of autoantigen after cellular injury and apoptosis could suffice, as shown recently in our laboratory[80].

Perpetuation

Perpetuation involves particular consideration of genetically-based tolerance deficits in PBC, and here more data are sorely needed. Consideration was given to this aspect in a recent review from this laboratory[14]. We can refer here to studies on the critical role of $CD4^+$ $CD25^{high}$ regulatory T cells (Tregs) in the prevention of autoimmune disease in murine models. It is postulated that Tregs are important for the prevention of autoimmunity and maintenance of self-tolerance, and studies have demonstrated that the transfer of T cells lacking the Treg subset into athymic nude mice results in the development of various T cell–mediated autoimmune diseases[61,81]. PBC patients display significantly lower frequencies of Tregs as percentages of total TCR-$\alpha\beta^+$/$CD4^+$ T cells, which may contribute to the failure in tolerance in PBC[66,82,83].

Destruction

Destruction involves a multilineage attack by $CD4^+$ and $CD8^+$ T cells, and B cells, on the vulnerable biliary

ductile, and here our hypothesis would state that the immunodominant AMA autoantigen PDC-E2, which is normally located in the mitochondrial inner membrane, is aberrantly expressed on the cell surface of the BEC and thus is immunologically recognized. Several possibilities have been visualized. First, although *in situ* hybridization studies of *PDC-E2* mRNA showed no significant difference in the amount of PDC-E2 transcript present in PBC liver compared with other liver diseases, PDC-E2 may be selectively overexpressed in small bile duct BECs perhaps as a result of aberrations of apoptosis[14]. Second, variants of PDC-E2 may cause an abnormal turnover of the molecule, leading to the accumulation of PDC-E2 in these subpopulations of cells. It is possible that toxic substances disposed of by the liver may accumulate in the biliary epithelium and potentially modify the PDC-E2 molecule locally, leading to the production of such variants. Third, altered *PDC-E2* mRNA could be produced by the abnormal transcription of PDC-E2. For example, it is possible that abnormal splicing during synthesis of *PDC-E2* mRNA would substitute an endoplasmic reticulum targeting signal instead of a mitochondrial targeting signal, thereby enabling PDC-E2 to be delivered into the endoplasmic reticulum and Golgi apparatus via a secretory route to be expressed on the cell surface of biliary ducts, instead of into mitochondria. Although direct evidence supporting these mechanisms is currently lacking, it remains possible that the molecules that are expressed and identified on the ductular surface of BECs, and recognized by anti-PDC-E2 antibodies, may not be PDC-E2 itself, but are PDC-E2 mimics that cross-react with human PDC-E2. Some experimental data seem to support this hypothesis.

Another hypothesis that might explain the selective targeting of bile ducts in PBC is that the autoantigen-specific immunoglobulin A (IgA) antibody plays a role. IgA is the principal isotype of immunoglobulin in epithelial surfaces, including biliary epithelium. If AMA-IgA autoantibodies are responsible for the specific destruction of BECs in PBC, it is possible that this occurs by disrupting cell metabolism of the cells i.e. the AMA-IgA bound to the mitochondrial antigen induces cellular dysfunction and so accounts for the tissue specificity. Interestingly, IgA from PBC patients colocalized with PDC-E2 inside the cells and on the apical membrane of BECs[84]. These data support the idea that both the aberrant polar expression of PDC-E2 and the trafficking of IgA in BEC are possible mechanisms for selective damage of BECs. Thus, the apical staining of BECs revealed by anti–PDC-E2 monoclonal antibodies could also be accounted for by the presence of an immune complex formed from secreted IgA and mitochondrial enzyme autoantigens.

CONCLUSIONS AND FUTURE PERSPECTIVES

There have been many substantial advances in the understanding of PBC since the molecular identification in 1988 of PDC-E2 as the major reactant for characteristic AMA response. Possible initiators of PBC have emerged as environmental chemical xenobiotics, or microorganisms that utilize the shared culprit autoantigen PDC-E2. Strong genetic predisposition is certain from case study data, noting here the female predisposition and family clustering, but formal genome wide studies are not yet available. It is highly likely that multiple deficits in immune tolerance will prove important, and that these will be genetically based; recent mouse models are certainly pointing in this direction. Other unexplained features of PBC include susceptibility to infections, likely associated with the aberrant humoral and cellular reactivities[85,86]. It is crucial to ascertain whether there is a pathogenic role of AMA in the bile duct damage of PBC and, if so, how this is mediated. Further investigation of innate immune mechanisms in PBC is called for, as is the role of the BEC itself in stimulation and perpetuation of the peribiliary inflammatory process.

It is almost a truism that only knowledge of the etiopathogenetic mechanisms will open the door to effective therapies for diseases such as PBC[87], yet the major advance in the therapy of PBC with UDCA[88] has come without insight into primary causes of the disease, just as successful biotherapies of rheumatoid arthritis have emerged without knowledge of the primary cause of that disease. Currently, we should press on with development and study of animal models, encourage the application of predictably informative genomic studies, and seize on the clues we have to environmental provocations.

At the practical level, clinicians should be alert to the need for early diagnosis, during the long latent period of the disease, in the susceptible middle-aged female population to ensure that such subjects do gain benefit from disease-retarding therapy, at whatever stage their disease may be[88]. To a degree, this is already happening if we compare what PBC was like 20 years ago[89] to what it is today[90].

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TOPIC HIGHLIGHT

Pietro Invernizzi, MD; Ian R Mackay, MD, *Series Editors*

Clinical features and management of primary sclerosing cholangitis

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Abstract

Primary sclerosing cholangitis is a chronic cholestatic liver disease characterized by inflammation and fibrosis of the bile ducts, resulting in cirrhosis and need for liver transplantation and reduced life expectancy. The majority of cases occur in young and middle-aged men, often in association with inflammatory bowel disease. The etiology of primary sclerosing cholangitis includes immune-mediated components and elements of undefined nature. No effective medical therapy has been identified. The multiple complications of primary sclerosing cholangitis include metabolic bone disease, dominant strictures, bacterial cholangitis, and malignancy, particularly cholangiocarcinoma, which is the most lethal complication of primary sclerosing cholangitis. Liver transplantation is currently the only life-extending therapeutic alternative for patients with end-stage disease, although recurrence in the allografted liver has been described. A PSC-like variant attracting attention is cholangitis marked by raised levels of the immunoglobulin G4 subclass, prominence of plasma cells within the lesions, and steroid responsiveness.

Key words: Sclerosing cholangitis; Diagnosis; Therapy; Cholestasis; Cholangiocarcinoma; Liver transplantation

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INTRODUCTION

Primary sclerosing cholangitis (PSC) is a chronic cholestatic liver disease characterized by fibrosing inflammatory destruction of the intrahepatic and/or extrahepatic bile ducts[1], leading to bile stasis, hepatic fibrosis, and ultimately to cirrhosis, end-stage liver disease, and need for liver transplantation. The majority of cases occur in association with inflammatory bowel disease (IBD), which often precedes the development of PSC[2]. The etiology of PSC is undefined, apart from an increasing body of evidence that points to an immunologic disturbance as a component of the disease. However, PSC lacks the features of a typical autoimmune disease and responds poorly, if at all, to typical immunosuppressive therapies[3]. No effective medical therapy for halting disease progression has been identified, but ursodeoxycholic acid is being assessed. A median duration of 12 to 18 years from the time of diagnosis before patients develop end-stage liver disease has been observed. Among eligible patients, liver transplantation (LT) is currently the only life-extending therapy for patients with end-stage PSC, although the disease can recur in the allografted liver and be a cause of morbidity post-transplant[4].

CLINICAL FEATURES

Clinical manifestations

PSC affects primarily young and middle-aged men, especially patients with underlying inflammatory bowel disease. Approximately 70% to 80% of PSC patients in the United States have ulcerative colitis (UC)[5-10].

Conversely, approximately 2% to 7.5% of patients with UC[11] and 1.4% to 3.4% of patients with Crohn's disease[12] develop PSC. IBD can be diagnosed at any time during the course of PSC, and PSC can occur at any time during the course of IBD[13]. In general, however, IBD is diagnosed several years earlier than PSC[13]. PSC may also develop many years after proctocolectomy for colitis and IBD can develop many years after liver transplantation due to advanced PSC[11]. Whether PSC is a distinct entity in patients with and without IBD might be a clinically significant issue[14,15], but at present, there are not sufficient data to conclude that PSC occurring in patients without IBD is an entity separate from PSC found in association with IBD[11,16].

Asymptomatic patients represent about 15% to 40% of the patients at time of diagnosis in early studies[2]. More recently, more patients are identified at an earlier stage of the disease with fewer symptoms. One study showed that the majority of patients (greater than 55%) initially present with asymptomatically elevated liver enzymes[17]. Due to its close association to IBD, many cases come to medical attention when patients with IBD are screened for liver disease.

The clinical course of PSC is typically one of insidious worsening of cholestasis and eventual development of jaundice and end-stage liver disease[3]. As such, asymptomatic patients with PSC are at increased risk for developing symptoms over time. Fatigue and pruritus are reported as the most common symptoms. Jaundice, pain, fever and weight loss, cholangiocarcinoma, or manifestations of portal hypertension in advanced stages of liver disease are uncommon initial manifestations. In one recent study, the most common presenting symptoms were described as abdominal pain (20%), pruritus (10%), diarrhea (8%), jaundice (6%), fatigue (6%) and fever (4%)[17]. Another recent study from Sweden suggested that more patients without IBD are identified and the patients are older at diagnosis[18]. Symptoms of bacterial cholangitis usually are not manifested until patients undergo endoscopic intervention or surgical exploration of the biliary tract[19]. Cholangiocarcinoma develops in up to 23% of patients[20] and can occur relatively early and before onset of cirrhosis[3].

Impairments in health-related quality of life among individuals with PSC compared to the general population were confirmed in two independent populations[21,22]. Patients with cirrhosis form primary hepatocellular disease, however, reported lower health-related quality of life scores compared to patients with cholestatic liver disease[21].

Biochemical features

A cholestatic picture of liver function with elevations in serum alkaline phosphatase values are the biochemical hallmark of PSC. Increases between 3 and 10 times the upper limit of normal occur in 95% of cases. Serum alanine and aspartate aminotransferase levels are usually 2-3 fold higher than normal levels. The serum total bilirubin level is normal in 60% of individuals at diagnosis[2]. The liver function tests, however, may be normal and can fluctuate during the course of the disease[23].

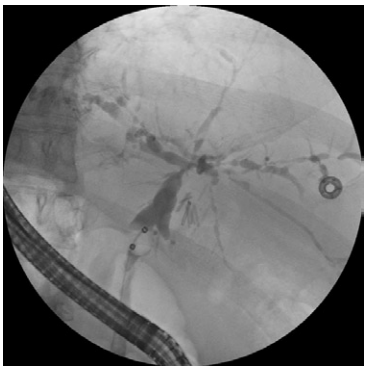

Figure 1 Cholangiographic finding in PSC. Cholangiogram demonstrating multifocal strictures with intervening saccular dilatation of both intrahepatic and extrahepatic bile duct characteristic of PSC. (Photograph courtesy of Dr. Rahul Pannala).

Several prognostic models for PSC have been developed, most of which include age, serum bilirubin and histologic staging[5,7,9,10,24]. Most recently, a Mayo model for predicting the survival has been refined[25]. This uses the age of the patient, total serum bilirubin, aspartate aminotransferase levels, presence or absence of variceal bleeding and serum albumin as independent variables, and can be used in early stages of PSC, before onset of cirrhosis. The limitations of prognostic models include the inability to account for the development of cholangiocarcinoma and health-related quality of life[19]. Once decompensated cirrhosis is present, the Model for End-Stage Liver Disease (MELD) score[26] more accurately predicts survival and is more appropriately used in prioritizing patients for liver transplantation[3].

Serologic features

Currently, testing for specific autoimmune antibodies does not contribute to the diagnosis of PSC. The prevalent autoantibody reactivity is a perinuclear antineutrophilic autoantibody (pANCA), present in approximately 80% of patients but lacking in diagnostic specificity[27-30]. The unidentified antigenic reactant is not the proteinase (myeloperoxidase) of conventional pANCA. Other autoantibodies such as antinuclear antibodies and smooth muscle antibodies occur in 20% to 60% of patients, usually in lower titers than those observed in autoimmune hepatitis[31]; their fine antigenic specificity has not been established. Antimitochondrial antibodies are rarely found in patients with PSC[6], in keeping with the lack of overlap between PSC and primary biliary cirrhosis (PBC). The serological markers of autoimmune liver diseases are covered in more detail in other articles in this series.

Radiographic features

Cholangiography is considered to be the gold standard for the diagnosis of PSC[6]. In experienced hands, endoscopic retrograde cholangiopancreatography (ERCP) is successful in demonstrating the intra- and extra-hepatic biliary tree in 95% of the cases[3]. Segmental fibrosis of intrahepatic and/or extrahepatic bile ducts with saccular dilatation of normal intervening areas results in the characteristic beads-on-a-string appearance (Figure 1). Intrahepatic duct involvement is nearly universal with most patients affected

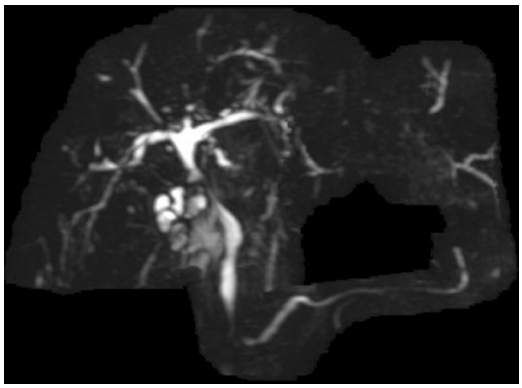

Figure 2 Cholangiographic findings in PSC. Magnetic resonance cholangiography demonstrating findings of PSC.

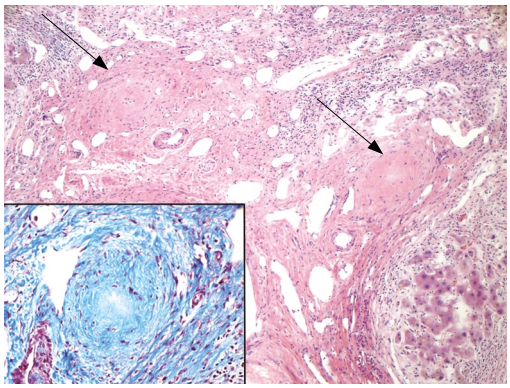

Figure 3 Fibro-obliterative lesions in PSC. Image shows an expanded portal area without two distinct fibro-obliterative lesions (arrows) in end-stage primary sclerosing cholangitis. There is no intact bile duct present in this portal area, only cross-sections of portal vein and hepatic artery branches (H&E, original magnification, 100 ×). Inset: Higher magnification of the fibro-obliterative lesion (Masson trichrome, × 400). (Photograph courtesy of Dr. Schuyler Sanderson).

by intrahepatic and extrahepatic disease[19]. Procedure-related complications from ERCP can occur in 3% to 8% of patients and include abdominal pain, pancreatitis, bleeding, common bile duct perforation, biliary sepsis and death[32-34].

The use of magnetic resonance cholangiography (MRC) for detecting PSC has been evaluated as a rapid, noninvasive examination of the biliary tract (Figure 2). MRC has no significant morbidity when performed in appropriately selected individuals, and avoids the potential adverse effects of radiation exposure and contrast media associated with ERCP[35]. For the detection of PSC, MRC has been found to be accurate and comparable to ERCP[36-39]. Moreover, one study has suggested it also results in cost savings when used as the initial test strategy for diagnosing PSC[34]. Factors that lead to difficulties in interpreting the MRC compared to ERCP include the presence of cirrhosis and PSC limited to the peripheral intrahepatic bile ducts[36]. The major disadvantage of MRC is that it is a purely diagnostic examination, although it can be used to identify patients who would benefit from subsequent therapeutic ERCP[19]. Although biliary tree changes on MRC aid in the diagnosis of PSC, they do not correlate with survival, as predicted by the Mayo Risk Score[38].

Histologic features

PSC is histologically characterized by damage, atrophy, and, ultimately, loss of medium- and large-sized bile ducts, within or outside the liver[40,41]. These are not typically captured in a percutaneous liver biopsy. The histological picture is complicated by the fact that separation of the disease process itself from the effects of distal obstruction of bile ducts can be challenging[41]. The smaller ducts are affected by the resultant obstruction and gradually disappear (ductopenia). The characteristic pathologic features of PSC are concentric periductal fibrosis ("onion-skinning") that progresses to a narrowing and then obliteration of the small bile ducts leaving a bile duct scar (Figure 3), but this is found in less than 15% of the patients with PSC[42]. Many of the biopsy changes, such as bile stasis, pseudoxanthomatous changes, Mallory bodies and copper accumulation, lack specificity for diagnostic purposes[41], and can occur with chronic extra-hepatic bile duct obstruction from any cause[43]. Several stages can be recognized histologically, ranging from stages Ⅰ to Ⅳ: cholangitis and portal hepatitis (stage Ⅰ); periportal fibrosis or periportal hepatitis (stage Ⅱ); septal fibrosis, bridging necrosis or both (stage Ⅲ); and biliary cirrhosis (stage Ⅳ)[44]. Sampling error is a significant limitation of liver biopsy[41].

Liver biopsy in patients with radiographic evidence of PSC is not needed for diagnosis, although it may help in excluding other diseases[45]. Histological staging may be complementary to ERCP evaluation, but most times is not necessary. Liver biopsy should probably be limited to patients with a challenging presentation or those being investigated for small duct PSC or possible overlap syndrome with autoimmune hepatitis[17]. The presence of biliary dysplasia on liver histology has been proposed as a marker for eventual cholangiocarcinoma in PSC[46,47], but its use in clinical practice is limited by poor reproducibility of findings on pathologic interpretation[47].

VARIANT FORMS OF PSC

Small duct PSC

Small duct PSC refers to disease that affects bile ducts that are too small to be identified by ERCP. This entity is characterized by a consistent liver histology and radiographically normal bile ducts[48,49]. The proportion of small duct PSC to "large duct PSC" has been described as approximately 5%-15%[50,51]. Small duct PSC is believed to be a distinct entity from the large duct form, with a less aggressive course and less likely to lead to cholangiocarcinoma[50,52].

Overlap with autoimmune hepatitis

PSC with overlap features of autoimmune hepatitis has been reported both in the pediatric and adult populations of patients with PSC. In adults, the therapeutic response to immunosuppressants, in particular the autoimmune

hepatitis- or hepatocellular component of the overlap syndrome, can be excellent, and can lead to complete remission of disease activity[53]. The response to therapy might be dependent on the predominance of AIH or PSC features. The overlap syndromes of autoimmune liver diseases, including overlap of AIH and PSC in childhood are covered in more detail in other articles in this series.

IgG4-related sclerosing cholangitis

During the past decade, patients with steroid responsive sclerosing cholangitis have been described, often but not always associated with autoimmune pancreatitis[54]. Histological findings of lymphoplasmacytic infiltration and infiltration of IgG4-bearing plasma cells, and high serum IgG4 levels have been consistently observed and frequently required for diagnosis of autoimmune pancreatitis[55,56], and have also been observed in the hepatobiliary system. Described hepatobiliary changes have included stenosis of the bile ducts, biliary duct wall thickening, stenosis of the portal vein, portal fibrosis[57], and hepatic inflammatory pseudotumor[58]. Hepatobiliary involvement without pancreatic involvement suggests that IgG4-related sclerosing cholangitis could be a distinct entity from autoimmune pancreatitis[58]. IgG4-related sclerosing cholangitis is similar to PSC with regard to cholangiographic features, but, in contrast to PSC, is susceptible to steroid therapy and is reversible[58]. Therefore, identifying patients with IgG4-related sclerosing cholangitis and distinguishing them from patients with PSC could have major therapeutic implications[59]. Although very limited data exists on the prognosis and natural history of IgG4-related sclerosing cholangitis, it seems that the prognosis of these patients is more favorable than that of patients with PSC[54].

DISEASE-MODIFYING TREATMENTS

Different forms of medical treatment have been tried, but until now, no treatment has been proven efficient in randomized controlled studies.

Ineffective or unproven therapies

Several drugs, such as penicillamine[60], methotrexate[61], budesonide[62], colchicine[63], cladribine[64], cyclosporine[65], mycophenolate mofetil[66,67], etanercept[68], oral and transdermal nicotine[69,70], silymarin[71], pirfenidone[72], and pentoxifylline[73], have been evaluated in the treatment of this condition, but none of them has demonstrated convincing evidence of benefit and some are associated with significant side effects[62,66].

Tacrolimus was shown in a pilot study to cause significant improvement in serum liver biochemistries including alkaline phosphatase[74]. A subsequent study from Mayo Clinic supports previous observations that oral tacrolimus is associated with significant reductions in alkaline phosphatase levels in PSC[75]. However, in this study, the drug was not well tolerated, and the clinical benefit with oral tacrolimus with respect to disease activity in PSC appears to be limited.

Biologic therapy has been a major advancement in the current therapy of inflammatory bowel disease. There are no controlled trials evaluating the role of biologic therapy (e.g. infliximab and adalimumab) in the management of PSC.

Ursodeoxycholic acid

Multiple controlled studies have suggested that ursodeoxycholic acid (UDCA) has beneficial effects on liver biochemistries of patients with PSC[76-85]. A few studies have documented an improvement in liver histological appearance[76,79,82]. Other studies have not included liver histology as an outcome mainly because of sampling issues[85]. However, UDCA has not yet proven to prolong survival or improve outcome of PSC. All the trials performed to date have been limited by small number of patients and relatively short follow-up periods.

In an open label study performed at Mayo Clinic, 30 patients with PSC received high-dose (25-30 mg/kg per day) UDCA[83], and substantial reduction not only in serum hepatic biochemistries but also Mayo risk score were observed after 12 mo of therapy. Differences in expected 4-year survival based on Mayo Risk Score were substantial between historical placebo and high-dose UDCA groups. A previous placebo-controlled study conducted at Mayo Clinic in which 51 patients received lower doses of UDCA (13-15 mg/kg per day)[77] had showed beneficial effects limited to serum hepatic biochemistries, but no difference in predicted survival. An independent, double-blind, placebo-controlled trial[82] of UDCA at 20 mg/kg per day involving 102 patients observed improvement in liver biochemistries, cholangiographic appearance and liver histology after 2 years of therapy, but failed to have any significant effect on survival. No significant UDCA related adverse events were reported from either study. On the other hand, a European study[85] with 219 patients who were randomized to receive either high-dose UDCA (17-23 mg/kg per day) or placebo for 5 years did not observe any significant decrease of serum alkaline phosphatase in the UDCA-treated patients. There was no significant benefit from UDCA on survival without liver transplantation or prevention of cholangiocarcinoma, but the study was too small to exclude a significant beneficial effect on survival. A large, multicenter National Institutes of Health sponsored randomized trial of high-dose UDCA is currently underway[86].

Combination therapy

Combination therapy is a relatively new avenue of clinical research in the treatment of chronic cholestatic diseases. Drugs in monotherapy are often limited by efficacy and dose-related toxicity; combination therapy may hold the potential for improved efficacy through additive or synergistic effects, with the potential minimization of drug toxicities[87]. A controlled but nonrandomized study with 12 patients treated with a combination of low-dose prednisolone and colchicine failed to find any benefit in PSC[88]. The combination of UDCA and methotrexate was studied in 19 patients with

PSC and no changes in biochemistries from baseline values were seen compared to patients receiving UDCA alone[89]. An 8-wk pilot study evaluating the combination of prednisone or budesonide combined with UDCA failed to demonstrate significant beneficial effects to justify its use in patients with PSC[90]. In a small study with 15 patients, positive results were obtained with the combination of UDCA, prednisolone and azathioprine in decreasing liver biochemistry values[91], but evidence supporting long-term use of this therapy is lacking. Most recently, a study with 80 PSC patients randomized to either UDCA alone or the combination of metronidazole and UDCA showed that combination therapy led to improved serum alkaline phosphatase and Mayo Risk Score, but no significant effect on disease progression compared to UDCA alone[92].

Innovative approaches to medical therapy

Trials of antibiotics such as metronidazole and minocycline have been promising but inconclusive. A small study of docosahexaenoic acid (DHA) which improves CFTR function[93] is currently underway. Most promising for the near future are inhibitors of TNF action, antifibrotic agents (such as angiotensin-converting enzyme [ACE] inhibitors, sirolimus/rapamycin), and inhibitors of formation of toxic bile (such as 24-norursodeoxycholic acid)[94].

Endoscopic therapy

Some patients present with clinical and biochemical deterioration and exhibit a dominant stricture that involves the larger extrahepatic biliary ducts. Such lesions may be amenable to endoscopic or radiologic dilatation with or without a biliary drainage procedure, such as sphincterotomy and stenting[45]. This leads to improvement of clinical symptoms, liver biochemistries and cholangiographic findings. However, the endoscopic treatment of PSC has generated controversy, not only with regard to optimal management, but also its overall influence on survival. The use of endobiliary stents has been compared to balloon dilatation alone in patients with PSC[95,96], and a greater frequency of intervention-related complications including acute cholangitis was observed in patients with endobiliary stent placement. Repeated balloon dilatations of dominant biliary strictures resulted in improved actual survival rates compared to survival rates predicted by Mayo risk score[97,98].

Biliary surgery

Dominant strictures can also be managed surgically by dilatation or choledochojejunostomy, but this treatment has become uncommon with more recent advancement of endoscopic techniques and growing success of liver transplantation. At present, biliary surgery in patients with PSC, other than simple cholecystectomy, should be minimized and reserved for the selected rare noncirrhotic patients who have marked cholestasis or recurrent cholangitis caused by a dominant extrahepatic or hilar stricture not amenable to endoscopic or percutaneous dilatation[45]. In patients who may undergo liver transplantation, prior biliary surgery has been associated with a significantly longer operation time, greater intraoperative blood loss, and a higher incidence of biliary complications post-liver transplantation compared with those patients with no history of biliary surgery[99-103].

Liver transplantation

Although PSC is an uncommon disease, advanced-stage PSC remains among the most common indications for LT in the United States and in Europe[20]. Unique circumstances that require evaluation for possible LT include recurrent bacterial cholangitis despite intensive medical and endoscopic therapy, severe extrahepatic biliary obstruction that precludes operative repair, and uncontrolled peristomal variceal bleeding. Intractable pruritus may also be an indication for liver transplantation. Liver transplantation should be considered before the disease is too advanced, in order to enhance the long-term survival rates post-liver transplantation[104]. Prognostic models can aid in the timing of liver transplantation. Reports from single centers performing LT in PSC patients have demonstrated excellent survival rates of 90%-97% at one year, and 83%-88% at 5 years[105,106]. However, retransplantation rates seem to be higher for patients with PSC than other diagnoses[3].

Recurrence of PSC in the liver graft has been documented. Diagnosis of recurrence can be challenging, as non-specific bile duct injuries and strictures caused by allograft reperfusion injury, ischemia, rejection and recurrent biliary sepsis can mimic the findings of PSC post-transplantation and need to be carefully excluded before the diagnosis of recurrence can be established[107,108]. The frequency of recurrent PSC after liver transplantation remains controversial. The frequency of recurrent disease is estimated between 10% to 20% of patients[109], but a recent systematic review has indicated that publication bias might be a concern regarding this topic[4]. PSC might recur earlier at a higher ratio after living donor liver transplantation, particularly when the liver graft is obtained from a biologically related living donor[110]. Proposed risk factors for recurrent PSC include inflammatory bowel disease, prolonged cold ischemia time, number of cellular rejection episodes, previous biliary surgery, cytomegalovirus infection, and lymphocytotoxic cross-match[4] but these require further investigation. As more liver transplant recipients survive longer, the recurrence of disease may become the primary cause of morbidity and mortality in PSC[4].

Liver transplantation in autoimmune liver diseases is covered in more detail in another article in this series.

DISEASE-RELATED COMPLICATIONS

Fatigue and pruritus

Pruritus is a prevalent problem in patients with chronic cholestatic disease[111]. Cholestyramine is effective in 80% to 90% of the patients, and represents the first-line of treatment[112]. Other drugs that have been commonly used for the treatment of pruritus include rifampin[113],

opioid antagonists[114,115] and ondansetron[116]. Sertraline has been demonstrated to have positive effects on pruritus in cholestatic liver disease in a small study[117]. Etanercept has also had positive effects on pruritus in patients with PSC in a small study not originally designed to primarily assess the effect of that drug on pruritus[68]. In controlled trials, UDCA has not been associated with improvement in pruritus, but those studies were not specifically designed for that purpose. Intractable pruritus may be an indication for LT[19].

Fatigue is also noted to be a prevalent problem in patients with chronic cholestatic disease, and a major determinant of impaired health related quality of life[118]. However, no medical therapy is available for treatment.

Metabolic bone disease

Severe bone disease in PSC patients is more common than expected, but less frequent than that reported in primary biliary cirrhosis[119]. Patients with longer duration of IBD, and more advanced liver disease were found to be at higher risk of severe osteoporosis[119]. In this same study, Angulo *et al*[119] found that the severity of osteopenic disease in PSC seems to increase as liver disease advances, but this finding was not confirmed in a subsequent study by Campbell *et al*[120]. Bone mineral densitometry measurements are the only test helpful in evaluating progression of osteopenia in patients with PSC, and the presence, severity and progression of the bone disease cannot be accurately evaluated by routine clinical, biochemical, or histological variables[119]. This is important since most patients with PSC and advanced liver disease undergo liver transplantation. Early post-transplant bone loss remains a clinically significant problem and frequently leads to fracturing in a third of patients with PSC when the pre-transplant bone mineral density is below the fracture threshold[119,121].

For treatment of osteoporosis and osteopenia, calcium and vitamin D supplementation are recommended, and in selected cases, bisphosphonates may be indicated. Many new drugs have become available for the treatment of post-menopausal osteoporosis, and more studies are needed to determine the role of these treatments in primary sclerosing cholangitis.

Gallbladder stones and polyps

Cholelithiasis has been noted in 26% of individuals with PSC, with the majority being asymptomatic[122]. Consideration should be given to performing a cholecystectomy if cross-sectional imaging results in the identification of gallbladder polyps given the potential for neoplastic transformation in PSC[123].

Peristomal varices

A special complication of portal hypertension in PSC patients with an ileal stoma is the development of peristomal varices[45]. Those develop within the adhesions between the ileal (portal) veins and the anterior abdominal wall (systemic) veins. Patients bleeding from peristomal varices often present with recurrent hemorrhagic episodes. Bleeding from the peristomal varices is more difficult to treat than bleeding from esophageal varices. Local treatment to control and prevent bleeding is usually unsuccessful in the long term. Liver transplantation should be considered for treatment. If that is not possible, peristomal variceal bleeding can be controlled with a portosystemic shunt[2].

Dominant stricture

A dominant stricture, defined by Stiehl *et al*[124] as a diameter in the common duct of less than 1.5 mm and in the hepatic duct of less than 1 mm, is a frequent finding and occurs in 45% to 58% of patients during follow-up. Stenotic lesions in PSC are thus far more often benign than malignant in nature[124]. Endoscopic treatment of dominant stenoses improves cholestasis and prolongs survival in comparison to predicted survival[97,125]. Prophylactic antibiotic administration prior to endoscopic manipulation of the biliary tree is recommended by the American Society for Gastrointestinal Endoscopy in the setting of bile duct obstruction to prevent contamination during the cannulation of the bile duct[126].

Bacterial cholangitis

Bacteriobilia is found in the majority of PSC patients[127], but, as previously mentioned, bacterial cholangitis usually is not manifested until patients undergo endoscopic intervention or surgical exploration of the biliary tract. Bacterial cholangitis is common in patients with dominant stricture and requires antibiotic treatment[45]. It may also occur after endoscopic procedures or in patients with bile duct stones or tight strictures[128], warranting prophylactic antibiotic administration prior to endoscopic manipulation of the biliary tree. Most biliary infections in patients with obstructive disease of the biliary tract are caused by aerobic enteric organisms such as *Escheria coli*, *Klebsiella* species, and *E. faecalis*[129]. Recurrent episodes of bacterial cholangitis can be an indication for liver transplantation in patients with otherwise preserved liver function. Prophylaxis with antibiotics has not been proven to be of benefit[128], but patients with recurrent cholangitis should be advised to seek medical attention rapidly and start antibiotics at the first sign of biliary infection.

Malignancy

Cholangiocarcinoma: Primary sclerosing cholangitis carries an increased risk of hepatobiliary malignancy, especially cholangiocarcinoma (CCA)[8,130]. The development of CCA is the most lethal complication of PSC. Cholangiocarcinoma can arise at any stage of PSC, although, in general, the incidence is higher in more advanced disease[5,131]. There are no clinical features that predict the diagnosis of CCA, and diagnosis can be challenging.

The cumulative life-time incidence of CCA is estimated as 6% to 23%[20]. The reported prevalence of CCA in explanted livers and autopsy is much higher, approximately 30% to 42%[9,132]. Overall, up to 50%

of CCA cases are detected synchronous with the PSC diagnosis or within one year of diagnosis of PSC[130,133-136]. Based upon the same reported series, the incidence of CCA during follow-up, starting at 1 year after the diagnosis of PSC, can be calculated as being between 0.5% and 1.5% per year. The malignancy usually develops in the fourth decade of life, whereas CCA in patients without PSC usually develops much later in life, in their seventh decade of life[20].

Risk factors for the development of CCA in PSC have not been clearly identified, but older age, longer duration of IBD and smoking behavior have been associated with an increased risk for development of CCA in patients with PSC. Finding biliary dysplasia on liver histology has also been proposed as a precursor in the development of cholangiocarcinoma[46,47].

The diagnosis of CCA can be challenging. The role of serum CA19-9 level in the diagnosis of CCA is controversial. There are no tumor markers which are specific for cholangiocarcinoma. In the context of PSC, a serum CA19-9 level greater than 100 U/mL has been reported to have a sensitivity of 75% and a specificity of 80% for presence of cholangiocarcinoma[137,138]. A recent study from the Mayo Clinic found that a serum level greater than 129 U/mL provided a sensitivity of 78.6% and specificity of 98.5% for CCA in PSC[139]. Even though these studies suggest CA 19-9 is an accurate test to diagnose cholangiocarcinoma, CA 19-9 was only found to identify patients with advanced, unresectable CCA, and thus its use is not appropriate as a screening test[139]. Ultrasonography, computed tomography, and magnetic resonance have inadequate sensitivity to distinguish CCA from PSC. Endoscopic biopsy and biliary brushing for cytology, digital image analysis, and fluorescent *in situ* hybridization are noted for good specificity but poor sensitivity in detecting CCA[19].

Patients with PSC and CCA have a very poor outcome, with median survival of approximately 5 to 11 mo[20,135,140]. Even though survival of patients in whom CCA was found incidentally by histological examination of the explanted liver has been reported to be good[5], in general, LT for patients with CCA results in a low success rate[141-144]. However, more recent data from investigational protocols have suggested better outcomes in highly selected individuals. The use of pretreatment radiotherapy and subsequent capecitabine for 2 to 3 wk prior to LT at Mayo Clinic has yielded a 3- and 5-year actuarial survival of 82%[145]. The use of brachytherapy and continuous 5-fluoracil infusion before liver transplantation in Nebraska resulted in 45% long-term cancer-free survival after follow-up for a median of 7.5 years[146]. Curative resection among individuals with early-stage cholangiocarcinoma may also be of benefit in PSC[133], although recent data suggest that transplant with neoadjuvant chemoradiation with localized, node-negative hilar CCA may achieve better survival with less recurrence than conventional resection[145,147].

Recent studies have suggested that the incidence of CCA in patients with PSC treated with UDCA is lower than expected and decreases with time of therapy[141,148]. Further studies are needed to confirm this finding.

Colonic dysplasia and carcinoma: Whether the presence of PSC increases the risk of colonic dysplasia and carcinoma in ulcerative colitis is controversial[149,150]. Patients with PSC and UC have been found to have an increased incidence of colonic carcinomas compared to patients with ulcerative colitis alone in a few studies[136,151-153], however, contradictory results have also been presented[154,155]. The size, design, end-points, and populations involved in these studies have varied, and critical review suggests that colorectal cancer is more common in the setting of PSC[150]. Furthermore, PSC patients with UC remain at an increased risk for developing colorectal dysplasia and carcinoma after they have undergone liver transplantation[11,156]. The immunosuppressive treatment after liver transplantation may have an impact on the development of cancer.

Two studies have indicated that UDCA reduced the incidence of colonic dysplasias and/or carcinomas[157,158].

Gallbladder neoplasia: Dysplasia, adenomas and carcinoma of the gallbladder have been described in PSC but are less common than cholangiocarcinoma. PSC is recognized as one of the major risk factors for both gallbladder and bile duct carcinoma[159]. A recent study reported statistically significant association between hilar/intrahepatic biliary neoplasia and gallbladder neoplasia, suggesting a "field effect" in the intrahepatic and extrahepatic biliary tree in PSC[160]. Identification of gallbladder polyps on cross-sectional imaging should lead to consideration for cholecystectomy[123]. Large studies on this subject have not been performed.

Hepatocellular carcinoma: Although patients with cirrhotic stage PSC may also be at risk for developing hepatocellular carcinoma, this malignancy occurs infrequently[131,136,161].

PREGNANCY AND PSC

Little is known regarding the natural history and potential complications of pregnancy in patients with PSC. There are very few case reports[162,163] and one small series of thirteen pregnancies in 10 patients with PSC[164] that describe the fetal and maternal outcome of pregnancy in PSC. Although previously described, it appears that hepatic disease activity is not significantly worsened during the gestational period[2]. Nonetheless, patients with PSC who become pregnant require close monitoring[162]. Regular blood tests, including serum bilirubin and aminotransferase levels, are essential. In the event that the patient develops symptoms worrisome for obstruction, an ultrasound is a safe diagnostic test and may detect the presence of dominant strictures or stones; it lacks sensitivity however. MRC might have an emerging role in pregnancy, and more invasive tests such as ERCP might be required.

CONCLUSION

Primary sclerosing cholangitis is a presumed immune-mediated liver disease of young men associated with significant morbidity and mortality. However, there is no proven medical treatment available for it. Further studies are needed for better understanding of the pathophysiology of the disease and for development of an optimal therapeutic strategy for patients with PSC to improve health related quality of life and halt progression of disease, thereby decreasing incidence of complications of advanced liver disease, and the need for transplantation.

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TOPIC HIGHLIGHT

Pietro Invernizzi, MD; Ian R Mackay, MD, *Series Editors*

Etiopathogenesis of primary sclerosing cholangitis

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Abstract

Primary sclerosing cholangitis (PSC) is a chronic cholestatic liver disease of unknown etiology but lymphocytic portal tract infiltration is suggestive of an immune-mediated basis for this disease. Associations with inflammatory bowel disease (IBD) especially ulcerative colitis (UC), and with particular autoimmune diseases, as well as the genetic associations further suggest PSC may be an immune-mediated disease. The immunogenetics of PSC have been the subject of active research and several HLA and non-HLA associated genes have been implicated in the development of the disease. Lymphocytes derived from the inflamed gut may enter the liver *via* the enterohepatic circulation to cause hepatic disease. PSC may be triggered in genetically susceptible individuals by infections or toxins entering the portal circulation through a permeable colon and hence evoking an abnormal immune response.

Key words: Autoantibody; Immunogenetics; Biliary epithelial cells; T cell receptor; Lymphocytes

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INTRODUCTION

Primary sclerosing cholangitis (PSC) is a chronic disease of the intra and/or extrahepatic bile ducts. It is characterized by a concentric obliterative fibrosis that leads to bile duct strictures (Figure 1). In many, this in turn progresses to biliary cirrhosis and hepatic failure. Approximately one third of patients will develop cholangiocarcinoma[1]. PSC is frequently associated with inflammatory bowel disease (IBD) usually ulcerative colitis (UC) and those with Crohn's have disease predominantly affecting the colon. Approximately three quarters of the Northern European population with PSC have concomitant IBD particularly extensive UC[2]. 4.0%-7.5% of patients with UC have PSC[3].

The term "secondary sclerosing cholangitis (SSC)" is used for a disease with similar clinical features to PSC but where a direct causative agent for the pathological process is known. Such agents include choledocholithiasis with intraductal stones, surgical damage to bile ducts, ischaemia from hepatic artery occlusion, infections, and chemical agents such as drugs. Table 1 comprises a full list of possible causes of SSC with a section also showing the conditions which can mimic sclerosing cholangitis on cholangiography. There is little good data on the natural history of SSC and very little information regarding the immunological processes occurring during the progression of SSC is known although liver biopsies often show similar changes to those of PSC with ductopenia and patchy inflammation. The remainder of this chapter will concentrate on the etiopathogenesis of PSC.

The etiology and pathogenesis of PSC remain very poorly understood. The insidious onset of the disease makes the identification of an aetiological agent very unlikely. As the disease is associated with autoantibodies and HLA haplotypes as well as being closely related to IBD it would appear to be immune mediated. An autoimmune mediated destructive process is also suggested by lymphocytic infiltration into areas of portal damage.

PSC is not however a classical autoimmune disease, as it occurs with a 2:1 male predominance compared with the female predominance found in classical autoimmune diseases such as primary biliary cirrhosis (PBC) and autoimmune hepatitis (AIH). Moreover PSC does not have the characteristic response to immunosuppressive treatment as seen in classical autoimmune disease (Table 2).

Circumstantial evidence that PSC may be immune mediated comes from the independent association of PSC with a number of autoimmune diseases. 119 patients with PSC were studied by Saarinen *et al*[4]. Each

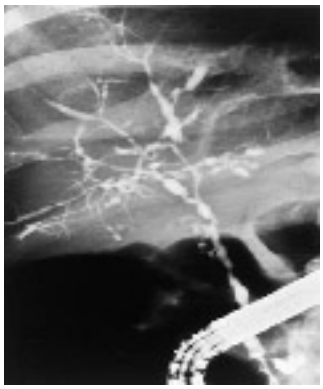

Figure 1 Cholangiogram showing beading and dilatation of the intra and extra hepatic bile ducts- the diagnostic features of primary sclerosing cholangitis (PSC).

Table 1 Causes and mimics of secondary sclerosing cholangitis (SSC)

	SSC
Causes	Surgical trauma to bile ducts
	Ischaemic injury eg after transplantation
	Hepatic arterial chemotherapy eg floxuridine
	Intraductal gallstones[3]
	Viral or bacterial infection eg CMV or cryptosporidiosis
	Caustic injury eg formalin treatment of hydatid disease
	Congenital abnormalities eg cystic fibrosis
Conditions mimicking sclerosing cholangitis on imaging	Malignancy eg metastatic carcinoma
	Hypereosinophilic syndrome
	Choledochal cyst

Table 2 The features of primary sclerosing cholangitis compared with classical autoimmune disease

Characteristic	Classical autoimmune disease	Immune-mediated inflammatory disease (such as IBD, psoriasis)	Primary sclerosing cholangitis
Age	Children and adults	Children and adults	Children and adults
Sex	Female predominance	No gender predilection	Male predominance
Autoantigens	Yes	No	No
Autoantibodies	Yes (pathogenic)	Yes (markers)	Yes (probably markers)
Associated autoimmune disease	Yes	Yes	Yes
HLA associations (class Ⅰ and Ⅱ)	Yes	Yes	Yes
Response to immunosuppression	Usually good	Often good	Good in children Poor in adults

PSC patient with IBD was matched to an IBD patient without PSC; 24% of the PSC patients had one or more autoimmune disorders outside the liver and colon compared with only 9% in the IBD group without PSC. Nine patients in the PSC group had 2 or more autoimmune diseases compared with only 2 in the IBD group. Diabetes mellitus and thyroid diseases were the most common in both groups. It is noteworthy that associated autoimmune disease did not seem to influence the outcome or clinical presentation of PSC[4].

Simultaneous or sequential occurrence of PSC and AIH has been described in both adult and pediatric populations[5]. The reported prevalence of this overlap syndrome is variable from 8%-54% and depends on the age of the study population, the type of scoring system used for diagnosis and the completeness of the analysed data.

In general, sclerosing cholangitis in children is characterized by more pronounced autoimmune features with a clinical overlap with AIH. This condition "autoimmune sclerosing cholangitis in childhood" has been addressed elsewhere in this issue (see Miele Verghani).

AUTOANTIBODIES

Atypical anti-neutrophil cytoplasmic antibodies (ANCA) are present in the serum of up to 88% patients with PSC (33%-88%)[5]. They are however not specific for PSC and are found in UC (60%-87%), and AIH (50%-96%)[6]. These ANCA are distinct from perinuclear-staining antineutrophil cytoplasmic antibody (p-ANCA) found in microscopic polyangiitis and cytoplasmic-staining antineutrophil cytoplasmic antibody c-ANCA in Wegener's granulomatosis.

Immunoblotting showed reactivity in 92% of IBD or hepatobiliary disease patients with an atypical p-ANCA to a myeloid specific nuclear protein with a molecular mass of 50 kDa[7]. The target antigen in PSC for these atypical ANCA is probably a neutrophil nuclear envelope protein. viz tubulin-beta isotype 5[8]. Terjung and colleagues have suggested that the term p-ANNA is therefore more appropriate as the recognised antigen is not cytoplasmic but originating in the nuclear membrane[7].

The importance of these autoantibodies in the develo-pment of PSC is unknown. Titres of ANCA correlate with disease activity in the systemic vasculitides, whereas in contrast there is a poor correlation between ANCA and clinical parameters in PSC[9-11]. Titres of ANCA remain unchanged after a transplant in PSC and after a colectomy in UC. Current evidence suggests that they are unlikely to play a role in the pathogenesis of PSC.

A high proportion of non-specific autoantibodies in addition to p-ANNA are found in patients with PSC (Table 3). They are of unclear relevance and unhelpful in diagnosis. These include anti nuclear antibodies (20%-67%), antimitochondrial antibodies (< 10%) and antithyroperoxidase antibodies (7%-16%)[5]. Anti-cardiolipin antibodies were found in 66% of PSC patients compared to 4% controls by Angulo but no

Table 3 Autoantibody prevalence in primary sclerosing cholangitis

Antibody	Prevalence (%)
Anti-nuclear antibody (ANA)	7-77
Anti-smooth muscle antibody (ASMA)	13-20
Anti-endothelial cell antibody (AECA)	35
Anti-cardiolipin antibody	4-66
Thyroperoxidase	7-16
Thyroglobulin	4
Rheumatoid factor	15

Anti-mitochondrial antibody is only rarely detected in PSC (< 10%). This is useful in differentiating PSC from primary biliary cirrhosis (PBC). Data taken from Angulo *et al*[12].

Table 4 Key HLA haplotypes in PSC[27]

	HLA haplotypes	Odds ratio
3 HLA haplotypes associated with an increased risk	B8-MICA*008-TNFA*2-DRB3*0101-DRB1*0301-DQB1*0201	2.69
	DRB3*0101-DRB1*1301-DQA1*0103-DQB1*0603	3.8
	MICA*008-DRB5*0101-DRB1*1501-DQA1*0102-DQB1*0602	1.52
3 HLA haplotypes associated with reduced risk (protective)	DRB4*0103-DRB4*0401-DQA1*03-DQB1*0302	0.26
	DRB4*0103-DRB1*0701-DQA1*0201-DQB1*0303	0.15
	MICA*002	0.12

resultant associations with thrombotic disease were demonstrated[12].

Significantly more PSC patients have autoantibodies to surface antigens expressed on biliary epithelial cells (BEC) than patients with PBC, AIH or normal controls. These induce increased expression of CD44 on the BEC and increased production of IL-6 by BEC[13]. Anti-BEC autoantibodies may be both IgM and IgG. IL-6 induces BEC proliferation *in vitro* and suppresses BEC apoptosis, and it is increased in the bile in cholangitis and in the serum in cholangiocarcinoma. Persistent IL-6 production may be in part, responsible for the bile duct changes seen in PSC.

Antibodies to the baker's yeast, Saccharomyces cerevisiae (ASCA) have been reported in IBD especially active Crohn's disease. ASCA are not autoantibodies but there does seem to be some genetic predisposition to their presence. ASCA has also been seen in autoimmune liver disease including PSC but no conclusions can be drawn from their presence[14].

IMMUNOGENETICS

PSC is not attributable to one gene locus and is a non-Mendelian (complex) disorder. A number of associations have been made with HLA haplotypes as well as a number of other genes. There is controversy as to whether there is a primary susceptibility allele but PSC is probably acquired through inheriting a combination of genetic polymorphisms that act together to cause susceptibility to disease. The genetics of PSC is still the subject of active research.

Major histocompatibility complex (MHC) genes in PSC

The MHC gene on the short arm of chromosome 6 encodes HLA molecules. Case control association studies have identified various HLA molecules and other immunoregulatory genes as determinants of disease susceptibility and progression in PSC. HLA molecules are highly polymorphic and have a central role in the T cell response. Class I molecules encode HLA A, B and Cw and class Ⅱ encode the *DR*, *DQ* and *DP* families. The Class Ⅲ region encodes a number of peptides which are active in the immune response including genes for TNFα and TNFβ, complement proteins C4, C2 and Bf and *MHC class I chain-related* (*MICA*) and *MICB* genes encoding the MHC class I chain related molecules α and β. Normal biliary cells express HLA class I and not class Ⅱ. HLA-DR, DQ and DP are aberrantly expressed on target cells in PSC.

There is an increased frequency of *HLA B8* and *DR3* (*HLA DRB1*0301*) in PSC compared with healthy controls as first described in 1982 and then confirmed in other studies[15-17]. A later study by Donaldson showed a secondary association with *DR2* in *DR3* negative patients[18]. An increase in *HLA-DR6* has also been observed in PSC patients[19,20]. *HLA B8* and *DR3* are in linkage disequilibrium. The *HLA B8, DR3* haplotype is also associated with several organ specific autoimmune diseases including lupoid chronic active hepatitis, type I diabetes mellitus, myasthenia gravis and thyrotoxicosis. There is no difference in class Ⅱ typing between PSC patients with and without autoimmune diseases outside the liver and colon suggesting association of PSC with autoimmune disease is not secondary to HLA but rather a primary phenomenon[4].

HLA DR4 is less common in PSC than in control populations and the significance of this is disputed[20]. Studies have suggested that although it has a protective effect against PSC development, when present it is associated with poor prognosis and possibly cholangiocarcinoma[19,21].

In rheumatoid arthritis (RA) more severe disease has also been seen with certain *DR4* alleles. Gow described the association of RA and PSC in 4 cases[22]. In three, the liver disease was unusually progressive, proceeding to cirrhosis in 14, 18 and 48 mo from diagnosis. It has been suggested therefore that RA in association with PSC may be a marker of patients at high risk of progression to cirrhosis. PSC also needs to be considered in all RA patients with cholestatic liver tests. The *DR3*, *DR2* heterozygote has been shown to be associated with an increased risk of death or liver transplant and a *DQ6* encoding haplotype in *DR3*, *DR2* negative individuals was associated with a reduced risk[19].

Molecular genotyping has identified 6 haplotypes that encode for peptides involved in the immune response in PSC (Table 4)[23].

The finding of multiple haplotypes associated with PSC indicates a complex relationship with the MHC. Susceptibility appears to involve either a combination

of *DR, DQ* and *MHC class I chain-like* (*MIC*) alleles or perhaps *MIC* alone. There is controversy concerning which allele or alleles within each haplotype may form the primary association.

MICA genes are a group of polymorphic genes on chromosome 6. They are localised in the class I region between *HLA-B* and *TNFA*. *MICA* molecules are stress and heat shock inducible and are expressed in non-diseased liver and on thymic and gastrointestinal epithelia. *MICA* has been identified as a ligand for γδ T cells, natural killer (NK) (CD56+) cells and cells expressing the NKG2D activatory receptor. Increased numbers of both γδ and NK cells have been documented in PSC livers[24,25].

An association between the *MICA*008* allele and PSC has been demonstrated by Norris *et al*[26] (which is due to an increased frequency of patients with 2 copies of this allele (i.e. homozygous). *MICA*008* is the main allele carrying the *MICA5.1* microsatellite allele. PSC has been found to be significantly associated with both the *MICA5.1* and the *MICB24* (MICB microsatellite) markers. The association was lost when stratified for *DR3* or *B8* positive and negative individuals. However, *B8* and *DR3* were associated with PSC only in the presence of these markers[27].

*MICA*002* has a strong negative association with disease and is the functional opposite of *MICA*008*. The *MICA*002* allele carries the *MICA9* microsatellite allele which is also therefore less common in PSC patients compared with controls as this allele has been shown to be protective. One copy of the *MICA*002* allele prevents PSC in most cases and so the resistant allele may be dominant[26,27].

Bernal first concluded that genetic susceptibility to PSC might be determined by polymorphism within the *TNF* genes[28]. The *TNF*-α gene is located in the class Ⅲ HLA region between the *HLA-B* and *DRB3* loci[29]. Increased frequency of the rare allele *-308A* (termed*TNF2*) of the *TNF* gene promotor has been reported in autoimmune disorders that include RA, systemic lupus erythematous and coeliac disease. Individuals with this allele may produce high levels of *TNF*-α. *TNF2* is in linkage disequilibrium with the extended *HLA-B8-DR3-DQ2* haplotype. The G to A substitution at position -308 in the *TNF*-α promotor has been shown by Mitchell *et al* to be associated with susceptibility to PSC, but this was secondary to the association with the *B8-DR3* haplotype[30].

Non-MHC genes in PSC

HLA haplotypes do not account for all of the susceptibility to develop PSC and genes outside the HLA region may also have a role in disease pathogenesis. Studies of non-MHC genes have failed to show an association between PSC and cytokine genes including IL-1β, IL-1RN and IL-10[30,31]. The *CD95* (*FAS*) gene (*TNFRSF6*), the gene encoding *CCR-5*, genes encoding *CTLA4* and the *Nod2* gene have also been examined in PSC. Karlsen *et al* have shown that genetic polymorphisms conferring susceptibility to IBD are not found in PSC/IBD patients. viz *CARD15*, *TLR-4*, *CARD4*, *SLC22A4*, *SLC22A5*, *DLG5* and *MDR1*[32]. The chemokine receptor-5 (CCR5) data are contradictory. *CCR5-Delta32* is a 32 base pair deletion associated with significant reduction in cell surface expression of the receptor. Melum *et al* showed no association of *CCR5-Delta32* with susceptibility or resistance to PSC contradicting earlier reports suggesting an association[33,34]. Cytotoxic T lymphocyte antigen-4 (CTLA-4) is expressed on activated T lymphocytes. It is a cell surface molecule that binds to the ligand CD80 (B.7) on antigen presenting cells. A *CTLA-4* gene polymorphism is described in several autoimmune diseases but in PSC this remains in question. The most recent and largest study was unable to demonstrate any effect in PSC[35].

PSC progression is related to periportal and septal fibrosis and this is associated with excess production and reduced degradation of extracellular matrix. This is regulated by a series of metalloproteinases (MMPs) and their naturally occurring inhibitors. There is a common polymorphism in the promotor sequence of the *stromelysin* (*MMP3*) gene with either a 5A or 6A repeat. The 5A allele is associated with increased transcription of stromelysin compared to the 6A variant. Satsangi *et al* in Oxford have found an association between the carriage rate of the 5A allele and susceptibility to PSC. 5A homozygosity was associated with development of portal hypertension[36]. This may suggest the MMP3 5A allele as a marker for fibrosis.

Wienke *et al* could not confirm the association of the *MMP-3 5A* allele with PSC and also found no general associations of the MMP-1 promotor polymorphism among Norwegian patients[37]. Patients with PSC who also had UC were found however to have an increased frequency of the *MMP-3* allele *5A* compared with PSC patients without UC (60% compared to 45%). All patients with cholangiocarcinoma were found to be carriers of the *MMP-1* allele *1G* compared with 72% of those with PSC who did not have cholangiocarcinoma.

Intracellular adhesion molecule-1 (*ICAM-1*, *CD54*) gene polymorphisms have been implicated in the susceptibility to a number of inflammatory conditions, including IBD. In PSC, studies have found that patients with advanced disease express ICAM on proliferating bile ductules and interlobular bile ducts. Increased soluble ICAM levels have been found in the serum of patients with PSC probably indicating activation of the immune system and inflammatory responses[38]. Yang *et al* have shown recently that, in British patients, the *ICAM-1* polymorphism K469E is associated with PSC and may be a protective allele. This association is independent of the coexistence of IBD. There is no relationship between the *ICAM-1* genotype and the rate of PSC progression[39]. These results were not confirmed in a Scandanavian population[40].

CELLULAR IMMUNE ABNORMALITIES IN PSC

There is a T cell predominant portal infiltrate in PSC

although the relative proportions and importance of the CD4 and CD8 cells are not known. CD4 cells are seen more commonly in the portal tracts and CD8 cells predominate in areas of interface hepatitis[41]. The cell infiltrate may change as the disease progresses. These cells are functional and are likely to be involved in the pathogenesis of disease. In the peripheral circulation there does appear to be a fall in CD8 cells as the disease progresses. This only occurs late in disease so is unlikely to be significant in disease pathogenesis[41-44].

Bo and colleagues showed that cell proliferation and function of liver derived T lymphocytes is impaired in PSC patients compared with liver derived T cells obtained from normal controls or patients with other autoimmune liver diseases[45]. They believe this is due to exposure to high levels of TNF *in vivo* and this exposure may be chronic.

Previously relatively high levels of TNF-α have been seen in T cell lines from liver biopsies in patients with different stages of PSC while decreased levels were observed in PBC patients. Therefore increased levels of TNF-α are present in PSC patients whether the disease be early or late stage[45-46].

T cells in PSC

PSC is characterized by a prominent T cell infiltrate in areas of portal damage. The T cell receptor (TCR) determines the specificity of T cells. It consists of two disulphide linked polypeptides, α and β. An alternative receptor, namely γδ has been identified. The predominant cell type is still αβ and the significance of T cells with γδ in PSC is not known[42]. TCR genes show genetic diversity but the *Vαβ* gene segment of the TCR can play a dominant role in recognition of certain peptide-MHC complexes. Expanded T cell populations using restricted sets of *TCR V* gene segments have been identified in areas of inflammation in the tissues affected in other immunopathic diseases such as RA and Sjogren's disease[47]. Broome reported the preferential expression in liver tissue of the Vβ3 region of the T cell receptor in PSC patients compared with liver tissue from PBC patients and healthy controls but no differences were seen in peripheral blood T cells[48]. This may indicate the presence of a specific antigen in the liver in PSC patients capable of driving the T cell production with this Vβ3 segment.

Oligoclonal T cell receptors that proliferate in culture with enterocytes and are cytotoxic to enterocyte cell lines were reported in PSC but this study is unconfirmed[49]. There are to date no studies of regulatory T cells (T regs) in PSC patients.

In summary, the available data do not as yet allow for any useful hypothesis on the T-cell contribution to the lesions of PSC.

BEC

BEC appear to act as the target for the immune response in PSC and are also an active participant in the immune reaction. They express a number of cytokines, enzymes, intracellular adhesion molecules (ICAM-1) and HLA molecules. Normal BEC express only HLA class I and not class Ⅱ whereas there is aberrant expression of class Ⅱ molecules on BEC in PSC[50-52], and also in PBC. Functionally important autoantibodies have been found to antigens on BEC in PSC. These induce BECs to produce IL-6 and increased expression of CD44. BEC however seem to lack the co stimulatory molecules necessary to activate T cells and unstimulated BEC inhibit T cell activation and this casts doubt upon the theory that BECs can act as antigen presenting cells[53,54].

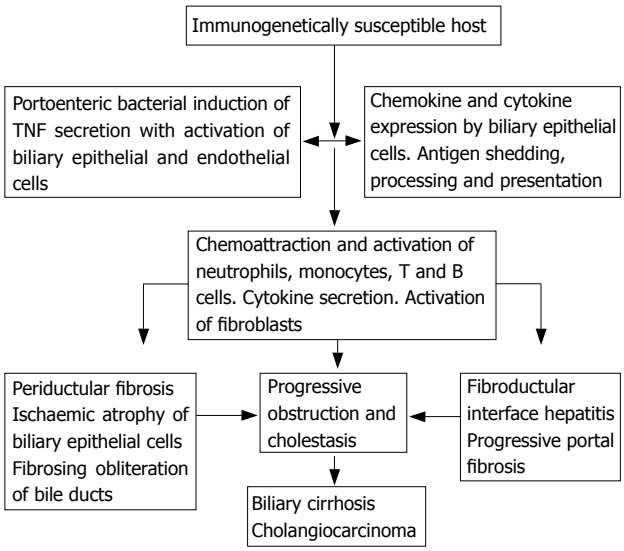

Figure 2 Vierling's hypothesis of the pathogenesis of primary sclerosing cholangitis[72].

However, it has become clear that cholangiocytes rather than being passive targets may play primary roles in the pathogenesis of peribiliary inflammation and periductular fibrosis in PSC[54,55]. Stimulation by proinflammatory cytokines induces cholangiocyte secretion of multiple chemokines, cytokines, and growth factors that immunomodulate inflammation and fibrogenesis[55]. The chemoattracted T cells include a population of PSC-specific T cells primed in the gut.

BACTERIA IN PSC

The association between PSC and IBD led to Vierling's hypothesis that colonic bacteria enter the portal circulation through a leaky mucosa in IBD thereby causing PSC (Figure 2)[55].

Bacterial antigens may act as molecular mimics in genetically susceptible people and cause an immune reaction responsible for initiating PSC. The bacteria are able to get through gut walls made permeable by colitis or in theory by any infective episode of acute infective or inflammatory colitis. Chemokines and cytokines are then released from Kupffer cells in the liver attracting macrophages/monocytes, lymphocytes, activated neutrophils and fibroblasts to the portal tracts. Vierling further suggested that the concentric fibrosis resulting could cause atrophy of the BEC secondary to ischaemia. The bile duct loss would lead to progressive cholestasis, further fibrosis and secondary biliary

cirrhosis. This does not explain however why there are fewer PSC patients with Crohn's colitis as compared with UC and why there can be an associated stricturing of the pancreatic duct.

Portal bacteremia has been described in UC patients undergoing colectomy[56]. A study looking at explanted livers showed higher bacterial positivity rates in bile and bile ducts in PSC patients compared with PBC patients, and α-haemolytic streptococci accounted for 46% of the bacterial strains found. Bile duct cannulation at endoscopic retrograde cholangiopancreatography (ERCP) could have accounted for this bacterial presence[57]. The study went on therefore to compare patients with PSC who had undergone ERCP to those who had not, in order to evaluate the potential role of these bacteria in the etiopathogenesis of PSC. Positive cultures were obtained from 3 of the naive PSC patients and from 6 of the PSC patients with previous ERCP. α-haemolytic streptococci were again the commonest bacteria seen. As most naive PSC patients were found to have negative bacterial cultures this bacteria is unlikely to play a primary role in etiopathogenesis but may be involved in disease progression[58].

Recent molecular studies have shown an increased prevalence of *H pylori* and other non-gastric *Helicobacter species* in cholestatic liver diseases compared with healthy controls and noncholestatic liver disease. In PSC positivity was significantly but weakly associated with UC[59].

Ponsioen *et al* have suggested an association between PSC and previous Chlamydia infection after the finding of an increase in seroprevalence of Chlamydia anti-lipopolysaccharide (LPS) antibodies in PSC patients, although no Chlamydia antibodies were found in liver tissue and thus the significance is unclear[60].

Among animal models, none has yet have been developed showing all the features of PSC, although a rat model in which there is small bowel bacterial overgrowth has shown hepatic injury somewhat similar to that seen in human PSC[61,62].

Abnormal accumulation of lipopolysaccharide (a bacterial endotoxin), presumably derived from portal blood, in the biliary epithelium has been shown in a rat model with a self-filling blind intestinal loop, and therefore may be involved in the pathogenesis of bile duct injury associated with intestinal injury[63]. Are these studies very persuasive?

LYMPHOCYTE HOMING

PSC is strongly linked to IBD but it also runs a course independent from the bowel disease illustrated by the fact that the disease can develop many years after colectomy. Grant *et al* hypothesized that T lymphocytes generated in the gut during active inflammation persist as long-lived memory cells and undergo enterohepatic circulation and can then trigger an inflammatory response in the liver when activated by an appropriate stimulus. The nature of the stimulus remains unclear; possibilities include hepatic expression of the original priming antigen or possibly mediation solely by the aberrant expression of gut specific adhesion molecules and chemokines[64].

There is overlapping expression of many molecules between the gut and liver including the two potential addressins vascular adhesion protein-1 (VAP-1) and mucosal addressin cell adhesion molecule-1 (MAdCAM-1). VAP-1 expression on liver endothelium is normally far stronger than that seen on mucosal vessels. In IBD gut expression is greatly increased, suggesting that lymphocytes from the liver may be able to enter the inflamed gut using VAP-1. MAdCAM-1 endothelial expression was thought to be restricted to the gut but has been recently seen on portal endothelium in inflammatory liver disease (including PSC) associated with IBD. Mucosal lymphocytes express $\alpha_4\beta_7$, which allows adhesion to hepatic MAdCAM-1 suggesting it may play a role in lymphocyte recruitment[65-67]. They propose that memory lymphocyte cells recirculate between liver and gut using either/both MAdCAM-1 and VAP-1[65].

The chemokine CCL21 activates lymphocyte adhesion to MAdCAM-1 dependent on $\alpha_4\beta_7$. CCL21, thought to only exist in secondary lymphoid tissue is upregulated in portal associated lymphoid tissue in PSC and plays an important role in recruiting lymphocytes. Expression of the gut-associated chemokine CCL25 (thymus-expressed chemokine (TECK)) has also been shown in PSC liver sinusoidal endothelium but was absent in liver in AIH/PBC. A significant population of CCR9+ mucosal lymphocytes (capable of binding CCL25) has been detected infiltrating PSC liver tissue compared with controls and matched peripheral blood, thus supporting the hypothesis of a T cell enterohepatic recirculation. CCR9 lymphocytes co-express the gut homing integrin $\alpha_4\beta_7$. Therefore CCL25 recruits CCR9+ lymphocytes to the liver in PSC by triggering adhesion to MAdCAM-1[66-67]. MAdCAM-1 and CCL25 are upregulated to the liver in inflammatory liver diseases whereas previously they were thought to be restricted to the gut. Conversely VAP-1, normally expressed in the liver, is up regulated in the gut in IBD[68].

However this does not explain why PSC is associated more with UC than Crohn's disease, as it would be predicted that just as many memory T cells are produced in Crohn's disease as in UC.

Hepatobiliary transporters in PSC

Defects in the hepatobiliary transport system have been shown to be the cause of a number hereditary cholestatic disorders eg progressive familial intrahepatic cholestasis and BSEP (bile salt export pump)[69]. This system is responsible for the hepatocellular uptake and excretion of bile salts into bile canaliculi. Defects in the transport system can result in bile duct injury.

Knockout mice for the *Mdr2* (*Abcb4*) gene, which corresponds to human *MDR3*/*ABCB4*, spontaneously develop sclerosing cholangitis with features similar to human PSC[70]. A non-functional multidrug resistance 3 (MDR3) protein leads to the formation of a "toxic" bile with increased concentration of free, non-micellar bile acids which cause BEC injury, pericholangitis, periductal fibrosis and, eventually, sclerosing cholangitis. Studies in

Table 5 Comparison of PSC and AIP-SC

	PSC	AIP-SC
Gender	M:F = 2:1	Probably some male predominance[81,85,87]
Clinical presentation	Usually insidious. Sometimes with obstructive jaundice secondary to cholangiocarcinoma.	Mild abdo/Back pain Sometimes with short history of obstructive jaundice due to CBD stricture
Associated inflammatory bowel disease	Yes	No
Cholangiographic findings	Diffuse changes throughout intra- and extrahepatic bile ducts. Abnormalities in pancreatic duct common.	Pancreatic duct strictures or narrowing. Often stricture of distal 1/3 of common bile duct. Intrahepatic duct changes less common.
Blood chemistry data	Often cholestatic but bilirubin usually near normal.	May be cholestatic. Bilirubin often high
Autoantibodies	Atypical pANCA plus range of others	Antibodies to carbonic anhydrase Ⅱ plus range of others[80,81,84]
Immunoglobulins	IgG4 levels normal	IgG4 levels usually elevated[82]
Histology	Absence of plasma cells positive for IgG4 on immunostaining	IgG4 positive plasma cells present in bile ducts and portal tracts[79]
Liver biopsy staging	Range of Ludwig staging including higher stages eg Ⅲ or Ⅳ	Ludwig staging usually only Ⅰ or Ⅱ[86]
Treatment	Ursodeoxycholic acid ± biliary drainage for dominant strictures	Systemic steroid therapy usually leads to complete resolution of symptoms and signs of disease. Occasionally patients relapse and require longer courses of steroids

Table 6 Evidence for the influence of immune mechanisms on the aetiology of PSC

	Evidence for the influence of immune mechanisms
Humoral immunity	Increased circulating immune complexes Elevated immunoglobulin levels (IgG and IgM) Low titres of non-organ specific autoantibodies (ANA and SMA) High titres of antineutrophil nuclear antibody (ANNA)
Cell mediated immunity	Decreased levels of circulating peripheral CD8+ve Tcells Portal T cell and NK cell infiltrate Increased activated and memory T cells Restricted T cell receptor repertoire (Vβ3) Aberrant expression of HLA-DR on BEC Coexpression of costimulatory molecules and HLA-DR on BECs Abnormal expression of adhesion molecules on biliary epithelial cells Abnormal expression of chemokine ligands on biliary epithelial cells
Immune effector mechanisms	Enhanced cytokine expression in the liver
Immunogenetic mechanisms	HLA associations

PSC patients, however, did not find MDR3 variations[71]. Similarly, the role of the cystic fibrosis transmembrane conductance regulator (CFTR) remains controversial[72-74]. The potential role of other hepatobiliary transporters eg BSEP, AE2 in the pathogenesis of PSC remains to be explored. As defects in these systems are known to cause bile duct injury and cholangitis, they are excellent candidates for further investigation.

The nuclear receptor SXR is a nuclear bile acid receptor which plays an important role in endogenous bile acid homeostasis and cholesterol synthesis. A recent study of PSC patients has shown that functional *SXR* gene variants modify the disease progression and affect survival[75].

Autoimmune pancreatitis (IgG4 associated sclerosing cholangitis)

Sarles *et al*[76] in 1961 provided the first description of what was later identified as autoimmune pancreatitis, an increasingly recognised benign inflammatory disease of the pancreas[77]. Abnormalities and sclerosing changes in both the intra- and extra hepatic bile ducts are well recognised in AIP (see pp this issue), and can cause diagnostic confusion with PSC. Correct diagnosis is important as AIP responds well to corticosteroid therapy and tends to have a significantly better outcome than PSC[71-81]. The association of AIP and sclerosing changes in the bile ducts has been termed AIP-SC[81-85]. Diagnositic criteria for AIP have been proposed and developed by the Japan Pancreas society[86]. These criteria consist of the finding of a diffuse narrowing of the pancreatic duct on imaging studies, and either a laboratory finding of an abnormally elevated serum gamma globulin, IgG, or more particularly IgG4 or the presence of autoantibodies or classical histopathological features of the disease ie fibrotic changes with a lymphocyte and, characteristically, plasma cell infiltration. The differences between the two conditions are summarised in Table 5.

CONCLUSION

Immune mechanisms play an important role in the pathogenesis of PSC, although it is remains unclear whether it is a classical autoimmune disease (Tables 2 and 6). There are strong MHC genetic associations including HLA molecules and the MIC molecules. HLA haplotypes however do not account for all the genetic susceptibility in the development of PSC and there is uncertainty about the importance of genes outside this region. Bacterial antigens may act as molecular mimics in hosts who are genetically susceptible and therefore cause an immune reaction leading to PSC initiation.

Lymphocytes may move from the inflamed gut in IBD *via* the enterohepatic circulation and cause inflammation of the liver when activated by a specific stimulus such as bacterially derived antigens.

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TOPIC HIGHLIGHT

Pietro Invernizzi, MD; Ian R Mackay, MD, *Series Editors*

Autoimmune paediatric liver disease

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Abstract

Liver disorders with a likely autoimmune pathogenesis in childhood include autoimmune hepatitis (AIH), autoimmune sclerosing cholangitis (ASC), and *de novo* AIH after liver transplantation. AIH is divided into two subtypes according to seropositivity for smooth muscle and/or antinuclear antibody (SMA/ANA, type 1) or liver kidney microsomal antibody (LKM1, type 2). There is a female predominance in both. LKM1 positive patients tend to present more acutely, at a younger age, and commonly have partial IgA deficiency, while duration of symptoms before diagnosis, clinical signs, family history of autoimmunity, presence of associated autoimmune disorders, response to treatment, and long-term prognosis are similar in both groups. The most common type of paediatric sclerosing cholangitis is ASC. The clinical, biochemical, immunological, and histological presentation of ASC is often indistinguishable from that of AIH type 1. In both, there are high IgG, non-organ specific autoantibodies, and interface hepatitis. Diagnosis is made by cholangiography. Children with ASC respond to immunosuppression satisfactorily and similarly to AIH in respect to remission and relapse rates, times to normalization of biochemical parameters, and decreased inflammatory activity on follow up liver biopsies. However, the cholangiopathy can progress. There may be evolution from AIH to ASC over the years, despite treatment. *De novo* AIH after liver transplantation affects patients not transplanted for autoimmune disorders and is strikingly reminiscent of classical AIH, including elevated titres of serum antibodies, hypergammaglobulinaemia, and histological findings of interface hepatitis, bridging fibrosis, and collapse. Like classical AIH, it responds to treatment with prednisolone and azathioprine. *De novo* AIH post liver transplantation may derive from interference by calcineurin inhibitors with the intrathymic physiological mechanisms of T-cell maturation and selection. Whether this condition is a distinct entity or a form of atypical rejection in individuals susceptible to the development of autoimmune phenomena is unclear. Whatever its etiology, the recognition of this potentially life-threatening syndrome is important since its management differs from that of standard anti-rejection therapy.

Key words: Autoimmune hepatitis; Autoimmune sclerosing cholangitis; Liver transplant; Children

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INTRODUCTION

Autoimmune liver disorders of childhood are inflammatory liver diseases characterized histologically by a dense mononuclear cell infiltrate in the portal tract and serologically by the presence of non-organ and liver specific autoantibodies and increased levels of transaminases and IgG, in the absence of a known etiology. They usually respond to immunosuppressive treatment, which should be instituted as soon as diagnosis is made. In children, as well as in young adults, autoimmune hepatitis (AIH) often presents acutely and has a more aggressive course than in older patients. The previously accepted requirement of 6 mo duration of symptoms before diagnosis can be made has been abandoned. In children, there are two liver disorders in which the liver damage is likely to arise from an autoimmune attack: classical AIH and AIH/sclerosing cholangitis overlap syndrome (autoimmune sclerosing cholangitis, ASC). A possible autoimmune pathogenesis has also been postulated for the so called post liver transplantation "*de novo* AIH", a condition originally described in children and later confirmed in adults. According to data collected at the Kings College Hospital tertiary center, there is an increase in

the yearly prevalence of AIH and ASC in childhood, although referral bias may play a role. Thus, in the 1990s these conditions were diagnosed in 2.3% of about 400 children older than 4 mo referred during one year, while in the 2000s their incidence increased to 12%.

AUTOIMMUNE HEPATITIS (AIH)

Clinical features

Two types of childhood AIH are recognized: AIH type 1 is characterized by the presence of smooth muscle (SMA) and/or antinuclear (ANA) antibodies; AIH type 2 is positive for anti liver kidney microsomal type 1 (anti-LKM-1) antibody[1]. Type 1 AIH represents two thirds of the cases and is a disease of children and adults, while type 2 AIH is mainly described in children. Severity of disease is similar in the two types of AIH[1]. In both, there is a predominance of girls (75%-80%). Anti-LKM-1 positive patients are younger and have a greater tendency to present with acute liver failure, but the duration of symptoms before diagnosis and the frequency of hepatosplenomegaly are similar in both groups. Both have a high frequency of associated autoimmune disorders (about 20%) and a family history of autoimmune disease (40%). Associated autoimmune disorders include thyroiditis, inflammatory bowel disease, vitiligo, insulin-dependent diabetes, nephrotic syndrome in both types[1]. Type 2 AIH can be associated to autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), an autosomal recessive genetic disorder in which the liver disease is reportedly present in about 20% of the cases[2].

There are three clinical patterns of disease onset[1]: (1) in at least 40% of patients, the presentation is indistinguishable from that of an acute viral hepatitis (non-specific symptoms of malaise, nausea/vomiting, anorexia, and abdominal pain, followed by jaundice, dark urine, and pale stools), some children, particularly anti-LKM-1 positive, develop acute hepatic failure with grade Ⅱ to Ⅳ encephalopathy 2-8 wk from onset of symptoms; (2) in 25%-40% of patients, the onset is insidious, with an illness characterized by progressive fatigue, relapsing jaundice, headache, anorexia, and weight loss, lasting from several months and even years before diagnosis; (3) in about 10% of patients, there is no history of jaundice, and the diagnosis follows presentation with complications of portal hypertension, such as splenomegaly, hematemesis from esophageal varices, bleeding diathesis, chronic diarrhea, and weight loss. The mode of presentation of AIH in childhood is therefore variable, and the disease should be suspected and excluded in all children presenting with symptoms and signs of prolonged or severe liver disease. The course of the disease can be fluctuating, with flares and spontaneous remissions, a pattern which may result in delayed referral and diagnosis. The majority of children, however, even those presenting with acute hepatitis, on physical examination reveal clinical signs of an underlying chronic liver disease, i.e. cutaneous stigmata (spider nevi, palmar erythema, leukonychia, striae), firm liver and splenomegaly; at ultrasound the liver parenchyma is often nodular and heterogeneous.

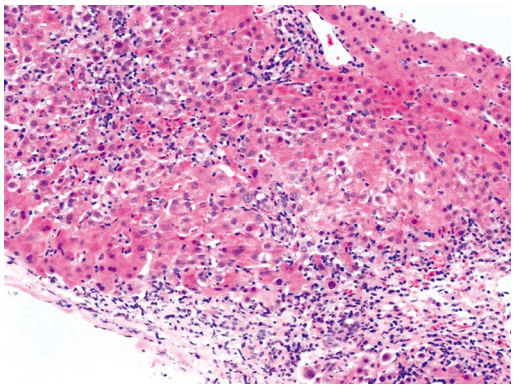

Figure 1 Portal and periportal lymphocyte and plasma cell infiltrate, extending to and disrupting the parenchymal limiting plate (interface hepatitis). Swollen hepatocytes, pyknotic necroses, and acinar inflammation are present. HE staining (Picture kindly provided by Dr. Alberto Quaglia).

Diagnosis and laboratory findings

Diagnosis of AIH is based on a series of positive and negative criteria defined by the International AIH Group (IAIHG)[3,4]. Though these criteria have been produced mainly for research purposes, they have been validated also in the clinical practice. Liver biopsy is necessary to establish the diagnosis of AIH, the typical histological picture include: a dense mononuclear and plasma cell infiltration of the portal areas, which expands into the liver lobule; destruction of the hepatocytes at the periphery of the lobule with erosion of the limiting plate ("interface hepatitis"); connective tissue collapse resulting from hepatocyte death and expanding from the portal area into the lobule ("bridging collapse"); hepatic regeneration with "rosette" formation (Figure 1). In addition to the typical histology, other positive criteria include elevated serum transaminase and IgG/gammaglobulin levels, and presence of ANA, SMA, or anti-LKM-1. Negative criteria relevant to the paediatric age are evidence of infection with hepatitis B or C virus, Wilson disease, and drug or alcohol consumption.

Autoantibodies: A key criterion for the diagnosis of AIH is the detection of ANA, SMA, and anti-LKM-1 by indirect immunofluorescence. Autoantibody detection not only assists in the diagnosis but also allows, as mentioned above, differentiation of AIH in type 1 and type 2. ANA and SMA and anti-LKM-1 are practically mutually exclusive[5]; in those rare instances when they are present simultaneously, the child is classified as having AIH type 2. Recognition and interpretation of the immunofluorescence patterns is not always straightforward[5]. The operator dependency of the technique and the relative rarity of AIH explain the non-infrequent occurrence of errors in reporting, particularly of less frequent specificities such as anti-LKM-1. Problems do exist between laboratory reporting and clinical interpretation of the results that are partly dependent on clinicians' unfamiliarity with the disease spectrum of AIH, but also partly dependent on

insufficient standardization of the tests. This problem is being addressed by the autoimmune serology committee of the IAIHG[5].

The basic technique for the routine testing of autoantibodies relevant to AIH is indirect immunofluorescence on a freshly prepared rodent substrate that should include kidney, liver, and stomach to allow the detection of ANA, SMA, anti-LKM-1 as well as anti liver cytosol type 1 (anti-LC-1, see below), but also of anti-mitochondrial antibody (AMA), the serological hallmark of primary biliary cirrhosis, a disease affecting almost exclusively adults. Since a high proportion of healthy adults may show ANA or SMA reactivity at the conventional starting serum dilution of 1/10, the arbitrary dilution of 1/40 is considered clinically significant by the IAIHG in the adult population. In contrast, in healthy children autoantibody reactivity is infrequent, so that titers of 1/20 for ANA and SMA and 1/10 for anti-LKM-1 are clinically relevant. Hence, the laboratory should report any level of positivity from 1/10, and the attending physician should interpret the result within the clinical context and the age of the patient.

ANA is detectable as a nuclear staining in kidney, stomach, and liver. Its pattern can be homogeneous, or coarsely or finely speckled. In most cases of AIH, but not in all, the pattern is homogeneous. For a clearer and easier definition of the nuclear pattern, HEp2 cells that have prominent nuclei can be used. These cells, however, should not be used for screening purposes, because nuclear reactivity to HEp2 cells is frequent at low serum dilution (1/40) in the normal population[6]. ANA reactivity is not specific to AIH, being detectable in chronic viral hepatitis B and especially C, though at lower titre, and in non-hepatic autoimmune disorders.

SMA is detected on kidney, stomach, and liver. On the renal substrate, it is possible to visualize a V (vessels), G (glomeruli), and T (tubules) staining[7]. VG and VGT patterns are the most frequently detected in AIH[8]. The VGT pattern corresponds to the so called "F actin" or microfilament (MF) pattern observed using cultured fibroblasts as substrate. Though "anti-actin" reactivity is present in the majority of patients with AIH type 1, some 20% of SMA positive AIH type 1 patients do not have the F-actin/VGT pattern[8]. The absence, therefore, of anti-actin SMA does not exclude the diagnosis of AIH[8]. As for ANA, SMA can be found in chronic viral hepatitis B or C and extrahepatic autoimmune disorders.

Anti-LKM-1 stains brightly the liver cell cytoplasm and the P3 portion of the renal tubules, but does not stain gastric parietal cells. Anti-LKM-1 is often confused with AMA, since both autoantibodies stain liver and kidney, though AMA stains the liver more faintly and the renal tubules more diffusely with an accentuation of the small distal ones and, in contrast to anti-LKM-1, it also stains the gastric parietal cells. In the context of childhood AIH, patients reported to be AMA positive are almost invariably positive for anti-LKM-1, since AMA positive AIH in children is extremely rare[9] and PBC even rarer, only two cases having been documented histologically and immunoserologically, both being teenage girls[10]. The identification of the molecular targets of anti-LKM-1, i.e. cytochrome P4502D6, and of AMA, i.e. enzymes of the 2-oxo-acid dehydrogenase complexes, has led to the establishment of commercial immunoassays based on the use of the recombinant or purified antigens[11] that can resolve any doubts remaining after immunofluorescence examination. Anti-LKM-1 is highly specific for AIH type 2, being found outside this condition in a small proportion (-5%) of patients chronically infected by the hepatitis C virus, that usually possess the human leukocyte antigen (HLA) allele *DRB1*07*.

Other autoantibodies less commonly tested but of diagnostic importance in paediatric AIH include those to liver cytosol type 1 (LC-1), anti-neutrophil cytoplasm (ANCA) and soluble liver antigen (SLA). Anti-LC-1, which can be present on its own, but frequently occurs in association with anti-LKM-1, is an additional marker for AIH type 2 and targets formimino-transferase cyclodeaminase (FTCD)[12]. In AIH type 1, as well as in inflammatory bowel disease and sclerosing cholangitis, ANCA is frequently found and targets a peripheral nuclear perinuclear antigen (hence the suggested name of pANNA, i.e. peripheral anti nuclear neutrophil antibody). pANNA is virtually absent in type 2 AIH[7]. Anti-SLA that was originally described as the hallmark of a third type of AIH[13], is also found in some 50% of paediatric patients with type 1 and type 2 AIH, where it defines a more severe course[14].

There are a small proportion of children with AIH without detectable autoantibodies. The prevalence and the clinical characteristics of this rare seronegative form of AIH, which responds to immunosuppression similarly to the seropositive forms, remain to be defined.

Comparison between type 1 and type 2 AIH: Clinical, laboratory and histological features of type 1 and 2 AIH are summarized in Table 1. In Northern Europe, type 1 AIH is associated with the presence of human leukocyte antigen (HLA) *DRB1*03*[1,11], while type 2 AIH is associated with the presence of *DRB1*07* and, less frequently, with *DRB1*03*[15]. In South America, the HLA *DRB1*1301* allele is reported to predispose to paediatric AIH type 1 and is also associated with persistent infection with the endemic hepatitis A virus[16,17]. Interestingly, in Northern European children HLA *DRB1*1301* is associated with autoimmune sclerosing cholangitis (see below). It is conceivable that some South American children diagnosed as having AIH type 1, but in whom routine cholangiograms were not performed, indeed had ASC.

Paediatric patients with AIH, whether anti-LKM-1 or ANA/SMA positive, have isolated partial deficiency of the HLA class Ⅲ complement component C4, which is genetically determined[18].

Anti-LKM-1-positive patients have higher levels of bilirubin and transaminases at presentation than those who are ANA/SMA positive and present significantly more frequently with fulminant hepatic failure[1]. Excluding children with the fulminant presentation, a

Table 1 Clinical, laboratory, and histological features at presentation of autoimmune hepatitis type 1, autoimmune hepatitis type 2, and autoimmune sclerosing cholangitis[1,20]

	Type 1 AIH	Type 2 AIH	ASC
Median age in year	11	7	12
Females (%)	75	75	55
Mode of presentation (%)			
Acute hepatitis	47	40	37
Acute liver failure	3	25	0
Insidious onset	38	25	37
Complication of chronic liver disease	12	10	26
Associated autoimmune diseases (%)	22	20	48
Inflammatory bowel disease (%)	20	12	44
Family history of autoimmune disease (%)	43	40	37
Abnormal cholangiogram (%)	0	0	100
ANA/SMA (%)	100	25	96
Anti LKM1 (%)	0	100	4
pANCA (%)	45	11	74
Anti SLA (%)[1]	58	58	41
Increased IgG level (%)	84	75	89
Partial IgA deficiency (%)	9	45	5
Low C4 level (%)	89	83	70
Increased frequency of HLA *DR*0301*	Yes	No[2]	No
Increased frequency of HLA *DR*0701*	No	Yes	No
Increased frequency of HLA *DR*1301*	No	No	Yes
Interface hepatitis (%)	66	72	35
Biliary features (%)	28	6	31
Cirrhosis (%)	69	38	15
Remission after immunosuppressive Treatment (%)	97	87	89

AIH: Autoimmune hepatitis; ASC: Autoimmune sclerosing cholangitis; ANA: Anti-nuclear antibodies; SMA: Anti-smooth muscle antibody; LKM1: Liver kidney microsomal type 1 antibody; pANCA: Perinuclear anti-neutrophil cytoplasmic antibody; SLA: Soluble liver antigen; C4: C4 component of complement; HLA: Human leukocyte antigen. [1]Measured by radioligand assay; [2]Increased in HLA *DR*0701* negative patients.

severely impaired hepatic synthetic function, as assessed by the presence of both prolonged prothrombin time and hypoalbuminemia, is more common in ANA/SMA-positive than in anti-LKM-1 positive patients. The vast majority of patients have increased levels of IgG, but some 20% do not[1], indicating that normal IgG values do not exclude the diagnosis of AIH. Partial IgA deficiency is significantly more common in LKM1-positive than in ANA/SMA-positive patients[1,19].

The severity of interface hepatitis at diagnosis is similar in both types, but cirrhosis on initial biopsy is more frequent in type 1 than in type 2 AIH, suggesting a more chronic course of disease in the former. Of note is that most patients already cirrhotic at diagnosis present with a clinical picture reminiscent of that of prolonged acute viral-like hepatitis. Multiacinar or panacinar collapse, which suggests an acute liver injury, is more frequently seen in type 2 AIH. The question as to whether the acute presentation in these patients represents a sudden deterioration of an underlying unrecognized chronic process or a genuinely acute liver damage remains open. Progression to cirrhosis during treatment is more frequent in type 1 AIH. As mentioned above, in both a more severe disease and a higher tendency to relapse is associated to the possession of antibodies to soluble liver antigen (SLA), which are present in about half of the patients with AIH type 1 or 2 at diagnosis[11].

Differential diagnosis: Since positive autoimmune serology can be present in conditions other than AIH, in particular ASC[20], chronic hepatitis B[21] or C[22] virus infections, and Wilson disease[23], all these disorders must be considered in the differential diagnosis and excluded. ASC, described below, shares the same serological profile as type 1 AIH, but has typical bile duct lesions on cholangiography. Up to 50% of children with hepatitis B and C are positive for ANA and/or SMA, usually at low titres[21,22], and some 5% of patients with chronic hepatitis C have anti-LKM-1 antibodies. In these patients the histology can also mimic AIH, though usually the degree of inflammation is milder. Detection of the typical viral markers allows a correct diagnosis. ANA, and at times SMA, can be present in Wilson disease, in association with high IgG and an inflammatory liver histology, which can make the differential diagnosis with AIH type 1 difficult. Urinary, serum, and liver tissue copper studies and search for Kayser Fleischer rings should be performed in all cases.

APECED: APECED is a monogenic disorder[24,25] with a variable phenotype. About 20% of the cases develop AIH that resembles AIH type 2[2]. This condition, also known as autoimmune polyendocrine syndrome 1 is an autosomal recessive disorder caused by homozygous mutations in the *AIRE1* gene and characterized by a variety of organ-specific autoimmune diseases, the most common of which are hypoparathyroidism and primary adrenocortical failure, accompanied by chronic mucocutaneous candidiasis.

Etiology and pathogenesis

The etiology of AIH is unknown, though both genetic and environmental factors are involved in its expression. Etiological hypotheses and possible mechanisms leading to the liver autoimmune attack are described under "Etiopathogenesis of AIH" in this issue.

Management and prognosis

AIH is exquisitely responsive to immunosuppression. The rapidity and degree of response depends on the disease severity at presentation. All types of presentations, apart from fulminant hepatic failure with encephalopathy, respond to standard treatment with prednisolone with or without azathioprine.

Standard treatment for AIH consists of prednisolone 2 mg/kg per day (maximum 60 mg/d), which is gradually decreased over a period of 4 to 8 wk with progressive normalization of the transaminases, and then the patient is maintained on the minimal dose able to sustain normal transaminase levels, usually 2.5 mg/d or 5 mg/d depending on age[1,26]. During the first 6 to 8 wk of treatment, liver function tests should be checked weekly to allow a frequent fine-tuning, avoiding severe steroid side effects. If progressive normalization of the liver function tests is not obtained over this period of time

or if too high a dose of prednisolone is required to maintain normal transaminases, azathioprine is added at a starting dose of 0.5 mg/kg per day, which, in the absence of signs of toxicity, is increased up to a maximum of 2-2.5 mg/kg per day until biochemical control is achieved. Azathioprine is not recommended as first-line treatment because of its hepatotoxicity in severely jaundiced patients, but 85% of the patients will eventually require azathioprine addition. A preliminary report in a cohort of 30 children with AIH suggests that the measurements of the azathioprine metabolites 6-thioguanine and 6-methylmercaptopurine are useful in identifying drug toxicity and non-adherence and in achieving a level of 6-thioguanine considered therapeutic for inflammatory bowel disease[27], though what is an ideal therapeutic level for AIH has not been determined. Although an 80% decrease of initial transaminase activity is obtained within 6 wk from starting treatment in most patients, complete normalization of liver function may take several months. In the King's series, normalization of serum transaminase activity occurred at median of 6 mo in ANA/SMA positive children and 9 mo in LKM-1 positive children[1]. Relapse while on treatment is common, occurring in about 40% of the patients and requiring a temporary increase of the steroid dose. An important role in relapse is played by non-adherence that is common, particularly in adolescents[28]. Moreover, the risk of relapse is higher if steroids are administered on an alternate-day schedule, often instituted in the belief that it has a less negative effect on the child's growth. Small daily doses are more effective in maintaining disease control and minimize the need for high-dose steroid pulses during relapses, with consequent more severe side effects[1].

A question frequently asked by the parents of teenaged girls is whether treatment can be safely continued during pregnancy. Although the experience is limited, there does not appear to be adverse events for mother and baby[29]. In particular, no teratogenic effects have been described with azathioprine in humans, though for women concerned about its use, treatment with steroids alone can be used.

Cessation of treatment is considered if a liver biopsy shows minimal or no inflammatory changes after at least one year of normal liver function tests. However, it is advisable not to attempt to withdraw treatment within three years from diagnosis or during or immediately before puberty, when relapses are more common. The reasons for this are unclear, though an important role may be played by non-adherence, as mentioned above. In the Kings experience, successful long-term withdrawal of treatment was achieved in 20% of patients with AIH type 1, but in none with AIH type 2[1].

In paediatrics, an important role in monitoring the response to treatment is the measurement of autoantibody titers and IgG levels, the fluctuation of which is correlated with disease activity[30].

Despite the efficacy of standard immunosuppressive treatment, severe hepatic decompensation may develop even after many years of apparently good biochemical control, leading to transplantation 10-15 years after diagnosis in 10% of the patients. In the Kings College Hospital series[1], over 97% of the patients treated with standard immunosuppression were alive between 0.3 and 19 years (median 5 years) after diagnosis, including 8% after liver transplantation. Side effects of steroid treatment were mild, the only serious complication being psychosis during induction of remission in 4%, which resolved after prednisolone withdrawal. All patients developed a transient increase in appetite and mild cushingoid features during the first few weeks of treatment. After five years of treatment, 56% of the patients maintained the baseline centile for height or went up across a centile line, 38% dropped across one centile line, and only 6% dropped across two centile lines[31]. Moreover, it has recently been shown that long-term daily treatment with prednisolone in children with autoimmune liver disease does not affect their expected final adult height according to parental stature[32].

Sustained remission, achieved with prednisolone and azathioprine, can be maintained with azathioprine alone in some children with AIH type 1, akin to the experience in adults[33], but not in AIH type 2.

In those patients (up to 10%) in whom standard immunosuppression is unable to induce stable remission or who are intolerant to azathioprine, mycophenolate mofetil at a dose of 20 mg/kg twice daily can be successfully used[31]. In case of persistent no response or of intolerance to mycophenolate mofetil (headache, diarrhea, nausea, dizziness, hair loss, or neutropenia), the use of calcineurin inhibitors (cyclosporine A or tacrolimus) should be considered.

Children who present with acute hepatic failure pose a particularly difficult therapeutic problem. If not encephalopathic, they usually benefit from conventional immunosuppressive therapy, but only one of the six children with acute liver failure and encepaholpathy in the Kings series responded to immunosuppression and survived without transplantation[1].

AUTOIMMUNE SCLEROSING CHOLANGITIS

ASC has the same prevalence as AIH type 1 in childhood[18]. This has been shown in a prospective study conducted over a period of 16 years, in which all children with serological (i.e. positive autoantibodies, high IgG levels) and histological (i.e. interface hepatitis) features of autoimmune liver disease underwent a cholangiogram at the time of presentation. Approximately 50% of these patients had alterations of the bile ducts characteristic of sclerosing cholangitis, though generally less advanced than those observed in adult primary sclerosing cholangitis (Figure 2). A quarter of the children with ASC, despite abnormal cholangiograms, had no histological features suggesting bile duct involvement and the diagnosis of sclerosing cholangitis was only possible because of the cholangiographic studies. Virtually all patients were seropositive for

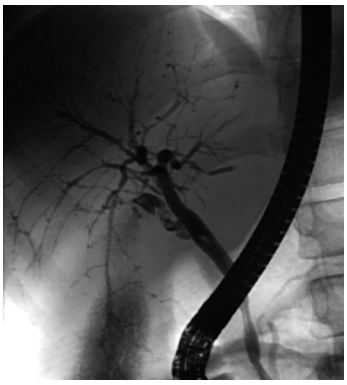

Figure 2 Retrograde cholangiogram of a child with autoimmune sclerosing cholangitis showing widespread bile duct strictures and dilatations (Picture kindly provided by Dr. Maria Sellars).

Table 2 Laboratory parameters at presentation in children with autoimmune hepatitis and autoimmune sclerosing cholangitis[20]

	AIH	ASC
Bilirubin (nv < 20 micromol/L)	35 (4-306)	20 (4-179)
Albumin (nv > 35 g/L)	35 (25-47)	39 (27-54)
AST (nv < 50 IU/L)	333 (24-4830)	102 (18-1215)
INR (< 1.2)	1.2 (0.96-2.5)	1.1 (0.9-1.6)
GGT (nv < 50 IU/L)	76 (29-383)	129 (13-948)
AP (nv < 350 IU/L)	356 (131-878)	303 (104-1710)

AST: Aspartate aminotransferase; INR: International normalized prothrombin ratio; GGT: Gamma glutamyl transpeptidase; AP: Alkaline phosphatase; nv: Normal values.

ANA and/or SMA. Fifty-five percent were girls, and the mode of presentation was similar to that of typical AIH. Inflammatory bowel disease was present in about 45% of children with ASC compared to about 20% of those with AIH, and 90% of children with ASC had greatly increased serum IgG levels. At the time of presentation, standard liver function tests did not help in discriminating between AIH and ASC (Table 2), though the alkaline phosphatase/aspartate aminotransferase ratio was significantly higher in ASC. pANNA were present in 74% of patients with ASC compared to 45% of patients with AIH type 1 and 11% of those with AIH type 2. Susceptibility to ASC in children is conferred by the presence of HLA *DRB1*1301*[32]. Clinical, laboratory, and histological features of type 1 and 2 AIH and ASC are compared in Table 1.

Children with ASC respond to the same immunosuppressive schedule described above for AIH[18], liver test abnormalities resolving within a few months after starting treatment in most patients. Steroids and azathioprine, however, though beneficial in abating the parenchymal inflammatory lesions, appear to be less effective in controlling the bile duct disease. Following favorable reports in adult primary sclerosing cholangitis[33,34], ursodeoxycholic acid is added at the dose of 20-30 mg/kg per day, though there is no information as to whether it is helpful in arresting the progression of ASC. Akin to AIH, measurement of autoantibody titers and IgG levels is useful in monitoring disease activity and response to treatment[20]. The medium-term prognosis is good, with a reported 7-year survival of 100%, though 15% of the patients required liver transplantation during this period of follow-up[18]. Evolution from AIH to ASC has been documented suggesting that AIH and ASC are part of the same pathogenic process[18].

The prospective study conducted at Kings College Hospital shows that in childhood ASC and AIH have a similar prevalence[20]. It also shows that ASC is more frequent than sclerosing cholangitis without autoimmune features[20], autoantibody negative sclerosing cholangitis having been observed in only 9 children referred over the 16-year study period[20].

Whether childhood ASC and adult PSC belong to the same disease spectrum remains to be established, since no prospective study in a large patient cohort has investigated at the time of presentation the presence of bile duct damage in adults with features of autoimmune liver disease. Interestingly, in a retrospective study, a high proportion of adult patients originally diagnosed as having AIH type 1 were found to have sclerosing cholangitis on magnetic resonance cholangiography[34]. The long term follow up of the Kings paediatric ASC series will provide important information about the possible links between ASC and PSC.

DE NOVO AIH AFTER LIVER TRANSPLANTATION

In the late 1990s, it was observed that AIH can arise *de novo* after liver transplantation in children who had not been transplanted for autoimmune liver disease[35]. Characteristic of this condition is a histological picture of interface hepatitis and multilobular collapse associated with increased IgG levels and positive autoantibodies. These include ANA, SMA, and classical anti-LKM-1, but also atypical anti-LKM-1, staining the renal tubules but not the liver. After this original report, *de novo* AIH after liver transplantation has been confirmed by several studies both in adult and paediatric patients[36,37]. Importantly, treatment with prednisolone and azathioprine using the same schedule for classical AIH, concomitant with reduction of the calcineurin inhibitor dose, is highly effective in *de novo* AIH, leading to excellent graft and patient survival. It is of interest that these patients do not respond satisfactorily to standard anti-rejection treatment, making it essential to reach an early diagnosis to avoid graft loss.

Whether the liver damage observed in these patients is a form of rejection or the consequence of an "autoimmune" injury, possibly triggered by drugs or viral infection, remains to be established. The administration of cyclosporin A or tacrolimus to rodents after bone marrow transplantation can result in a "paradoxical" autoimmune syndrome in which the immunosuppressive drugs interfere with maturation of T lymphocytes and favor the emergence of autoaggressive T-cell clones[35-37]. This experience in animals may explain, in part, the development of this disorder in immunosuppressed children after liver transplantation.

The manifestations of the autoimmune condition in rodents vary in different strains and depend on genetic

factors possibly encoded by the major histocompatibility complex[35]. Analysis of the HLA phenotypes of the recipients and donors in the original report did not show an association between the development of autoimmune features, the presence of either HLA *DRB1*03* or *-DRB1*04*, or the degree of donor-recipient HLA mismatch[28]. Five of the seven patients, however, had received livers from donors with HLA markers known to be associated with susceptibility to AIH, including two with *DRB1*04*, one with *DRB1*03*, and two with both *DRB1*03* and *DRB1*04*[38].

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TOPIC HIGHLIGHT

Pietro Invernizzi, MD; Ian R Mackay, MD, *Series Editors*

Overlap syndromes among autoimmune liver diseases

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Abstract

The three major immune disorders of the liver are autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC). Variant forms of these diseases are generally called overlap syndromes, although there has been no standardized definition. Patients with overlap syndromes present with both hepatitic and cholestatic serum liver tests and have histological features of AIH and PBC or PSC. The AIH-PBC overlap syndrome is the most common form, affecting almost 10% of adults with AIH or PBC. Single cases of AIH and autoimmune cholangitis (AMA-negative PBC) overlap syndrome have also been reported. The AIH-PSC overlap syndrome is predominantly found in children, adolescents and young adults with AIH or PSC. Interestingly, transitions from one autoimmune to another have also been reported in a minority of patients, especially transitions from PBC to AIH-PBC overlap syndrome. Overlap syndromes show a progressive course towards liver cirrhosis and liver failure without treatment. Therapy for overlap syndromes is empiric, since controlled trials are not available in these rare disorders. Anticholestatic therapy with ursodeoxycholic acid is usually combined with immunosuppressive therapy with corticosteroids and/or azathioprine in both AIH-PBC and AIH-PSC overlap syndromes. In end-stage disease, liver transplantation is the treatment of choice.

Key words: Autoimmune hepatitis; Immunosuppressive agents; Primary biliary cirrhosis; Primary sclerosing cholangitis; Ursodeoxycholic acid

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INTRODUCTION

The term "overlap syndrome" is used to describe variant forms of autoimmune hepatitis (AIH) which present with characteristics of AIH and primary biliary cirrhosis (PBC) or primary sclerosing cholangitis (PSC). Standardization of diagnostic criteria for overlap syndromes has not been achieved so far, since these disorders are uncommon. It remains unclear whether these overlap syndromes form distinct disease entities or are only variants of the major immune hepatopathies[1,2].

Overlap syndromes should always be considered once an autoimmune liver disease has been diagnosed[1]. Patients with overlap syndromes usually present with nonspecific symptoms, including fatigue, arthralgias, and myalgias. A hepatitic biochemical profile typically coexists with cholestatic laboratory changes[3,4]. Interestingly, transitions from one to another autoimmune hepatopathy have also been reported and are discussed together with the overlap syndromes[5,6]. Overlap of autoimmune cholangitis (AMA-negative PBC) and AIH has been described anecdotically and is discussed together with AIH-PBC overlap syndromes. Although combined features of both PBC and PSC have been reported in single cases[7], there is no clear evidence for the existence of an overlap of PBC and PSC.

It appears inappropriate to use the term overlap syndrome for coexistence of AIH and other chronic liver diseases like chronic hepatitis C. Autoantibodies are detected in up to 65% of patients with chronic hepatitis C and LKM1 antibodies, the hallmark of AIH type 2 was also observed in 7% of the patients with chronic hepatitis C[8]. Conversely, in patients with AIH and hypergammaglobulinemia, anti-HCV tests in the past turned out to be false positive in many cases[9]. Thus, the term overlap syndrome should not be used for patients with AIH and concomitant chronic hepatitis C.

This article is an extension of a recent review[10] and discusses current views and controversies on overlap syndromes. A case report is included to exemplify the typical features of an AIH-PBC overlap syndrome.

DIAGNOSIS OF AUTOIMMUNE LIVER DISEASES

The diagnostic criteria of AIH, PBC and PSC are discussed in detail in this issue of the *World Journal of Gastroenterology* and are therefore just summarized briefly, since they are the basis for the diagnosis of the respective overlap syndrome.

AIH

The diagnosis of AIH depends on several descriptive criteria which were summarized and updated by the International AIH Group (IAIHG) in 1999[11]. A definite diagnosis requires exclusion of other major causes of liver damage, including alcoholic, viral, drug- and toxin-induced, and hereditary liver disease. The scoring system includes characteristic laboratory features (hepatitic serum liver tests, the presence of elevated serum IgG or γ-globulins and of serum autoantibodies), histocompatibility leucocyte antigen (HLA) associations, a portal mononuclear cell infiltration and interface hepatitis in the liver tissue and a positive treatment response to corticosteroids[11].

PBC

The diagnosis of PBC is based on a cholestatic serum enzyme pattern, serum antimitochondrial antibodies (AMA) and/or PBC-specific AMA-M2, and a compatible histology. Although elevated serum IgM is characteristic for patients with PBC, it is not regarded mandatory to establish the diagnosis[12,13]. PBC is frequently associated with other autoimmune disorders, like Sjögren's syndrome, Hashimoto thyroiditis, and celiac disease.

PSC

PSC is a rare chronic cholestatic disease of the liver and bile ducts that is generally progressive and leads to end-stage liver disease. In contrast to PBC, twice as many men as women are affected. PSC is diagnosed most frequently in patients aged between 25 and 40 years[14]. Criteria for the diagnosis of PSC include cholestatic serum enzyme pattern, typical cholangiographic findings of bile duct stenoses and dilatations and histologic findings compatible with PSC showing mild to moderate portal infiltration[14,15]. Concomitant inflammatory bowel disease is found in 70%-90% of the patients and atypical perinuclear antineutrophil cytoplasmatic antibodies (pANCA) are detected in more than 70% of the patients[16].

AIH-PBC OVERLAP SYNDROME

PBC and AIH are the most frequent autoimmune hepatopathies with a prevalence of 25-40/100 000[17,18] and 17/100 000[19], respectively, in recent epidemiologic studies in Europe and the United States and female gender predominates in both AIH (80%) and PBC (90%-95%). Serum liver tests typically show a hepatitic pattern in AIH and a cholestatic pattern with marked elevation of aP and γ-GT, but mild elevation of serum transaminases in PBC. While serum IgG is the predominant immunoglobulin elevated in AIH, serum IgM is elevated in most patients with PBC.

Table 1 Diagnostic criteria of AIH-PBC overlap syndrome proposed by Chazouillères *et al* in 1998[5]

AIH (2 out of 3 criteria)
(1) Alanine aminotransferase (ALT) levels > 5 × ULN
(2) Serum immunoglobulin G (IgG) levels > 2 × ULN or a positive test for smooth muscle antibodies (ASMA)
(3) Liver biopsy showing moderate or severe periportal orperiseptal lymphocytic piecemeal necrosis
PBC (2 out of 3 criteria)
(1) Alkaline phosphatase (AP) levels > 2 × or γ-glutamyltranspeptidase (GGT) levels > 5 × ULN
(2) Positive test for antimitochondrial antibodies (AMA)
(3) Liver biopsy specimen showing florid bile duct lesions

ULN: Upper limit of normal value.

Patients presenting with clinical, biochemical, serological and histological features of both diseases have been reported since the 1970s[20,21]. Later, the term "overlap syndrome" was used to describe these conditions, although there was no common definition or uniformly accepted diagnostic criteria for this[22,23]. Two extended analyses provided evidence for AIH-PBC overlap in 8% of 199 patients with AIH (n = 162) or PBC (n = 37)[1] and in 9% of 130 patients with PBC[5]. In the latter study, an AIH-PBC overlap syndrome was accepted when 2 or 3 criteria for PBC and AIH were fulfilled[5] (Table 1). Although these diagnostic criteria for an AIH-PBC overlap syndrome are not validated and their sensitivity has not been established, they provide a diagnostic template that can be consistently applied[4].

In a comparative study, patients with AIH-PBC overlap syndrome presented with typical features of PBC (AMA-M2 positive, bile duct damage compatible with PBC), but a more hepatitic picture than a cohort of PBC patients[24]. Patients with AIH-PBC overlap syndrome showed a predominant HLA type B8, DR3, or DR4 similar to AIH and a good response to corticosteroid treatment, and this was, therefore, named "PBC, hepatitic form"[24]. Autoantibodies are generally believed to present a hallmark for the diagnosis of AIH, but up to 20% of patients with AIH present without antinuclear antibodies (ANA), anti-smooth muscle antibodies (ASMA), or antibodies against liver-kidney-microsomes (LKM) 1[11]. ANA represent the least specific serum autoantibodies for the diagnosis of chronic liver diseases and are also found in 30% of elderly healthy controls, 10% of pregnant women, and 30% of patients with malignancies[25]. Serum ANA in patients with PBC are not a marker of AIH-PBC overlap syndrome, but often found in PBC patients without further signs of AIH[26]. In contrast, ANA with a specific immunofluorescence pattern of multiple nuclear dots directed against Sp100 (5-10 dots) or Coilin p80 (2-6 dots) are rather specific although less sensitive for PBC[25]. In 3.9% of 233 patients with PBC, the presence of soluble liver antigen (SLA) autoantibodies was found to be a marker of AIH-PBC overlap syndrome with a good

response to immunosuppressive therapy[27].

In addition to AIH-PBC overlap syndrome, some patients presented with typical features of PBC or AIH[5,28]. A well-defined series of 12 patients with PBC followed by AIH was described in 282 PBC patients[29]. The time interval between the diagnosis of PBC and the diagnosis of AIH varied from 6 mo to 13 years. Of importance, patients with multiple flares of hepatitis at the time of diagnosis of AIH had already developed cirrhosis on liver biopsy[29]. Remission was achieved in 80% of the patients who received additional immunosuppressive therapy. This case series emphasizes the possible role of AIH in the deterioration of liver function in PBC patients unless diagnosis is made early and steroid therapy is administered. This study suggested that these patients may have two coincident autoimmune diseases rather than a variant of PBC or AIH[29]. A recent retrospective analysis indicated that patients with AIH-PBC overlap syndrome might have worse clinical outcomes compared to patients with PBC alone[30]. However, this conclusion is somewhat controversial, since the treatment was not standardized and no difference was found when the diagnostic criteria proposed by Chazouilleres *et al*[5] were applied in this cohort of patients.

Therapy

Randomized controlled trials are the best method to address therapeutic issues. However, the low prevalence of AIH-PBC overlap syndrome has made controlled therapeutic trials in these patients impossible so far. Thus, therapeutic recommendations still rely on the experience in the treatment of either AIH or PBC, and on retrospective, non-randomized studies with inherent limitations. It remains controversial if patients with AIH-PBC overlap syndrome require immunosuppressive treatment in addition to ursodeoxycholic acid (UDCA). In a strictly defined cohort of 16 patients with AIH-PBC overlap syndrome, the response to UDCA therapy (13-15 mg/kg daily) and the survival of the patients were similar to patients with classical PBC[31]. However, other groups reported that a combined therapy of UDCA and corticosteroids is required in most patients to obtain a complete biochemical response[5,24]. This question was addressed again recently in a retrospective study of 17 patients with AIH-PBC overlap syndrome[32]. In this study, patients received UDCA alone or UDCA in combination with immunosuppressors and were followed up for 7.5 years. In the patients treated with UDCA alone, biochemical response was observed in only 3 patients whereas 8 patients were non-responders and 50% of them showed increased fibrosis. All but one of the non-resonders subsequently received combined therapy, and 85% of the patients achieved biochemical remission[32]. In the second group of patients who received combined therapy throughout the study, fibrosis did not progress and 67% achieved biochemical remission. Thus, it appears appropriate to start treatment with UDCA (13-15 mg/kg daily). However, if this therapy does not induce an adequate biochemical response in an appropriate time span (e.g. 3 mo) or in patients with predominantly hepatitic serum liver tests, a glucocorticosteroid should be added. Prednisone has been used at an initial dose of 0.5 mg/kg daily and should be progressively tapered once ALT levels show a response[32]. The role of other immuno-suppressants, e.g. azathioprine (1-1.5 mg/kg daily) in the long-term management of patients with AIH-PBC overlap syndrome is unclear, but its successful use in AIH makes azathioprine an attractive alternative to corticosteroids for long-term immunosuppressive therapy[3,32]. Budesonide, a synthetic corticosteroid with a high first pass metabolism that reduces its systemic side effects, is a promising treatment option for patients with AIH and has also been used in patients with AIH-PBC overlap syndrome with success[33,34]. For corticosteroid-resistant patients with AIH-PBC overlap syndrome, intermediate treatment with other immunosuppressants such as cyclosporine A has been considered[23]. Liver transplantation is regarded as the treatment of choice for end-stage disease.

A case report of AIH-PBC overlap syndrome

A 57-year-old woman presented to our outpatient clinic in May 2007 for evaluation of abnormal serum liver tests. She reported fatigue and slight pruritus, but was otherwise in good general health. In January 2007, elevated serum liver tests were detected for the first time when she went for a routine medical examination. At presentation, serum liver tests revealed elevated γ-GT (2 × N), elevated transaminases (AST 2.5 × N, ALT 5.5 × N), and normal bilirubin. Serum AMA (1:3840) and AMA-M2, ASMA and SLA were positive, whereas ANA, LKM1 and ANCA were all negative. Her immunoglobulins showed elevated IgG (23.2 g/L) and IgM (3.3 g/L). Metabolic and viral liver diseases were ruled out. A liver biopsy disclosed an interface hepatitis and mild portal fibrosis without evidence of cirrhosis (Figure 1). AIH-PBC overlap syndrome was diagnosed and a combined therapy of UDCA (13-15 mg/kg daily) and budesonide (6 mg/d) was initiated. Two weeks later, her transaminases decreased by 50% and azathioprine (100 mg/d) was administered. After 3 mo of combined therapy, all serum liver tests were normal and budesonide was tapered to 3 mg/d. At the last follow-up visit in August 2007, the patient reported an improved general condition, and fatigue and pruritus disappeared.

AUTOIMMUNE CHOLANGITIS (AIC) AIC-AIH OVERLAP SYNDROME

AIC shares many features with PBC and is therefore also called AMA-negative PBC. Like PBC, it is characterized by a female preponderance, a cholestatic serum enzyme pattern and florid bile duct lesions on histology and it slowly progresses to fibrosis and cirrhosis of the liver if left untreated[35]. Patients with AIC are AMA negative and often present with serum ANA and/or ASMA. Several studies support the view that AIC and PBC are variants of one single disease only differing in serum autoantibody pattern[36-38]. Twenty-two of 30 patients

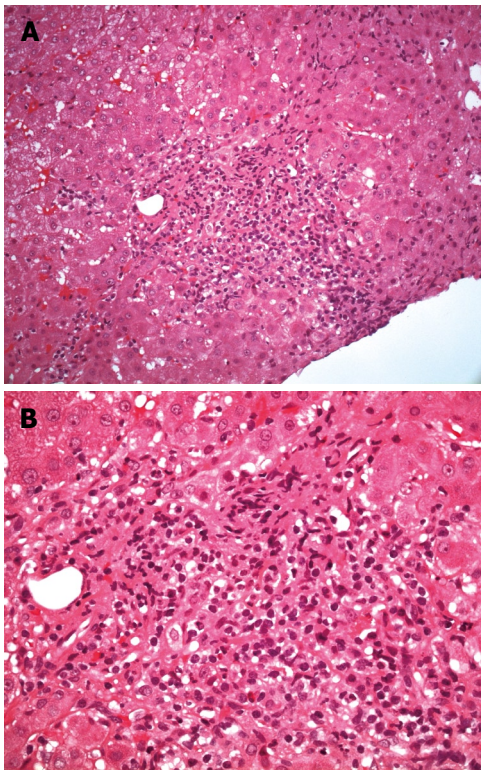

Figure 1 Overlap syndrome autoimmune hepatitis-primary biliary cirrhosis. A 57-year-old woman presented with elevated γ-GT (2 × ULN) and transaminases (AST 2.5 × ULN, ALT 5.5 × ULN), and normal bilirubin. Serum AMA (1:3840), AMA-M2,ASMA and SLA were positive. Her immunoglobulins showed elevated IgG (23.2 g/L) and IgM (3.3 g/L). A liver biopsy disclosed an interface hepatitis and mild portal fibrosis without evidence of cirrhosis. **A**: HE, × 20; **B**: HE, × 40 (Courtesy of Prof. Dr. Müller-Höcker, Munich).

with AIC (AMA-negative PBC), but none of the 316 controls, were positive in a new AMA-M2 recombinant assay which detected autoantibodies directed against human E2 members of the 2-oxo acid dehydrogenase complex family[36]. In addition, immunohistochemical studies showed that PDC-E2 immunoreactivity was expressed on apical membranes of biliary epithelial cells not only in patients with PBC, but also in 7 of 9 patients with AIC[37]. Treatment response to UDCA (13-15 mg/kg daily) and outcome of liver transplantation in end-stage disease were also similar in patients with AIC and those with PBC[39,40]. Thus, these data indicate that a majority of AIC patients (when defined as AMA-negative PBC) suffer from "true" PBC.

Concomitant features of AIH and AIC have been reported in single cases. An AMA-negative woman presented with features of an AIH-AIC overlap syndrome based on the presence of hepatitic and cholestatic biochemical changes and interface hepatitis as well as bile duct lesions on histology. In analogy to the therapy of AIH-PBC overlap syndrome, this patient responded to a combined treatment with UDCA and immunosuppressors[41].

AIH-PSC OVERLAP SYNDROME

While AIH-PBC overlap syndrome is predominantly found among adults, AIH-PSC overlap syndromes have mainly been described in children, adolescents and young adults[42-44]. Use of the modified AIH score led to the diagnosis of an overlap syndrome in 8% of 113 PSC patients and 1.4% of 211 PSC patients, respectively, when evaluated retrospectively[45,46]. However, diagnostic criteria are not defined for AIH-PSC overlap syndrome which makes comparability of these studies difficult. In a recently published prospective study, 41 consecutive patients who were diagnosed with PSC were evaluated for an AIH-PSC overlap syndrome[47]. The diagnosis of AIH-PSC overlap syndrome was established when the following criteria were met: (1) revised AIH score > 15; (2) ANA or ASMA antibodies present in a titre of at least 1:40; and (3) liver histology with piecemeal necrosis, lymphocyte rosetting, moderate or severe periportal or periseptal inflammation[47]. By applying these criteria, 17% of the PSC patients were diagnosed with AIH-PSC overlap syndrome. Patients with AIH-PSC overlap syndrome were treated with UDCA (15-20 mg/kg daily), prednisolone (0.5 mg/kg daily, tapered to 10-15 mg/d) and 50-75 mg azathioprine with good biochemical response[47]. Of interest, the survival probability of the patients with AIH-PSC overlap syndrome was better than those with classical PSC as assessed by the Mayo score, a prognostic index.

The largest case series of AIH-PSC overlap syndromes in children and adolescents was published by colleagues from the Kings' College in London[44]. In this prospective study, a group of 55 children was followed up for 16 years who showed clinical, biochemical, and histological signs of AIH. In 27 of the 55 children, cholangiographic findings were typical of sclerosing cholangitis, whereas other signs and symptoms were characteristic of AIH. Therefore, the term "autoimmune sclerosing cholangitis" (ASC) was proposed for this AIH-PSC overlap syndrome. Patients with ASC more commonly suffered from inflammatory bowel disease and more often were positive for ANCA in serum than those with AIH. Serum transaminases tended to be higher in AIH, but serum alkaline phosphatase although mostly elevated in PSC was normal at several occasions in both diseases. Thus, AIH and ASC may belong to the same disease process and may overlap with PSC.

Increasing awareness for the AIH-PSC overlap syndrome has led to the observation that AIH and PSC may be sequential in their occurrence, and this has first been described in children[44]. More recently, a similar observation has been reported in a small case series of 6 adults (mean age 31 years; 4 male; 3 with ulcerative colitis) who developed biochemical and cholangiographic features of PSC after an average of 4.6 years of a diagnosis of AIH and became resistant to immunosuppressive therapy[6]. Thus, in patients with AIH who become cholestatic and/or resistant to immunosuppression, PSC should be considered and ruled out.

Therapy

Ursodeoxycholic acid (UDCA) is widely administered in PSC due to its beneficial effects on serum liver tests,

histological features, prognostic surrogate markers, and development of colonic dysplasia associated with accompanying ulcerative colitis, although long-term efficacy of UDCA still remains unproven[48-52]. UDCA at higher doses (> 20 mg/kg daily) may be superior to standard doses for patients with PSC[53] and has also been used in the treatment of AIH-PSC overlap syndrome[47,52]. UDCA has been used in combination with immunosuppressive drugs in AIH-PSC overlap syndrome, and the long-term course was considered favorable[44,47]. Thus, UDCA in combination with an immunosuppressive regimen may be an adequate medical treatment for most patients with AIH-PSC overlap syndrome although data from controlled trials are lacking. Liver transplantation should be considered in late-stage diseases.

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TOPIC HIGHLIGHT

Pietro Invernizzi, MD; Ian R Mackay, MD, *Series Editors*

Autoimmune liver serology: Current diagnostic and clinical challenges

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Abstract

Liver-related autoantibodies are crucial for the correct diagnosis and classification of autoimmune liver diseases (AiLD), namely autoimmune hepatitis types 1 and 2 (AIH-1 and 2), primary biliary cirrhosis (PBC), and the sclerosing cholangitis variants in adults and children. AIH-1 is specified by anti-nuclear antibody (ANA) and smooth muscle antibody (SMA). AIH-2 is specified by antibody to liver kidney microsomal antigen type-1 (anti-LKM1) and anti-liver cytosol type 1 (anti-LC1). SMA, ANA and anti-LKM antibodies can be present in de-novo AIH following liver transplantation. PBC is specified by antimitochondrial antibodies (AMA) reacting with enzymes of the 2-oxo-acid dehydrogenase complexes (chiefly pyruvate dehydrogenase complex E2 subunit) and disease-specific ANA mainly reacting with nuclear pore gp210 and nuclear body sp100. Sclerosing cholangitis presents as at least two variants, first the classical primary sclerosing cholangitis (PSC) mostly affecting adult men wherein the only (and non-specific) reactivity is an atypical perinuclear antineutrophil cytoplasmic antibody (p-ANCA), also termed perinuclear anti-neutrophil nuclear antibodies (p-ANNA) and second the childhood disease called autoimmune sclerosing cholangitis (ASC) with serological features resembling those of type 1 AIH. Liver diagnostic serology is a fast-expanding area of investigation as new purified and recombinant autoantigens, and automated technologies such as ELISAs and bead assays, become available to complement (or even compete with) traditional immunofluorescence procedures. We survey for the first time global trends in quality assurance impacting as it does on (1) manufacturers/purveyors of kits and reagents, (2) diagnostic service laboratories that fulfill clinicians' requirements, and (3) the end-user, the physician providing patient care, who must properly interpret test results in the overall clinical context.

Key words: Autoantigen; Autoimmune hepatitis; Autoantibody; Primary biliary cirrhosis; Primary sclerosing cholangitis; Liver disease

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INTRODUCTION

The presence of autoantibodies plays a central role in the diagnosis and classification of autoimmune liver diseases (AiLD)[1,2], but their nature and significance remain challenging in regard to pathogenesis. Such antibodies discriminate between distinct subtypes of the AiLD and facilitate diagnosis of the overlap syndromes[3]. AiLD represent a broad range of disorders that can affect one or the other of the two cellular components, namely hepatocytes in autoimmune hepatitis (AIH), and cholangiocytes in primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC) and the autoimmune hepatitis/sclerosing cholangitis overlap syndrome of childhood, designated as autoimmune sclerosing cholangitis (ASC)[4], and discussed elsewhere in this issue.

Antibody to nuclei (ANA) and/or to smooth muscle (SMA) characterizes type 1 AIH (AIH-1) and antibody to a liver kidney microsomal constituent (anti-LKM) defines patients with type 2 AIH (AIH-2)[5]. Usually the two patterns of serology are mutually exclusive, but in the rare cases in which they coexist, the disease features resemble those of AIH-2[6]. ASC is a third form of AiLD

which is similar clinically, histologically and serologically to AIH-1, but is associated with radiological changes of sclerosing cholangitis[7]. SMA, ANA and to a lesser extent anti-LKM can be found in post-transplantation *de novo* AIH[8]. The presence of anti-mitochondrial antibodies (AMA) with a specificity for the E2 subunit of the pyruvate complex (PDC-E2), and certain PBC-specific ANA, characterise PBC[1,9]. Perinuclear anti-neutrophil cytoplasmic antibody (p-ANCA) is the most frequent antibody reactivity in primary sclerosing cholangitis (PSC)[1,3], but *per se* has low specificity for diagnosis.

HISTORICAL NOTES ON AUTOIMMUNE LIVER SEROLOGY

The evolution of knowledge on AIH is discussed in another article in this issue. Here we provide a brief historical survey of the serological tests currently used by diagnostic laboratories.

Anti-nuclear antibody (ANA)

Serum antibodies with specificity for cell nuclear antigens were first described by Miescher *et al* in 1954[10] following the discovery of the lupus erythematosus (LE) cell by Hargraves and colleagues[11] and the recognition that the LE cell phenomenon was related to a serum factor reacting with nuclear antigens, subsequently termed "antinuclear factor" (ANF), and later antinuclear antibody (ANA). Deoxyribonucleic acid (DNA) and deoxyribonucleoprotein (DNAP) were identified in 1957 as "ANF" target antigens[11,12] and it was further shown that antibodies responsible for the LE-cell phenomenon reacted with DNA and gave a "homogenous" pattern of nuclear staining by immunofluorescence[13]. In 1956 a positive test for LE cells in blood was reported in young women with a chronic liver disease then called chronic active hepatitis (CAH), leading to the designation of "lupoid hepatitis", an early label for what is now known as AIH-1[14,15]. Testing for ANF/ANA by immunofluorescence (IFL) supplanted the cumbersome LE cell test in the early 1960s.

Smooth-muscle autoantibody (SMA)

Antibodies binding to smooth muscle of rat stomach were initially detected in serum samples of patients with liver diseases by Johnson *et al*, in 1965[16]. The presence of SMA in patients with AiLD was confirmed by Whittingham *et al*[17]. Patients with non-AiLDs were reported as seronegative for SMA and, notably, also negative were patients with SLE. The antibody was often found in association with ANA, which was already a known marker of AIH, and tended to fade with steroid induced remission. Bottazzo *et al*[18] reported that the SMA staining arterial vessels (V), glomerular mesangium (G) and fibers surrounding the kidney tubules (T), responsible for the VGT pattern, was confined to an aggressive form of hepatitis now known to be AIH-1. The antigenic moiety mainly but not exclusively responsible for SMA activity in what in the 1970s was called CAH was identified as filamentous (F) actin[19-21].

Liver kidney microsomal antibody (anti-LKM)

Cytoplasmic antibodies in "CAH" were described in the laboratory of Deborah Doniach[22,23] whose group first used the expression anti-liver kidney microsomal (anti-LKM) antibodies[24]. "Microsomal" is something of a misnomer as "microsomes" are the *in vitro* equivalent of particles of the endoplasmic reticulum wherein the antigen is located. Other nosological entities in which anti-microsomal antibodies were evident included drug induced hepatitis, leading to the use of LKM1, LKM2, LKM3 to designate the different immunofluorescent patterns, which reflect the different targeted autoantigens[25]. The ability of anti-LKM1 antibodies to define a second serological type of AIH, i.e. AIH type 2, was proposed by Homberg *et al*[26]. Three groups independently identified cytochrome P450 IID6 (CYP2D6) as the molecular target of anti-LKM1 antibodies[27-29]; the group of Alvarez[27] was the first to publish its data in the form of a full-length paper.

As mentioned, other LKM antibody patterns were subsequently described. LKM2 antibodies were recognised in patients with hepatitis induced by tienilic acid[24], a uricosuric diuretic withdrawn from clinical use in 1980 and Rizzetto's group described LKM3 antibodies in a proportion of cases of chronic hepatitis D infected patients[30]. In contrast to anti-LKM1 and LKM2 antibodies, anti-LKM3 stained human exocrine pancreas and thyroid. Anti-LKM2 reacted with CYP2C9 and anti-LKM3 with uridine diphosphate glucuronosyl transferases (UGT)[25]. A fourth type of LKM antibodies recognising CYP1A2 and CYP2A6 has been described in patients with AIH associated with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED)[31]. The IFL pattern of the antibody is indistinguishable from that of anti-LKM1. An anti-liver microsomal antibody (anti-LM) staining the centrolobular hepatocytes but not the kidney and which recognises CYP1A2 has been described in dihydralazine-induced hepatitis and in a few cases of AIH[32-34].

Liver cytosol antibody (anti-LC1)

Anti-LC1 were originally described in association with anti-LKM1, or in isolation, by Martini *et al* in patients with AIH-2[35]. Lenzi *et al* have also found anti-LC1 antibodies in 14% anti-LKM-1 antibody positive patients suffering from chronic hepatitis C virus infection[36]. The enzyme formiminotransferase cyclodeaminase (FTCD) has been identified as the molecular target of anti-LC1 antibodies[37,38].

Mitochondrial antibody (AMA)

The first indication that PBC could be an autoimmune disease was obtained in 1958 when the serum of a woman with PBC was found to contain high titres of complement-fixing antibodies directed to tissue homogenates[39], that later, by absorption studies, were shown to be absorbed by a rat liver mitochondrial fraction[40]. A breakthrough for the clinical hepatologist was the observation in 1965 by Walker, Doniach,

Roitt and Sherlock that human tissue sections rich in mitochondria give a characteristic immunofluorescence pattern when they are incubated with sera from patients with PBC but not with controls which, in that study, included patients with extra-hepatic bile duct obstruction, drug induced cholestasis and viral hepatitis[41]. In 1967, Berg *et al*[42] demonstrated that PBC sera reacted *in vitro* with a trypsin-sensitive mitochondrial antigen that was named M2 antigen, in contrast to M1, the target of anti-cardiolipin antibody. Subsequently Berg developed a nomenclature based on the types of anti-mitochondrial reactivity that spanned M3-M9, but this is no longer used. The M2 antigen was located at the inner surface of the inner mitochondrial membrane of all mitochondria tested[42-45]. The target antigens of M2 were identified in the 1980s as components of the 2-oxo-acid dehydrogenase complexes, the predominant target being the E2 subunit of pyruvate dehydrogenase complex, as judged by molecular cloning[46,47]. PBC-specific AMA were later shown to recognise other enzymes of the 2-OADC, including the E2 subunits of branched chain oxoacid dehydrogenase complex (BCOADC), the oxoglutarate dehydrogenase complex (OGDC) and the PDC-E3 binding protein[1,48].

Antibodies against soluble liver antigen/liver-pancreas antigen

Two autoantibodies, anti-soluble liver antigen (SLA) and anti-liver-pancreas (LP), both described in AIH by two independent German groups, have been shown to target the same antigen, hence the current name of anti-SLA/LP antibodies[49-51]. The LP antigen has first been reported by Berg's group in the supernatant of liver and pancreas homogenates[50]. The SLA antigen was described by Manns and colleagues in 1987 as a component of the supernatant of liver and kidney homogenates[49]. Anti-SLA antibodies detected by a competitive ELISA were then proposed as markers of a third type of severe AIH seronegative for the conventional AIH-1 autoantibodies[49].

Anti-asialoglycoprotein receptor antibodies

Attempts to identify antigens specifically expressed on the hepatocyte surface which could serve as self targets in AiLD have led to the description of a crude liver extract preparation known as the liver specific protein (LSP) and its major component, the asialoglycoprotein receptor (ASGPR)[52,53]. ASGPR, also designated as hepatic lectin, is a type II transmembrane glycoprotein. It is the only known liver-specific autoantigen, and is constitutively expressed on the hepatocellular membrane.

RECOMMENDATIONS FOR AUTOANTIBODY DETECTION BY IMMUNOFLUORESCENCE (IFL)

IFL is the main technique for the screening of autoantibodies diagnostically relevant to liver disease. The methodology is practically unchanged from that introduced by Weller and Coons in 1954[54]. It uses unfixed, air-dried, tissue sections which are incubated with a test serum potentially containing an antibody. After removing unbound serum by washing, a fluorochrome labelled second antibody, raised in animal and specific for human immunoglobulins, is applied to detect the first tissue-bound antibody[55]. Specific patterns can then be recognised using an ultraviolet microscope. A consensus statement in 2004 from the Committee for Autoimmune Serology of the International Autoimmune Hepatitis Group (IAIHG) provided guidelines on how to test for autoantibodies relevant to AIH and concluded that indirect IFL on fresh sections of multi-organ (liver, kidney, stomach) from rodents (usually rat) should be the first line screening[55]. The recommendations of the Committee include detailed guidelines for the preparation of substrate, application of the test serum samples, optimal dilution of samples and fluorochrome-labelled revealing agents, selection of controls and identification of diagnostically relevant staining patterns[55]. The use of the three tissues enables the simultaneous detection of virtually all the autoantibodies relevant to liver disease, namely SMA, ANA, anti-LKM1, AMA and anti-LC1[55]. The first serum dilution recommended for autoantibody detection (before titration) is for adults 1:40, and for children 1:20 for ANA and SMA and 1:10 for anti-LKM1 in children[55].

Autoantibodies detected by IFL and their reactants

ANA: This autoantibody is readily detectable as nuclear staining in all the three tissues of the composite substrate. On the liver it is also possible to identify different patterns, the homogenous being typical of AIH-1[55]. A clearer definition of the different ANA patterns seen in PBC is best achieved by the use of the human larynx epithelioma cancer cell line (HEp-2) because these cells have large nuclei, and the mitotic phase of these cells permits the easy detection of anti-centromere antibodies (ACA) because they stain the chromosomes of cells in mitosis[56,57]. HEp-2 permit ready detection of the IFL patterns called multiple nuclear dot (MND) and rim-like membranous (RLM) typical of PBC[58,59]. Anti-MND stains 5-20 dots of variable size, distributed all over the nucleus but sparing the nucleoli[58]. The pattern can be confused with that of ACA but anti-MND do not stain the chromosomes of cells in mitosis whereas ACA do so[58]. Moreover, the dots of ACA are all of the same size while those of MND vary in size and number between individual cells[58]. In addition to homogenous ANA, speckled and nucleolar patterns are seen in AIH, and to a lesser extent in PBC, but are not disease-specific.

SMA: SMA of the VGT pattern is considered specific for AIH-1, though some 20%-40% of patients with AIH-1 do not have it[55]. SMA can also be detected, always by IFL, using fibroblasts or HEp-2 cells. The VGT pattern corresponds to the microfilament staining of isolated fibroblasts and represents a cable pattern across the cell[18]. Both patterns have been termed "anti-

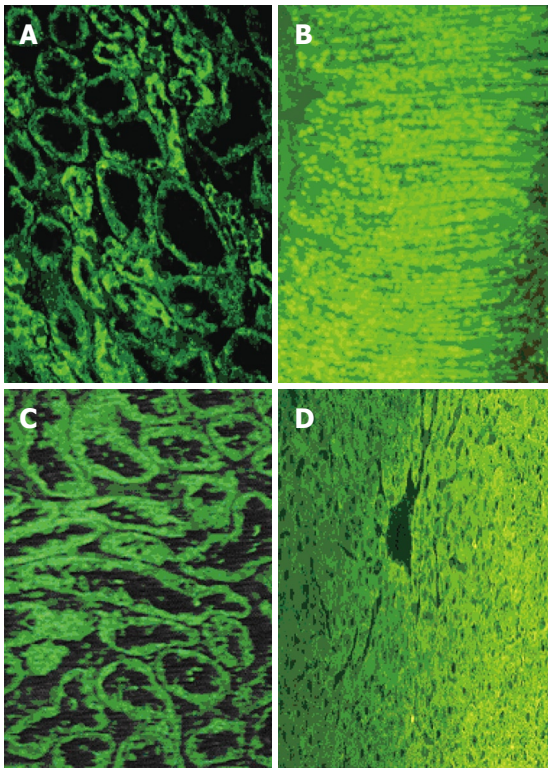

Figure 1 Immunofluorescence of anti-mitochondrial (**A** and **B**), and anti-liver kidney microsomal antibody (anti-LKM1) (**C** and **D**). AMA stain (**A**) stronger the smaller, distal tubules while anti-LKM1 the proximal tubules of the rat kidney (**C**). These specificities are frequently misdiagnosed, especially when only the kidney substrate is used and the sections do not contain both proximal and distal tubules. Thus, the use of rat stomach (**B**) and liver (**D**) is strongly recommended to prevent misinterpretation; AMA characteristically stain the gastric parietal cells while anti-LKM1 stain the rat liver but not the stomach.

actin" though there is no molecular proof as yet that actin is indeed the only or indeed the main target of VGT SMA.

Anti-LKM1: Anti-LKM1 brightly stains the third portion of the proximal renal tubules and the cytoplasm of the hepatocytes but it spares cells of the gastric mucosa[55]. Anti-LKM1 is a frequently undiagnosed autoantibody, being commonly misinterpreted as AMA[1,60]. AMA is extremely rare in pediatric patients and PBC is extremely rare in childhood[61,62]. So, when AMA is reported in a child with clinical and histological characteristics of AIH, the serological report is almost certainly incorrect.

AMA: The confusion between AMA and anti-LKM1 occurs because both autoantibodies stain the renal tubules, though with a pattern different to a trained eye and readily appreciated when the kidney tissue section contains both distal and proximal tubules (Figure 1). AMA stains strongly the mitochondria-rich distal tubules which are smaller than the proximal tubules stained by anti-LKM1 antibodies. AMA also stains the gastric parietal cells within the stomach, which are spared by anti LKM1, whereas AMA stains hepatocytes much less brightly than does anti-LKM1. The analysis therefore of the three-tissue substrate should allow a correct serological interpretation. Some serodiagnosticians claim a utility of HEp-2 cells for recognition of AMA which gives a "string of pearls" pattern of cytoplasmic staining. Unfortunately interpretative problems are still frequent especially in those laboratories where only kidney is used as substrate, and particularly when the tissue is poorly oriented. Advice on how to orient and cut the kidney has been issued by the Autoimmune Serology Committee of IAIHG[55].

Anti-LC1: This antibody stains the cytoplasm of hepatocytes with a zonal distribution within the liver, being particularly abundant on perivenous hepatocytes and the renal tubules. In most cases, however, anti-LC1 is obscured by the simultaneous presence of anti-LKM1[35,36]. Anti-LC1 can be also detected by gel diffusion techniques such as double dimension immunodiffusion and counter immunoelectrophoresis, techniques in which the cytosol of liver homogenate is used as antigen and the test serum is run with a positive control[63].

ANCA: ANCA is detected by indirect IFL using neutrophils as substrate and can give a cytoplasmic (c-ANCA) or perinuclear (p-ANCA) pattern[64,65]. The pattern of p-ANCA is an artifact caused by the ethanol fixation of the neutrophils which leads to the migration of some positively charged cytoplasmic antigens to the negatively charged nuclear envelope, so giving the characteristic perinuclear fluorescence staining. An atypical p-ANCA staining, unaffected by ethanol fixation, gives a perinuclear staining subtly different from the classical p-ANCA. It recognizes components of the nuclear envelope and has been described, especially in patients with PSC[66]. In view of the location of the antigen, some groups are now describing these antibodies as perinuclear anti-neutrophil nuclear antibodies (p-ANNA)[67,68].

AUTOANTIGENS OF LIVER-RELATED AUTOANTIBODIES

Nuclear antigens

No single AIH-1-specific nuclear antigen has been identified so far. A number of nuclear molecular targets has been detected, including centromere, histones, double-stranded DNA, chromatin, and ribonucleoprotein complexes with no single pattern or combination thereof being characteristic of AIH[3], although most typical is a homogenous pattern attributable to anti-chromatin.

Smooth muscle antigens

SMA giving the "anti-actin" IFL pattern has long been considered highly diagnostic for AIH type 1, its target deemed to be F-actin (noting that purified actin is a monomer G-actin, which is polymerized in the presence of ATP)[3,20,55,69]. The advent of commercial kits using highly purified F-actin as target has provided the opportunity both to test the molecular specificity of the SMA giving the IFL actin pattern and to assess the diagnostic performance of antibodies directed to molecularly pure F-actin (anti-FA)[70-75]. In Granito and

Villalta's studies, the IFL anti-actin pattern was strongly associated with AIH-1 and so was anti-FA, this latter being marginally more sensitive[70,74,75]. When disease specificity of the two reactivities was analysed the IFL pattern was found to be highly specific, being absent or extremely rare in diseases other than AIH-1. In sharp contrast, anti-FA was detectable in patients with viral hepatitis, PBC, primary sclerosing cholangitis, AIH-2 and celiac disease[70,74,75]. In a paper by Frenzler, positivity for anti-FA was found in some 75% of patients subsequently diagnosed as having AIH-1 but also in 24% non-AIH patients[71]. In an attempt to address the relatively high non-specificity of the molecular assay, Villalta *et al* performed a receiver operating curve (ROC) analysis, from which they deduced for this assay a cut off point giving a specificity similar to IFL: the cut off point had to be increased from the 30 arbitrary units (AU) suggested by the manufacturer to 53 AU[75]. At this cut-off point the specificity of the molecular assay was indeed comparable to that of IFL, but the sensitivity dropped by more than 10% below that of IFL.

The results obtained with the IFL and molecular assays overlap considerably, but by no means completely, with several instances of positivity with one test and not with the other[72,73,75]. With the availability of highly purified F-actin the question as to whether the antibody responsible for the anti-actin IFL pattern is directed against actin could be tested directly[70-75]. Three anti-SMA positive sera containing both reactivities were absorbed with solid phase F-actin: the reactivity against F-actin was abolished (absorbed out) but that giving the fluorescent pattern was unaltered in two of the 3 sera and reduced, but not abolished, in the third[72]. In summary, detection of the IFL anti-actin pattern continues to provide to date the best specificity/sensitivity compromise[55]. The antibody responsible for the IFL "actin" pattern targets, in addition to actin, molecules other than actin[3,72]. The question arises as to whether to maintain the tradition, and with it the term of "anti-actin" for the antibody recognised in IFL, or whether to call it anti-micro filament (MF) pattern as suggested by the Serology committee of the IAIHG[55].

LKM1 antigen

While the target antigens of ANA and SMA certainly need better molecular definition, that of anti-LKM1 in AIH-2 has been clearly identified as the microsomal enzyme cytochrome P450IID6 (CYP2D6)[5,26-28]. Its identification has enabled the establishment of assays based on the use of recombinant antigens which have proven useful in solving diagnostic uncertainties between AMA and anti-LKM1[1,60,76]. Such ELISAs, however, are not always able to detect anti-LKM1 antibodies in patients with chronic hepatitis C virus infection whereas IFL and radioligand assays can do so possibly because of their ability to identify conformational epitopes undetectable by ELISA[77-80]. Short CYP2D6 peptides used as antigenic preparations perform less well than those using full-length CYP2D6 and their diagnostic use is limited.

LC1 antigen

ELISAs for detection of antibodies to FTCD, the target of anti-LC1, have been developed and used in diagnostic laboratories and their diagnostic and clinical relevance is under investigation[37,38].

SLA/LP and ASGPR

Progress has been made in the definition of other autoantibodies frequently present in AIH but undetectable by IFL including antibodies against SLA/LP[51,81-85] and ASGPR. Most of anti-SLA/LP positive patients are also positive for ANA, SMA or anti-LKM1, but occasionally anti-SLA is present in isolation and, in this case, its detection is of diagnostic importance[81,86]. The identification of the molecular target of anti-SLA/LP antibodies as the UGA serine tRNA-associated protein has led to the development of ELISA or dot-blot assays increasingly replacing the conventional inhibition ELISA originally used for anti-SLA antibody detection[51,83]. Recent studies investigating the exact role of this protein have shown that SLA/LP is a selenocysteine synthase but how the biosynthesis of selenocysteine may relate to the pathogenesis of AIH is not known[87].

Anti-ASGPR antibody detection requires either purified or recombinant antigen. The lack of disease-specificity and the difficulty in developing a reliable molecular based assay for the detection of anti-ASGPR has limited its wider applicability in diagnostic practice.

Mitochondrial antigens

The most recent advance in the immunodiagnosis of AMA is the availability of an ELISA using the triple MIT3 hybrid antigen preparation, developed in the Gershwin laboratory. This preparation contains all three immunodominant mitochondrial antigenic epitopes, namely PDC-E2, BCOADC-E2 and OGDC-E2[88]. Although assays based on MIT3 are reported to give positive results for PBC sera that test negative for AMA by conventional IFL techniques[89,90], IFL testing for AMA should remain the screening procedure.

PBC-specific nuclear antigens

As mentioned above, major target antigens of PBC-specific ANA have been identified. These include the nuclear body speckled 100 kDa (sp100), promyelocytic leukaemia (PML), and small ubiquitin-like modifier (SUMO) proteins corresponding to the MND pattern, and proteins within the nuclear pore complex (anti-NPC) including the 210 kDa glycoprotein (gp210) and the 62 kDa nucleoporin (NUP62), the major target antigens of anti-RLM antibodies and responsible for the RLM pattern[58,59,91]. New immunoassays testing autoantibodies to sp100, PML, gp210 and NUP62 have been developed using short peptides, polypeptides or full-length proteins as targets, but they have not been fully evaluated nor standardized[91-98]. They may be of diagnostic assistance, especially in those cases where it is difficult to interpret the IFL staining patterns due to concurrent autoantibody reactivities or in true AMA-negative PBC cases[1,92,99,100]. We note also the presence

of ACA reactivity in the combined PBC/CREST disease. Assays to detect multiple reactivities (multiplex) and to provide a full autoimmune serological profile of relevance to PBC are being developed[89]. At present, a lack of guidelines for the detection of PBC-specific autoantibodies by scientific bodies responsible for the standardization of autoimmune serological tests is a significant handicap and perpetuates uncertainties on which are the clinically relevant tests (see below).

Atypical p-ANCA (pANNA) antigens

These are under current investigation[66-68]. The original description of a 50 kDa neutrophil-specific nuclear protein of the nuclear pore complex as the target antigen recognised by 90% of atypical p-ANCA from patients with PSC was followed by a study from the same group suggesting that the identity of the antigen is tubulin beta chain 5 (TBB5)[101]. However, when using the molecular target for their detection anti-TTB5 antibodies were found not only in PSC but also in other AiLDs.

DIAGNOSTIC RELEVANCE OF LIVER-RELATED AUTOANTIBODIES

ANA, SMA, anti-LKM1, AMA and p-ANCA should be determined in all patients with biochemical, clinical and/or histological features suggestive of AiLD[3,5]. Autoantibody titres usually vary during the course of the disease. Hence seronegativity or low autoantibody titres on a single test cannot exclude the diagnosis of AiLD and repeat tests may allow autoantibody detection and correct disease classification. Conversely, the presence of autoantibodies even at high titres in the absence of any other clinical and laboratory features suggestive of AiLD is insufficient to make a diagnosis though a patient with high titre autoantibodies needs to be seen at regular intervals. Titres of ANA, SMA and LKM1 antibodies contribute in calculating the IAIHG diagnostic score for patients with a probable or definite diagnosis of AIH[5]. IFL titres of > 1:80 attract a +3 score; 1:80 a +2 score and 1:40 +1 score. A negative score of -4 is given to cases with hepatitic features but detectable AMA at a titre of $\geqslant$ 1:40; such mixed serology points to "overlap syndrome", discussed in another article in this issue. In children, titres of 1:20 for ANA or SMA and 1:10 for anti-LKM1 are sufficient to support the diagnosis of AIH if accompanied by other suggestive features[5,55].

In AIH-1, ANA alone are present in 15% of patients, SMA alone in 35%, and ANA and SMA co-occur in 60%[3]. In the 5% or so of cases negative for these reactivities, anti-SLA/LP may be positive. In AIH-2 at presentation anti-LKM1 and/or anti-LC1 antibodies are positive in more than 90% of patients[25,35,36,63]. In PBC, AMA are detectable in more than 95% of patients and disease-specific ANA occur in 30%-70% of PBC patients according to different reports[9,58,59,100]. In PSC, atypical p-ANCA are present in up to 90% of patients but this reactivity also occurs in AIH (up to 70%) and PBC (5%), as well as frequently in patients with inflammatory bowel disease[66-68,102,103]. In what is termed "*de novo*" AIH and in post-liver transplant patients, ANA, SMA, AMA and anti-LKM have been reported, at varying frequencies[85,94]. A diagnosis of AIH-2 is strongly supported by seropositivity for anti-LKM1 and/or anti-LC1, particularly in the absence of viral hepatitis C[5]. For PBC, the presence of AMA is one of the three widely accepted diagnostic criteria[9].

Autoantibody positivity is part of the criteria used for the diagnosis of AiLD, though it is not diagnostic on its own. Elevated titres and certain patterns carry significant diagnostic connotations.

We are aware of various reports that, at first sight, might appear prejudicial to the diagnostic utility of liver-related autoantibodies[104]. Thus ANA and/or SMA are reported in PBC, PSC, *de novo* AIH, chronic viral hepatitides B, C and D, acute liver failure, drug-induced hepatitis, non-alcoholic steatohepatitis, alcohol-induced liver disease, hepatocellular carcinoma, and also in a variety of non-liver related diseases. Hence, the diagnostic significance of antibody positivity depends on the associated clinical features[3], as well as the level of reactivity. Anti-LKM1 and anti-LC1 are reported in a proportion of adult (0%-6%) or pediatric (0%-11%) cases with chronic hepatitis C infection[36,105-107]. AMA are present (expectedly) in patients with AIH/PBC overlap syndrome, and also in chronic hepatitis C virus infected patients[1], and most recently were described in patients with acute liver failure[108]; AMA occur also in various rheumatological disorders which may co-exist with PBC notably Sjögren's syndrome and systemic sclerosis[1,48,108-111] and are described in non-liver related conditions with asymptomatic recurrent bacteriuria in women, pulmonary tuberculosis and leprosy[112-114]. However we would submit that in the index disease (AIH or PBC) the frequency and titre of the relevant liver-related autoantibody is substantially higher than for the contrast disease.

Anti-ASGPR antibodies are found particularly in AIH-1 (approximately 90%) but are also present in patients with PBC (14%), chronic hepatitis B and C (7%) and alcoholic hepatitis (8%)[3,52,115]. Anti-SLA antibodies can be found in occasional seronegative AIH patients i.e. those who are negative for ANA, SMA or anti-LKM-1. Anti-SLA antibodies are also frequently present (up to 50%, depending on the sensitivity of the method used) in typical cases of AIH-1 and AIH-2, and also in ASC[86]. Their high specificity for AiLD is has been questioned by reports of anti-SLA being present in some 10% of chronically infected HCV patients[115]. More recently, anti-SLA antibodies have been described in 22% of patients with acute liver failure (ALF)[111]. Since in most cases of ALF we do not know the cause, the presence of anti-SLA can either detract from their disease specificity or, alternatively, suggest an autoimmune pathogenesis (or an autoimmune component to the pathogenesis) of ALF. Monitoring of autoantibodies may be useful in the case of AIH as disappearance or sharp decrease of ANA, SMA and anti-LKM1 can be an indicator of response to

immunosuppressive treatment[3,6]. AMA titres do not relate to the stage of PBC and their fluctuation over time does not seem to have pathogenic significance[1,9,116], although "activity" of the PBC process is not as readily measurable as that of AIH. Practically AMA are only tested at presentation to help establish the diagnosis and repeat tests are normally requested only in cases seronegative for AMA at presentation but with clinical or laboratory findings compatible with PBC[1,2,117].

PROGNOSTIC SIGNIFICANCE AND UTILITY OF LIVER-RELATED AUTOANTIBODIES

AIH

Both SMA and ANA tend to lower in titre and even disappear during immunosuppressive therapy in most patients with AIH-1 although neither their titre at diagnosis nor their fluctuations during the disease are thought to predict disease course and outcome[3]. However, in 2002 Gregorio *et al* found a positive correlation between SMA titre and AST levels over time in pediatric AIH-1 cases, suggesting a potential use of these antibodies, together with IgG levels, to monitor disease activity[118]. There are no comparable adult sequential studies; this may be a reason why no correlation has been ascertained. Nevertheless, Czaja and colleagues have suggested that adult AIH-1 patients with antibodies to anti-actin have a disease onset earlier in life, respond less well to corticosteroids and progress to liver failure or require liver transplantation more frequently compared to those without anti-actin antibodies[69]. The presence of antibodies to double stranded DNA (dsDNA) has been associated with higher levels of immunoglobulin G and higher relapse rates during immunosuppressive treatment compared to seronegative cases[119]. Seropositivity for anti-ASGPR in patients with AIH correlates with histological activity with persistence indicating unresponsiveness to immunosuppressive treatment, and re-appearance being highly suggestive of relapse especially after corticosteroid withdrawal[3,52,115,120]. Anti-SLA antibodies denote patients with a more severe course of AIH and a propensity for relapse after corticosteroid withdrawal compared to their negative counterparts[49,81,121,122]. AIH-2 patients with anti-LC1 antibodies have histologically more severe disease compared to those without anti-LC1 antibodies[35,123,124].

PBC

AMA titres do not seem to be associated with disease severity but those of the IgG3 subclass may identify patients prone to develop more severe disease compared to those without AMA-IgG3[116,125]. PBC-specific ANA have been found more frequently in patients with advanced disease in a number of cross-sectional studies. Anti-NPC seropositivity is associated with accelerated progression to advanced disease and death[94,96,100,126-129] and also, ACA may identify patients with more severe PBC according to studies from USA and Japan[96,130]. These data have obvious implications for the clinical management of PBC given that the only accepted index for estimating survival has been obtained and validated in patients with advanced PBC and hence is of limited use in early disease. Thus, anti-NPC and ACA testing may be important for identifying asymptomatic patients with a likely unfavourable disease course. Once PBC has progressed to advanced histological stages, and serum bilirubin levels have become abnormal, anti-NPC determinations do not appear to offer any additional advantage over other prognostic models such as the Mayo risk score.

PATHOGENIC RELEVANCE OF LIVER-RELATED AUTOANTIBODIES

Despite their undoubted clinical relevance in diagnosis and classification of AiLD, the pathogenic role of autoantibodies and the mechanisms through which they may cause liver damage remains a topic for further research, mainly because of the difficulty in discriminating those actively involved in the immunopathogenic cascade, from those secondary to liver cell damage. The mechanism(s) responsible for the induction of liver-related autoantibodies is currently unknown; several possibilities including molecular mimicry and immunological cross-reactivity have been suggested[78,93,106,131-145]. Most liver-related autoantibodies have limited organ specificity and this notion militates against a direct pathogenic role in highly organ-specific autoimmune injury. For antibodies with a pathogenic potential, complement-dependent and/or antibody-dependent cell-mediated cytotoxicity (ADCC) are the likely effectors of damage[131,146].

EMERGING ISSUES: DIAGNOSTIC ACCURACY, QUALITY ASSURANCE AND STANDARDIZATION PROGRAMMES FOR LIVER AUTOIMMUNE SEROLOGY

There are a number of open issues on serum autoantibodies in AiLD. Their diagnostic significance is unquestioned, but problems concerning autoantibody detection and interpretation have not yet been resolved and are not being addressed with sufficient vigour. Several laboratories ignore, for example, the IFL cut-off points recommended by the Committee for Autoimmune Serology of the IAIHG and use their own, thus undermining comparability between different laboratories/centres. Worryingly, the cost per test seems a major reason for arbitrary elevation of cut-off points in routine practice: selecting 1:80 or even 1:160 as a screening dilution expands the number of "negatives" albeit reducing or eliminating the need for re-testing. In patients with AiLD and relatively low autoantibody titres, such as children with AIH, a report that is inaccurately indicative of negativity for autoantibodies can delay diagnosis and, harmfully, defer treatment[76,147]. Hence rigorously performed autoantibody testing may in fact

provide a more economical report than a "false negative" one if such leads the clinician to order additional costly diagnostic procedures.

Additional problems for autoantibody testing especially with IFL are intrinsic to the methodology itself. First, availability of tissue substrate comprised of freshly cut sections from cryostat blocks of unfixed liver, kidney stomach tissue is limited to relatively few specialised laboratories. Second, sections of commercial origin are of variable quality because, to lengthen shelf-life, they are treated with fixatives, which readily result in enhanced background staining[55]. Third, IFL requires highly-trained and experienced personnel, is time-consuming and cannot be automated, resulting in a low throughput and increased personnel costs leading to a significant shift from IFL towards ELISAs or blot assays based on liver-autoantibody profiles; these compared to IFL are less-time consuming, easy to perform and amenable to automation. However, the authors of this review reiterate the recommendations of the Committee for Autoimmune Serology of the IAIHG stating that the current ELISAs should complement but not replace IFL. Either technique has their *pros* and *cons*, and gives answers to different questions, such that results are not directly comparable[148-150]. Most liver-related autoantibodies can be detected by IFL when using a triple rodent tissue. HEp-2 cells can help to differentiate ANA patterns and ethanol-fixed neutrophils can be used for the detection of ANCAs. In contrast, ELISAs give answers for (usually) pre-selected individual autoantibody specificities. While the analytical sensitivity of ELISAs is satisfactory, their specificity varies according to the manufacturer[150] whereas such problems are rather infrequent by IFL testing based on a triple rodent tissue substrate[151].

Over the last decade there has been a steady increase in the use of the liver-related autoantibody tests to assist both diagnosis and clinical research into AiLD[55]. This increase has been attributed mainly to the introduction of molecularly based assays for the testing of antibodies to F-actin[70-75], CYP2D6[152] and SLA[83,122,153] in AIH, and for evaluating antibodies to sp100 and gp210 in PBC[92,94-96,154]. Of concern, results for these antibody specificities may be promulgated by laboratories without authentication from externally or independently monitored quality assurance programmes (QAP).

Quality assurance (QA) can occur at three levels. The first is at the level of commercial providers of assay kits, reagents etc who would establish QA "in house" before marketing but who often elect to participate also in QAPs for routine laboratories. The second are the formalised QAPs, run by semi-governmental agencies or other organizations, as described below. The third level, which scarcely exists, involves the end-user, the responsible clinician, who must order tests advisedly with good clinical data and interpret these in the light of the clinical information to make wise evidence-based decisions. Thus it behoves the clinician to become fully aware of the many contributions (and shortcomings) of contemporary diagnostic immunoserology.

Table 1 Laboratories from various countries participating to the UK National External Quality Assessment Service (UK NEQAS)

Country	Number	Country	Number
Austria	3	Latvia	1
Belgium	7	Malaysia	1
Croatia	2	Malta	1
Cyprus	1	New Zealand	3
Denmark	2	Norway	9
Eire	15	Portugal	31
Estonia	1	Republic of Chile	1
Finland	5	Singapore	1
France	29	South Africa	3
Germany	9	Spain	68
Greece	16	Sweden	14
Hong kong	1	Switzerland	7
Hungary	5	The Netherlands	1
Israel	8	Turkey	2
Italy	65	UK	136
Kingdom of Saudi Arabia	1	United Arab Emirates	1
Kuwait	1	USA	3

REPRESENTATIVE QUALITY ASSURANCE PROGRAMMES FOR DIAGNOSTIC SEROLOGY IN LIVER DISEASE

USA

The College of American Pathologists (CAP, www.cap.org) runs survey programmes which allow laboratories to evaluate regularly their autoantibody testing performance. Of relevance to liver, CAP circulates coded anti-M2 AMA, anti-LKM1 and SMA samples for testing. The participating laboratories analyse the sera and return their results for evaluation. In return, each laboratory receives an anonymised report of the performance of all participating laboratories.

UK

A National External Quality Assessment Service (UK NEQAS) (www.ukneqas.org.uk) is responsible for the objective assessment of the performance of autoantibody testing. The UK NEQAS for General Autoimmune Serology incorporates one sample in each of six distributions annually for AMA, anti-LKM1 and SMA. The performance reports of the participating laboratories also provide information on kit suppliers. Participation is not limited to UK but is open to non-UK Countries (Table 1).

Germany

There are currently two regulatory and quality assurance agencies, namely INSTAND (Institut für Standardisierung, www.instandev.de) and DGKL (Deutsche Gesellschaft für Klinische Chemie und Laboratoriumsmedizin, www.dgkl.de). INSTAND circulates twice per year two samples to be tested for AMA, SMA and anti-LKM1 antibody testing. Participants (150) report results quantitatively and semi-quantitatively (from 0-4 to evaluate antibody titre; 0 = negative; 1 = borderline; 2 = low; 3 = middle; and 4 =

high). There is no reference to specific manufacturers but only to test methods and overall percentage of consistent results. DGKL has a similar approach but evaluations are divided on the basis of the methods used and they provide also information in relation to the kits manufacturers. Target values are determined in two reference laboratories.

France

Quality autoantibody assessment in France is organised by the French Health Products Safety Agency (AFSSAPS, Agence Française de Sécurité Sanitaire des Produits de Santé, http://agmed.sante.gouv.fr/). This Agency has the executive responsibility for proposing relevant QAPs to clinical laboratories, whether in the private or in the public sector. An autoantibody detection survey has been running on an annual basis since 1998.

Italy

In Italy there are no formal regulatory and quality assurance programmes with several laboratories participating in the surveys by UK NEQAS or CAP. Recently, a study group has been formed (Forum Interdisciplinare per la Ricerca nelle Malattie Autoimmuni-FIRMA-www.grouppofirma.com). FIRMA aims to provide guidelines for autontibody testing and to identify and collect sera of different autoantibody specificities that will be available for all of its member institutions.

Finland

Labquality at Helsinki offers twice per year three samples for SMA, AMA and anti-LKM1 assessment. Qualitative target values are determined in a reference laboratory and results are listed according to manufacturer and method. Evaluation reports are confidential.

Australia and New Zealand

QAPs have been established under the auspices of the Royal College of Pathologists of Australasia (RCPA) based on the selection by RCPA of expert organizing groups which distribute batches of sera to diagnostic laboratories that voluntarily elect to participate (www.rcpaqap.com.au). Diagnostic laboratories from Australia, New Zealand, and several South East Asian countries together with manufacturers and purveyors of kits participate in this programme. The Tissue Antibodies module includes AMA, SMA and anti-LKM1 antibodies. Feedback to the laboratories is by a report to all participants in which any single laboratory can identify its own performance versus that of all other participants. The RCPA issues certification of participation in this QAP. The reports sent back to laboratories are inspected by the National Association of Testing Authorities (NATA) during laboratory assessment visits. In order to be accredited, laboratories must participate and perform satisfactorily in the relevant proficiency testing programmes. There is a 'regulatory' element here in that NATA certification is required for access to fees under the Medicare rebate scheme.

As expected, quality assurance programmes have highlighted difficulties encountered by peripheral laboratories. In mid-2007, UK NEQAS distributed a serum with a typical anti-LKM1 antibody staining; a substantial proportion (53 out of 356, 15%) of the laboratories reported negativity for anti-LKM1 antibody test and, among these 53 laboratories, 43 incorrectly reported positivity for AMA instead (Peter White, UK NEQAS, personal communication). Also, rather worryingly, several additional laboratories did not return reports on anti-LKM1 either because they themselves do not offer this test or because they ignore its significance (Peter White, UK NEQAS, personal communication).

It is clear that exchange of calibrated reference sera and rigorous standardization programmes on liver-related autoantibody serology are urgently needed. Such initiatives will need to involve initially researchers and laboratories with a special interest in the respective antibody specificities and subsequently clinical laboratories performing routine screening tests. To this end, efforts have been made recently by the IAIHG to arrange an exchange of sera at international level but whether such an initiative will take off depends on securing financial support. Administrative sponsorship should initially come from the International Association for the Study of Liver (IASL), the American Association for the Study of Liver Diseases (AASLD) or the European Association for the Study of Liver (EASL) or from Clinical Immunology Societies of developed countries.

In conclusion, practice guidelines on liver autoimmune serology based on consensus of experts in the field have been issued and need to be steadily updated[55]. The more the clinician is aware of these guidelines, the greater the chance of correct and clinically relevant autoantibody diagnosis. It is in the best interest of the patient to obtain eventually the highest possible commitment and coordination of all organizations, agencies, industrial partners and networks working in the field.

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TOPIC HIGHLIGHT

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Transplantation in autoimmune liver diseases

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Abstract

Liver transplantation remains an effective treatment for those with end-stage disease and with intractable liver-related symptoms. The shortage of organs for transplantation has resulted in the need for rationing. A variety of approaches to selection and allocation have been developed and vary from country to country. The shortage of donors has meant that new approaches have to be adopted to make maximal use of the available organs; these include splitting grafts, use of extended criteria livers, livers from non-heart-beating donors and from living donors. Post transplantation, most patients will need life-long immunosuppression, although a small proportion can have immunosuppression successfully withdrawn. Newer immunosuppressive drugs and different strategies may allow a more targeted approach with a reduction in side-effects and so improve the patient and graft survival. For autoimmune diseases, transplantation is associated with significant improvement in the quality and length of life. Disease may recur after transplantation and may affect patient and graft survival.

Key words: Liver transplantation; Autoimmune disease; Recurrence; Immunosuppression

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INTRODUCTION

The three major autoimmune liver diseases that may require liver transplantation are primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC) and autoimmune hepatitis (AIH). In this review, we will discuss the role, timing and outcome of transplantation for these indications.

Criteria for liver transplantation for patients with autoimmune diseases are relatively well defined[1]. As with other indications, liver transplantation is indicated either to relive intractable symptoms of liver disease (such as pruritus or encephalopathy which do not respond to conventional therapy) or to prolong life. Life after transplantation is normally excellent but is never normal. Furthermore, survival is reduced when compared to an age and sex-matched population[2]. Reasons for the reduction in survival include the mortality of the procedure itself, the risks of recurrent disease and the consequences of immunosuppression which may be class related (such as an increased risk of sepsis and some malignancies) or more-specifically related to the individual drugs used (such as renal failure and cerebro- and cardio-vascular death). Thus, for most patients with chronic liver disease, timing of transplantation has to be done with consideration of the risks and balance of remaining with the native liver and of the procedure.

Some guidance is given by prognostic models, of which the most commonly used is the model for end-stage liver disease (MELD) formula[3]. MELD, initially used to predict short-term survival after stent insertion has been shown to be accurate in prediction of most patients with chronic liver disease. The score, which is derived from serum bilirubin, creatinine and prothrombin time, is useful; for the average patient, there is a survival benefit when transplanted with a score of 16 or more. Addition of other analytes to the formula, such as serum sodium, may increase the accuracy[4]. In some situations, the model does not predict outcome. For example, in those with a liver cell cancer where the prognosis without transplant is dependent on the cancer rather than parenchymal function. Other exceptions occur with hepatopulmonary syndromes, for example. There are several recent reviews of the general indications and contra-indications (see for example[5,6]).

There is an increasing gap between the number of patients who would benefit from a transplant and the availability of suitable organs for transplantation. In order to fulfil demand, differing strategies have been utilized: this includes use of split livers, non-heart beating donors, marginal donors and to a lesser extent,

living donor programmes. These strategies have, to some extent, masked the shortage of organs. Extended criteria or marginal livers are being utilized in greater numbers, (these are grafts where there is concerns that their use might impact on the outcome of the patient). These include those grafts where there is a greater risk of non-function (characterized by steatosis in the graft, older donors and prolonged cold ischemia times), technical problems (such as the use of split, partial or reduced grafts) or those grafts that carry a risk of transmission of viral infection or malignancy.

Living donor transplantation accounts for around 2%-5% of transplants in Europe and North America but for almost all transplants carried out in Asia, where donation rates from deceased donors are very low. Limitations on living donation focus on the risk to the donor: too much liver volume removed from donor may induce liver failure in the donor, too little may cause recipient graft failure. The mortality for the donor in left lobe is 0.05% rising to 0.4%-0.5% in right lobe transplants and donor morbidity is 20% with long-term outcomes unknown[7].

The shortage of grafts means that rationing must occur: the competing interests of equity, justice and utility have to be recognised. Thus, criteria for selection (that is admission to the list) and allocation (identification of the recipient for a graft) need to be agreed. Different health care systems have adopted different principles. Conflict may exist where transplantation is considered for some indications, such as liver disease from alcohol where the medical views are not in accordance with those of the public[8]. The immunological processes that operate in an allografted liver are complex, since the immune system of the host can react against alloantigens, human leucocyte antigen (HLA) molecules and "minor" transplantation antigens of the donor. Concurrently, passenger leucocytes of the donor may react against HLA or other antigens of the host, resulting in "two-way" immune responsiveness. The liver above all other organs has a propensity to generate a state of intra-hepatic immune tolerance that limits harmful immune reactivities, sometimes to the degree that immunosuppressant drugs become dispensable after a liver transplant. The immunological issues involved, which are beyond the scope of this article, are discussed informatively in several reviews[9,10].

Post transplant, most patients will need life-long immunosuppression. However, in the last decade there have been developments in the management of immunosuppression. In the early days of liver transplantation, the principles of immunosuppression after liver transplant were extrapolated from renal transplant programmes. However, there are some major differences: early acute rejection after liver transplantation is not associated with an adverse outcome; the requirement of immunosuppression is less and sometimes, as mentioned, it is even possible to withdraw immunosuppression completely. It is not possible reliably to identify those patients in whom immunosuppression can be safely withdrawn: however, those with good graft function at 5 years and with minimal inflammation on histology and were not transplanted for autoimmune diseases are most likely to benefit from a planned withdrawal of immunosuppression[11,12].

Tailoring immunosuppression to the individual is a much discussed but little practiced approach. For instance, those grafted for hepatitis C virus infection need to be protected against rejection since major changes in corticosteroids will increase the consequences of viral re-infection. The advent of newer biological agents including humanized monoclonal antibodies such as Campath-1H (alemtuzumab), antibodies to interleukin-2 receptor (IL-2R), and CLTA-4Ig, may permit more selective approaches to immunosuppression. Campath-1H is a humanized monoclonal antibody against CD52, a molecule expressed on the surface of human B and T lymphocytes. Antibodies to the IL2Ra chain target the CD25 molecule on activated T lymphocytes. Cytolytic T-lymphocyte-associated antigen 4 (CTLA-4) Ig is an immunoglobulin fusion protein with CTLA-4, a natural down-regulatory molecule expressed by T lymphocytes. There are other biologicals under investigation. Induction of full tolerance has long been the goal of solid organ transplantation but, despite advances in the laboratory, this goal has so far remained elusive in the human. The adoption of approaches allowing for early immunological engagement (the Window of Opportunity for Immunological Engagement) as suggested by Calne[13] or use of Campath-1 or other biologicals may offer a new and effective approach[14].

AIH

AIH is a relatively uncommon indication for liver transplantation, currently accounting for no more than 5% of cases[15]. As with cirrhosis from other causes, liver transplantation is indicated in those with end-stage disease characterized by a MELD score > 16, signs of decompensation on treatment such as hepatic encephalopathy, ascites or variceal haemorrhage or, rarely, with hepatocellular carcinoma development. In those who present with acute or fulminant liver failure, liver transplantation should be considered early in the course. Outcomes are good with 1 year and 5 years patient survival rates of about 87% and 80%-90%. Graft survival rates at 1 year and 5 years are 84% and 74%-76%[16-18].

Recurrence after transplantation

Diagnostic criteria for recurrent AIH (rAIH) have been developed and are summarised in Table 1. The reported recurrence rate of AIH following transplantation is variable 17%-42% at 5 years[19,20]. Table 2 shows the reports of recurrent AIH. Gautam`s systematic review of 13 papers concluded disease recurrence occurred in 22% of recipients at a median interval of 26.4 mo[21]. Czaja suggested that a loss of self-tolerance and molecular mimicry would explain the repopulation of the allograft with recipient antigen-presenting cells and that the already primed promiscuous recipient cytotoxic

Table 1 Criteria for the diagnosis of recurrent AIH

Criteria
Liver transplant for autoimmune hepatitis
Auto-antibodies in significant titre (> 1:40)
Sustained rise in serum aminotransferase activity (> 2 times normal)
Elevated serum immunoglobulins
Compatible liver histology (infiltration of portal tracts by plasma cells, piecemeal necrosis and bridging necrosis[21])
Corticosteroid dependency
Exclusion of other causes of graft dysfunction (such as rejection and HCV infection)

T cells are likely factors for recurrent disease[22].

Many studies have been published in the literature, but most include relatively small numbers, use different criteria for the diagnosis and are retrospective. Reich retrospectively reviewed 24 AIH transplant recipients; 6 patients developed biopsy proven recurrence at 15 mo, 3 proceeded to regrafting and 2 of these patients developed recurrent AIH in the second graft. No patient transplanted for fulminant hepatic failure developed recurrence compared to 1/3 of those with chronic disease[15]. Duclos-Vallee performed protocol biopsies and demonstrated histological recurrence preceded biochemical abnormality by 1-5 years in 23.5%[25]. There was no difference in survival or recurrence between the three sub-types of AIH. Rates of rejection were high both in the control and AIH groups but greater in those grafted for AIH. (50% and 88%)[27]. No patient required re-transplant because of recurrent disease and there was no difference in patient survival or graft survival[18].

There are no consistent risk factors for recurrence identified. Pre-transplant disease duration, donor/recipient gender distribution, HLA studies, and rejection episodes did not correlate with AIH recurrence but the degree of necro-inflammation in the native liver was significantly greater in those with recurrence in one study[24]. The choice of immunosuppression is controversial but a recent systematic review by Gautam found no difference in recurrence rates between recipients on tacrolimus (31%) or cyclosporin[21].

Khalaf reported a histological recurrence in 18.7% (median follow up of 530 d) which was successfully treated by optimizing immunosuppression. Steroid withdrawal failed in all recipients and was always accompanied by almost immediate elevation of liver enzymes[28]. A case of AIH recurrence 6 years after a living donor related liver transplant, in the absence of autoantibodies was reported. The patient had steroids discontinued 1 year post orthotopic liver transplant (OLT) whilst maintained on tacrolimus but became antinuclear antibody (ANA) positive again 3 years later, 2 years prior to the histological diagnosis but in the absence of abnormal LFTs[29].

De novo AIH

De novo AIH has features of a steroid responsive AIH in patients transplanted for other non-immune indications and is characterized by a biochemical hepatitis, circulating auto-antibodies, elevated immunoglobulins and an inflammatory infiltration with interface hepatitis. The first report of de novo AIH was in 7 children at a median of 2 years post-transplant[30]. Children are more at risk than adults but the condition is still relatively uncommon with an incidence of around 3%. There is usually a good response to additional immunosuppression with corticosteroids, but in some cases there is progression to cirrhosis and subsequent graft failure[31]. Whether this is truly a de novo autoimmune phenomenon or merely a form of rejection is not certain: early studies suggesting an immune response to graft antigens are controversial[32] and studies suggesting an immune response to graft isoforms of glutathione-S transferase remain unconfirmed.

Table 2 Reports of recurrent autoimmune hepatitis

Author	Follow up (mo)	*n*	Recurrence	Period recurrence occurred	Re-OLT/ Cirrhosis
Milkiewicz 1999[23]	29	47	13/47	29 mo	3/47
Ayata 2000[24]	67	12	5/12	35-280 d	2/12
Reich 2000[15]	27	24	6/12	At 15 mo	3/24
Molmenti 2002[18]	29	55	11/55	At end	
Duclos-Vallee2003[25]	120	17	7/17	2.5 yr[1]	2/17
Núñez-Martínez 2003[26]	38	15	1/15	At end	
Vogel 2004[27]	24	28	9/28	5 yr	4/28
Gautam 2006[21]			23%	2.4 mo[2]	

[1]Mean; [2]Median.

Conclusion

The outcome for OLT in AIH is good and is merited in those with chronic disease and a much smaller cohort will have an acute or fulminating course the prognosis of which is relatively unaffected by corticosteroids. Recurrence of disease is relatively common in the allograft and may be detected on protocol biopsy at an asymptomatic stage before biochemical or clinical clues. Generally recurrent AIH responds well to increases in immunosuppression or addition of corticosteroids. This should be taken into account when considering long term immunosuppression and especially on reduction should be in conjunction with immunoglobulins, autoantibody profile and histology. Most data are retrospective with relatively small numbers and studies are lacking in long term reduction and withdrawal of immunosuppression and further controlled studies are required.

PBC

Indications

Indications for transplantation are listed in Table 3. Unlike pruritus, which is rapidly reversed after transplantation, lethargy is not an indication since often

Table 3 Indications for transplantation in PBC

Indications
Symptom based
Intractable pruritus refractory to medical therapy
Hepatic encephalopathy
End-stage liver disease
Recurrent variceal haemorrhage
Episode of spontaneous bacterial peritonitis
Pulmonary hypertension
Hepato-pulmonary syndrome
Diuretic resistant ascites
Progressive osteopaenia
Muscle-wasting
Hepatoma (Milan criteria)
Biochemistry
Serum bilirubin > 150 μmol/L
Serum albumin < 25 g/L

Table 4 Criteria for the diagnosis of recurrent PBC

Criteria
Transplantation for PBC
Characteristic histological features of PBC
Mononuclear inflammatory infiltrates
Lymphoid aggregates
Epithelioid granulomas
Bile duct damage
Persistence of anti-mitochondrial antibodies
Elevated immunoglobulins
Exclusion of other causes of graft damage

Definite recurrent PBC is made when all 4 of these criteria are present, and in the presence of at least 3 of the 4 histological features. Probable recurrence when only 2 histological features are present[40].

it does not improve with transplantation[33]. The need for transplantation for PBC is falling (United Network for Organ Sharing (UNOS) data shows, of 2391 cadaveric liver transplants in 1991, 18% were for cholestatic liver disease compared with 10% of 4579 in 2000 and was the second most common indication for transplantation) and the impact of ursodeoxycholic acid (UDCA) is a tempting but controversial explanation.

Timing of transplantation

A variety of disease-specific prognostic models have been developed but for short term survival a MELD score is effective and a score > 16 indicates a survival benefit from transplantation. Serum bilirubin > 100 μmol/L[34] as well as significant poor liver function with length of life attributed to disease limited to 1 year are indicators for transplantation assessment[35]. A Mayo risk score > 7.8 has also been validated to indicate survival in the absence of transplantation[36,37].

Survival after OLT

The 1, 3 and 5 year actuarial patient and graft survival was 94%, 91%, and 82%, and 89%, 83%, and 75%, respectively in a series of 301 PBC transplant recipients in the UK[38] which is comparable to European transplant registry data. The commonest indication for re-transplantation in the first year is chronic rejection[39]. Survival rates remain consistently better than other indications, even after adjusting for case-mix and other risk factors. Immunosuppression is usually a standard triple regimen of calcineurin inhibitor (tacrolimus or cyclosporin), corticosteroids (withdrawn over 3 mo) and azathioprine or mycophenolate mofetil.

Recurrent disease

Recurrent disease (Table 4) is diagnosed by characteristic histology and absence of other causes of graft damage. The histology of recurrence is comparable to pre-transplant PBC[40]. Patients with anti-mitochondrial antibodies and normal liver function tests in the presence of normal histology may develop recurrence with hallmark granulomatous cholangitis[41]. Elevated serum immunoglobulins and persisting anti-mitochondrial antibodies do not in themselves indicate recurrent disease. Recurrent PBC is seen in 17% of patients at a mean of 36 mo[42] rising to 30% at 10 years. Recurrence rates on biopsy as high as 35% at 1 year have been reported[43]. The reported median time to recurrence is between 3.7 and 5 years[44,45]. Recurrence may not be diagnosed unless a protocol biopsy is taken as the liver tests may be normal[42]; indeed only half will have biochemical abnormality[44]. Liver tests may remain normal for 5 years after histological diagnosis[45].

The role of UDCA in the treatment or prevention of recurrent PBC remains uncertain. A retrospective review of 154 PBC liver transplant recipients followed at the Mayo Clinic for least 1 year reported that recurrent PBC was not associated with death or liver re-transplantation. 38 patients with recurrent PBC received UDCA at an average dose of 12 mg/kg per day for a mean duration of 55 mo. Over a 36-mo period, an estimated 52% of UDCA-treated patients experienced normalization of serum alkaline phosphatase and alanine aminotransferase compared to 22% of untreated patients but no significant difference in the rate of histological progression was noted between subgroups. UDCA did not influence patient and graft survival[46]. It should be noted that this experience does not concord with our own unpublished data where graft loss from recurrent PBC is 4%.

Should all those transplanted for PBC be offered UDCA? The agent is safe and improves all serological parameters and may retard progression so, even in the absence of clear evidence, we would advocate its routine use.

Risk factors for recurrence

Many studies have evaluated risk factors for recurrence. The literature is mixed concerning donor and recipient age as well as cold and warm ischaemia time[42,43,47,48]. The type of immunosuppression used is also controversial[45,49]. In a study of 485 recipients followed up over 79 mo, the recurrence rate with tacrolimus was 23% with OR 2.73 and time to recurrence 62 mo compared to 123 mo on cyclosporin ($P < 0.001$)[47]. Guy found similar results with OR 2.5 for tacrolimus[43]. No differences between cyclosporin and tacrolimus were

seen in other trials, though protocol liver biopsies were not performed or were only done in the context of graft dysfunction[50,51]. Sanchez reported a 156 patient cohort using protocol biopsies at 1, 2, 5, 10 and 15 year intervals with recurrence in 8.4% of recipients taking cyclosporin, azathioprine and steroids, compared with 12.2% of those receiving cyclosporin and steroids alone and 16.7% of patients taking tacrolimus and steroids ($P = 0.11$)[52]. Thus the evidence does suggest that cyclosporin is, compared with tacrolimus, associated with a slower rate of progression of recurrent disease. Whether this indicates that those grafted for PBC should be offered cyclosporin-based immunosuppression rather than that based on tacrolimus, and whether those with recurrent PBC should be switched from tacrolimus to cyclosporin is uncertain.

Implications of recurrence

The consequences of recurrent disease appear to be relatively small[53]. In our series of 486 PBC transplant recipients, 3 were re-grafted as a consequence of recurrent disease, all of whom have recurrence in the re-graft[54].

Quality of life issues

Pruritus may resolve within days of transplantation. Fatigue persists and does not appear to improve post liver transplant[33] although there is a great improvement in quality of life[55]. Gross studied 157 adult patients with PBC or PSC before and 1 year after liver transplantation. The quality of life following transplantation was significantly better than before transplantation in all aspects but at 1-year follow-up, was not predictable by the pre-transplant subjective health status or clinical factors[56].

PSC

Survival after transplantation

Indications for transplantation are as for other end-stage liver disease complications. European data show patient survival at 1, 3, 5 and 10 years was 86%, 79%, 76% and 66% respectively from Jan 1988-June 2006 (www.eltr.org).

PSC recurrence

Recurrent PSC (Table 5) must be distinguished from secondary sclerosing cholangitis; Characteristic histological features are not always present so the diagnosis may be made on imaging the biliary tree. PSC recurrence is relatively common with figures of 37% at 36 mo and 60 % at 5 years[57,58] Gautam's systematic review of 14 reports revealed a recurrence rate of 17% but was unable to comment upon possible risk factors[21]. Sheng studied the prevalence of stricturing disease in 100 patients who underwent transplantation for PSC and 543 controls without PSC. 27% PSC liver recipients compared to 13% of controls showed intra-hepatic strictures by cholangiography. Intra-hepatic and non-anastomotic extra-hepatic strictures were significantly more frequent in the PSC group[59]. In a small cohort who underwent living donor liver transplantation with a median follow up of 3.5 years, half developed recurrent PSC with the mean time to recurrence 3.3 years (1.1-5.4 years).There was no direct comparison to their cadaveric cohort[60]. Khettry retrospectively analysed 51 PSC patients with a follow-up of 2 to 14 years. Of the remaining 42 patients, 6 had recurrent PSC with typical histological and cholangiographic findings, 12 had autoimmune liver disease that was not otherwise specified with histology of AIH/overlap syndrome, 3 had chronic rejection, 4 had ischemic cholangiopathy, and 17 had no recurrence. Post-transplant malignancies were significantly more common in the non-recurrent cases compared with all others combined ($P = 0.031$) and caused death in four. The majority of deaths (11/13) in other groups were due to sepsis. In conclusion, allograft autoimmune liver disease was seen in 18 (43%) of 42 long-term post-LT PSC patients, with progression in 5 of 18 patients. Features of PSC were seen in 6 (33%) of 18[61].

Table 5 Criteria for the diagnosis of recurrent primary sclerosing cholangitis[72]

Criteria
Transplant for PSC
Multiple non-anastomotic strictures, headings and irregularity more than 90 d post OLT
Characteristic liver histology (fibrous cholangitis and/or fibro-obliterative lesions) with or without ductopenia, biliary fibrosis or biliary cirrhosis may be seen (but absence of characteristic features does not exclude the diagnosis).
Exclusion of other causes of secondary sclerosing cholangitis & stricturing (due to surgery, trauma, ischaemia, hepatic artery stenosis/thrombosis, established ductopaenic rejection, blood type ABO incompatibility and infections)
Cholestatic liver tests

Many factors have been associated with recurrence including steroid-resistant rejection, OKT3 use, preservation injury, ABO incompatibility, cytomegalovirus infection, male sex, donor-recipient gender mismatch and steroid resistant rejection but not specific calcineurin inhibitor use or frequency of rejection[61-67].

Although there is some controversy as to the effect of pre-transplant colectomy on the recurrence rate, our own data consistently show that colectomy either before or during transplant is not associated with recurrent disease whereas the incidence of recurrence in those who had a colectomy post transplant is no different to those with an intact colon. Overall, recurrence of PSC leads to patient and graft loss.

Colitis and colonic neoplasia after transplantation

Evidence linking immunosuppression with inflammatory bowel disease-free survival is mixed[68,69]. Colitis is variable and may present *de novo* after transplantation, with an incidence of 6% at 1 year and 20% at 5 years[70]. In a study of 20 PSC transplant recipients with coexisting ulcerative colitis followed over a median period of 11.9 years before OLT and 4.4 years after OLT, there was a significantly higher relapse rate after OLT than pre-transplant. 35% of recipients went onto colectomy after OLT (3 for disease severity and 4 for neoplasia/

dysplasia)[71]. These results were mirrored in a study from Birmingham which looked at 152 patients with PSC (100 with coexisting IBD). The incidence of colorectal cancer after transplant was 5.3% compared with 0.6% in non-PSC cases. All cancers in the PSC group were in patients with IBD and an intact colon. The cumulative risk of developing cancer in the 83 patients with an intact colon and IBD was 14% and 17% after 5 and 10 years, respectively. The multivariate analysis identified colonic dysplasia after transplant ($P < 0.0003$), duration of colitis more than 10 years ($P < 0.002$), and pancolitis ($P < 0.004$) as risk factors[69]. Colonoscopy is thus recommended annually following transplant.

FUTURE PROSPECTS

Over the last three decades, liver transplantation has evolved from an experimental, high risk procedure to a routine operation with a high success rate. Indications have widened and contra-indications decreased. Currently, the major limitation remains the shortage of organs so that not everyone who might benefit from the procedure can receive a graft and surgeons have to use extended criteria organs. The use of stem cell therapy and liver cell transplants, remain in their infancy. There still remain considerable challenges ahead: major causes of graft loss include recurrent disease, especially Hepatitis C but also autoimmune diseases, and the side-effects and complications of immunosuppression. The goal of achieving tolerance remains elusive but the development of new agents, especially biologicals, may allow for more effective strategies. The stimulus and challenges of liver transplantation have advanced our understanding of the mechanisms of alloantigen immune recognition and target cell damage and helped introduce new immune-modifying agents and strategies. They have also helped our understanding of the anatomy, physiology and pathophysiology of the liver. However, the long-term goal of clinical research must be the treatment of disease in the native liver so that transplantation becomes redundant.

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REVIEW

Why, who and how should perform liver biopsy in chronic liver diseases

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Abstract

Chronic viral hepatitis is a common disease in the general population. During chronic hepatitis, the prognosis and clinical management are highly dependent on the extent of liver fibrosis. The fibrosis evaluation can be performed by FibroTest (using serological markers), by Elastography or FibroScan (a noninvasive percutaneous technique using the elastic properties of the hepatic tissue) and by liver biopsy (LB), considered to be the "gold standard". Currently, there are three techniques for performing LB: percutaneous, transjugular and laparoscopic. The percutaneous LB can be performed blind, ultrasound (US) guided or US assisted. There are two main categories of specialists who perform LB: gastroenterologists (hepatologists) and radiologists, and the specialty of the individual who performs the LB determines if the LB is performed under ultrasound guidance or not. There are two types of biopsy needles used for LB: cutting needles (Tru-Cut, Vim-Silverman) and suction needles (Menghini, Klatzkin, Jamshidi). The rate of major complications after percutaneous LB ranges from 0.09% to 2.3%, but the echo-guided percutaneous liver biopsy is a safe method for the diagnosis of chronic diffuse hepatitis (cost-effective as compared to blind biopsy) and the rate of complications seems to be related to the experience of the physician and the type of the needle used (Menghini type needle seems to be safer). Maybe, in a few years we will use non-invasive markers of fibrosis, but at this time, most authorities in the field consider that the LB is useful and necessary for the evaluation of chronic hepatopathies, despite the fact that it is not a perfect test.

INTRODUCTION

Chronic viral hepatitis is a common disease in the general population. Chronic hepatitis B virus (HBV) infection affects 350 million individuals globally, and approximately 15%-40% may develop serious complications, including end-stage liver disease and hepatocellular carcinoma[1]. Chronic hepatitis C virus (HCV) infection is also an important cause of chronic hepatitis, data from World Health Organization (WHO) suggesting that 170 million people are infected world-wide with HCV, 10 million of them in Western Europe[2]. It is estimated that at least 3.9 million persons (1.8% of the population) in the United States are anti-HCV seropositive, and that 2.7 million are chronically viremic[3].

At the same time, alcoholic steatohepatitis (ASH) and non-alcoholic steatohepatitis (NASH) are frequent in the developed countries. Regarding NASH, long-term follow up studies on obese patients showed increased cirrhosis-related morbidity and mortality[4]. Also, studies on cohorts of diabetic patients showed increased incidence of non-alcoholic chronic liver disease and hepatocellular carcinoma[5].

The complete evaluation of a patient with diffuse liver diseases requires: clinical evaluation, biological evaluation and morphopathological exam-liver biopsy (LB), for the grading and staging of the liver disease. The clinical evaluation is often irrelevant, only the presence of spider naevi on the anterior thorax or an enlarged and firmer liver could suggest that the patient already has liver cirrhosis (suspicion that has to be confirmed or infirmed by further tests). The biological evaluation by means of

the usual tests is also often irrelevant, especially in chronic hepatitis C, known to induce sometimes severe hepatic lesions, while the aminotransferases are normal or only slightly elevated. Thus, it is considered that the liver biopsy has a key role for the diagnosis and follow-up of chronic diffuse hepatopathies, especially for the staging of chronic hepatitis C[6-9].

So, what is the utility of LB in chronic liver disease? Fontolliet[6] stated that LB has the following roles: to confirm the diagnosis of chronic hepatitis; to assess the necro-inflammatory activity (grading) and the severity of fibrosis (staging); to exclude another hepatopathy or an associated disease; to certify the diagnosis of cirrhosis (when present).

A review of the short history of LB shows us that: Paul Ehrlich is credited with performing the first percutaneous LB in 1883 in Germany; Sheila Sherlock described the percutaneous LB technique in 1945 and after Menghini reported a technique for the "one-second needle biopsy of the liver" in 1958, the procedure became more widely used[10].

But did the LB become a method of diagnosis unanimously accepted by the patients and the doctors? To answer this question we will present the results of a French study, performed on 1177 general practitioners that showed that 59% of the patients infected with HCV refused the LB, opinion shared by 22% of the general practitioners[11].

All these being said, we would like to discuss in this paper several aspects concerning the LB, trying to answer the following questions: (1) Why? (2) Who? (3) How to perform the LB?

WHY TO PERFORM A LB IN CHRONIC LIVER DISEASE?

During chronic hepatitis, the prognosis and clinical management are highly dependent on the extent of liver fibrosis[12]. The fibrosis evaluation can be performed by means of: FibroTest-using serological markers; Elastography or FibroScan-a noninvasive percutaneous technique using the elastic property of the hepatic tissue; liver biopsy-that seems to be the "gold standard".

FibroTest

The non-invasive tests for the assessment of the severity of chronic liver diseases are an interesting alternative, more and more evaluated in the last years, aimed to replace, maybe, the LB. After 2000, the non-invasive tests predictive of liver damage were studied more and more, especially in chronic hepatitis C[13] and, more recently, also in chronic hepatitis B[14] and NASH[15-17].

FibroTest-ActiTest (FT-AT) was developed using biochemical markers and repeatedly demonstrated a high predictive value for fibrosis and necroinflammatory histological activity, in patients with chronic hepatitis C[18-21]. In two separate studies the FT-AT has been proven valuable also in patients with chronic hepatitis B[14, 22].

FT-AT is a noninvasive blood test that combines the quantitative results of six serum biochemical markers (alfa2-macroglobulin, haptoglobin, gamma glutamyl transpeptidase, total bilirubin, apolipoprotein A1 and ALT) with patients' age and gender in a patented artificial intelligence algorithm (USPTO 6631330) in order to generate a measure of fibrosis and necroinflammatory activity in the liver[14]. Previously validated FT-AT are used (Biopredictive, Paris, France; Fibro-SURE LabCorp, Burlington, NC), that provide an accurate measurement of bridging fibrosis and/or moderate necroinflammatory activity with AUROC (Area Under Receiver-Operating Characteristic Curve) predictive value between 0.70 and 0.80, when compared to the liver biopsy[23].

It is recommended that FibroTest-ActiTest should not be performed during Ribavirin therapy, because it can induce hemolysis and low haptoglobin levels, nor in patients with Gilbert's syndrome, with acute hepatitis or extra hepatic cholestasis[23], cases in which falsely elevated fibrosis and activity scores can be obtained.

In a recently published editorial in the American Journal of Gastroenterology, Paul Thuluvath[24] discusses the FibroTest, stating that it was extensively studied only by Poynard *et al* and that there are only few independent studies. It is also considered that there are significant inter-laboratory variations, thus some studies demonstrated that significant fibrosis can be over-looked or over-rated in approximately 15%-20% of the cases. As a conclusion of this editorial, Paul Thuluvath considers that "we may be approaching a time when serum biomarkers may become an integral part of the assessment of patients with chronic liver disease, but published evidence suggests that these markers are not yet ready for prime time".

Elastography or FibroScan

Another non-invasive method of assessment of liver fibrosis is transient elastography. This technique enables the assessment the liver's stiffness and it is performed by a device called FibroScan (Echosens). The main component of the FibroScan is an ultrasound probe mounted on a vibrating device (piston). The patient to be examined lies down on his back and the ultrasound probe is applied to the skin surface between the ribs, thus examining the right liver lobe. The piston induces elastic vibrations, with low frequency and small amplitude that propagate through the liver. The reflected waves are captured by the transducer, their velocity being directly related to the elasticity (stiffness) of the liver. After several elastographic measurements are performed, the mean value must be calculated, thus enabling a correct assessment of the fibrosis.

This method of evaluation is totally painless and lasts only a few minutes. The stiffness of the liver is measured up to 2 cm in depth and on a surface with the diameter of approximately 1 cm (thus enabling the evaluation of a portion of the liver 500 times bigger than by LB)[25]. In the study performed by Foucher *et al*[26] the stiffness of the liver was measured up to a depth of 4 cm and on a surface with the diameter of 1 cm, so that 1/500 of the liver was evaluated. However the FibroScan device is ex-

ceedingly expensive rising to more than 60 000 Euros.

The value of the FibroScan method for the assessment of the severity of fibrosis in chronic hepatitis is under evaluation, but in the last 2 years several papers were published that demonstrate that this non-invasive method is precise enough to be compared to LB[12,25-28].

In the study performed by Castera *et al*, the elasticity of the liver, measured with the FibroScan device, varied between 2.4 and 75.4 kilopascals (kPa), with a median of 7.4 kPa[27]. When comparing the FibroScan to the LB in patients with chronic hepatitis C, the cut-off values were 7.1 kPa for F $\leqslant$ 2; 9.5 kPa for F $\leqslant$ 3 and 12.5 kPa for F = 4[27]. The area under the receiver operating characteristic curve (AUROC) of FibroScan was 0.83 for F $\leqslant$ 2; 0.90 for F $\leqslant$ 3 and 0.95 for F = 4[27]. The same authors demonstrated that, when combined with FibroTest, FibroScan was more precise than the LB, the AUROC values reaching 0.88, 0.95 and 0.95 respectively, for fibrosis $\leqslant$ 2, $\leqslant$ 3 or 4.

In a study performed by Ziol *et al*[12] the AUROC value of FibroScan as compared to the LB was 0.79 for F $\leqslant$ 2; 0.91 for F $\leqslant$ 3 and 0.97 for F = 4. Thus, the authors concluded that transient elastography appears reliable to detect significant fibrosis or cirrhosis in patients with chronic hepatitis C.

The majority of studies that compared the FibroScan to the FibroTest and to the LB showed a slight superiority of FibroScan *vs* FibroTest[27,28].

Starting from the encouraging results of FibroScan in patients with chronic hepatitis C, this method was also used to evaluate the severity of fibrosis in patients with chronic hepatitis B and in patients with primary billiary cirrhosis (PBC)[29,30].

It is probable that in a not too far future, the combination of FibroScan with FibroTest could avoid biopsy in most patients with chronic hepatopathies[27].

FibroTest and Elastography have a good value for the cases with no fibrosis or with important fibrosis (cirrhosis), but for the intermediate stages the value is low. This is the reason why LB is still the most used method of assessment of the severity of liver lesions in chronic hepatitis, several guidelines recommending that the decision to treat should be made after liver biopsy[31-32].

Liver biopsy

At this moment LB is still the "gold standard" for the evaluation of chronic hepatitis[33], but the method is not perfect. There are some problems regarding the diagnosis of cirrhosis by LB[33] and regarding the differences of the severity of fibrosis when twin LB are performed in both liver lobes[34]. This is why we consider appropriate a review of the advantages of LB, of its limits, of the best techniques to perform LB and also of the possible complications.

How representative can be a needle biopsy? The size of a biopsy specimen, which varies between 1 cm and 4 cm in length and between 1.2 mm and 1.8 mm in diameter, represents 1/50 000 of the total mass of the liver[31]. The British guideline about LB[31] considers that most hepatologists are satisfied with a biopsy specimen containing at least six to eight portal triads. A critical review of the literature reveals that biopsy samples 2 cm or more in length, containing at least 11 complete portal tracts, should be reliable for grading and staging chronic viral hepatitis[35]. Another study, concerning the dimensions of the biopsy specimen needed in order to perform an accurate pathological diagnosis, demonstrated that a fragment of at least 10 mm is enough for a correct staging and grading[36].

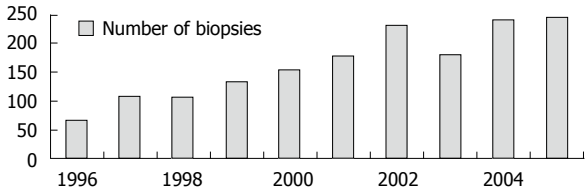

Figure 1 Liver biopsies performed in our department in the last years (1996-2005).

But which is the main reason to perform liver biopsy? The answer is: chronic hepatitis C. In our Department of Gastroenterology, the last 1500 LB were performed to evaluate: chronic C viral infection in 56.0% of the cases; chronic B viral infection in 34.2% of the cases; chronic viral coinfection in 3.2% of the cases; NASH and ASH in 4.5% of the cases and other liver diseases in 1.7% of the cases[37].

In France, in a nationwide survey, Cadranel *et al*[38] showed that 54% of the LB were performed for chronic C viral infection. The total number of LB performed each year in France is approximately 16 000[39].

The number of LB performed in every department increased in the last 15 years, mainly due to the increasing number of cases with chronic hepatitis C discovered in the last period, but we do not know the future trend, in connection to the introduction of non-invasive tests of fibrosis. The evolution of the number of ultrasound guided LB in our department in the last 10 years is shown in Figure 1.

WHO SHOULD PERFORM THE LIVER BIOPSY?

There are two main categories of specialists who perform LB: gastroenterologists/hepatologists and the radiologists. The specialty of the individual who performs the LB determines if the LB is performed under ultrasound (US) guidance or not.

In many countries the ultrasound examination is performed both by radiologists and by clinicians (Germany, Italy, Austria, Switzerland and Romania). In other countries, the ultrasound examination is performed only by radiologists (USA, UK, The Netherlands and Denmark).

Currently it is estimated that in the USA, 50% of the LB are performed by radiologists[40]. In the same country, a questionnaire regarding the LB practice, answered by 112 gastroenterologists/hepatologists,

showed that 30% of them do not perform LB (due to concern about risks, low reimbursement and logistical issues)[41]. Another important fact is that in countries in which gastroenterologists do not perform US examinations, the LB performed by the clinician are "blind", or done by the radiologist. It was suggested in USA that, by installing a US machine in the endoscopy unit, the cost of LB would decrease because a previous US examination in the Radiology Department would not be necessary before the LB, but that would require the gastroenterologist and hepatologist to become proficient in US technique and interpretation[40].

The European Diploma of Gastroenterology stipulates that, in order to become specialists, all the gastroenterologists should perform at least 300 US examinations[42]. In the opinion of Vautier's team, issued years ago, "the ideal liver biopsy may be one that is performed in the ward by a gastroenterologist using ultrasonographic guidance"[43].

HOW TO PERFORM LB?

Currently, there are 3 techniques for performing a liver biopsy[31]: percutaneous, transjugular and laparoscopic. The percutaneous liver biopsy (PLB) can be performed: blind, US guided or US assisted.

The most important question regarding the percutaneous liver biopsy that should be addressed is: *blind or echo-guided techniques?* The answer depends on the skills of the gastroenterologist (hepatologist) and on the technical possibilities (accessibility to the ultrasound machine).

However, it is still debatable whether ultrasound-guided LB has an advantage over the blind one or not[43,44]. In a prospective study in France, Cadranel *et al*[38] showed that from 2084 liver biopsies, only 56% were echo-guided. Also, many studies showed that the complications of LB seem to be related to the type of the technique, blind or echo-guided, respectively: (1) Younossi *et al*[45] showed that the complications appeared in 4% of the cases with "blind" biopsies and in 2% of the cases with "ultrasound-guided" biopsies (the study revealed the cost-effectiveness of echo-guided biopsy); (2) Farrell *et al*[46] showed complications in 1.8% of the cases with "ultrasound-guided" biopsies and in 7.7% of the cases with "blind" biopsies ($P < 0.05$); (3) Pasha *et al*[44] showed that severe complications occurred in 0.5% of the cases with "ultrasound-guided" biopsies and in 2.2% of the cases with "blind" biopsies ($P < 0.05$). The same author revealed that the pain appeared more often (50% of the cases) in the "blind" biopsy group as compared to the "ultrasound-guided" biopsy group (37% of the cases, $P = 0.003$).

But how often does the ultrasound guidance change the liver biopsy site? In a prospective study, Riley[47] showed that by ultrasound examination the site of biopsy was changed in 15.1% of the cases (21/165 patients). The reasons for changing the place of biopsy were the interposition of: lung, gall bladder, large central vessel, ascites, colonic loop, and slim liver edge.

Considering all these facts, it is reasonable and cost-efficient to perform the LB under US guidance[40,45], recent data suggesting a decrease in severe postbiopsy complications by up to 30% and less postbiopsy pain[40].

Type of needle

There are two types of biopsy needles used to perform a LB: "cutting needles" (Tru-Cut, Vim-Silverman) and "suction needles" (Menghini needle, Klatzkin needle, Jamshidi needle). Regarding how we use the needle, we can perform the LB manually or automatic, using spring-loaded devices (the so called "gun system").

Data from literature showed that there is a correlation between the rate of complications and the type of the needle used for biopsy: 3.5‰ for the Tru-Cut needle and 1‰ for the Menghini type needle[48].

Usually the choice of the biopsy instrument/needle is based on operator preference, instrument availability and clinical scenario[40]. The choice between the automatic biopsy gun *vs* manual activated needle depends on the experience of the center (operator). In a Dutch study[49] that compared standard Tru-Cut needle with a new automatic biopsy gun (Acecut), the performance of the automatic needle was superior and more consistent with respect to tissue yield, but post-biopsy pain and post-biopsy use of analgesics was superior after automatic biopsy gun. Thus, the authors[49] conclude that the automatic Tru-Cut needle offers an advantage, particularly for physicians with no or limited experience in liver biopsy.

Another group[50] did not find either type of needle to offer more safety when comparing the Tru-Cut needle with an automatic biopsy needle.

In a personal prospective study[37], we compared the number of portal spaces obtained after PLB performed with a modified Menghini needle (manual activated needle) to the number of portal spaces obtained by PLB performed with an automatic needle (Auto Vac). The mean number of portal spaces obtained by Menghini needle biopsy was 14.03 ± 7.48, and by automatic needle biopsy was 8.81 ± 4.35 ($P < 0.0001$).

The size of the needle

Usually the size of the biopsy needle used for LB in chronic hepatopathies varies between 1.2 and 1.8 mm. Gazelle's group[51] showed that larger needles produce more bleeding after LB in anaesthetized pigs (by comparing 2.1 mm with 1.6 mm needles and also by comparing 1.6 mm with 1.2 mm needles). Another study performed by Plecha *et al*[52], using cutting needles of 14, 18 and 22 gauge on porcine models, showed that the larger is the caliber of the needle, the greater is the absolute blood loss, but the conclusion of the study was that the use of larger-caliber needles is more efficient, despite the greater amount of blood loss, because more tissue can be recovered and because fewer passes are necessary, thus reducing the chance of complications.

However, other studies concerning the size of the

needle in connection with the rate of hemorrhagic complications, performed in humans, did not show any difference[46].

The number of passes of the needle into the liver

It has been demonstrated that taking more than one biopsy can increase the diagnostic value, but may have an effect on morbidity[38,39,47,53,54]. In a study performed by Riley[47] on 165 patients, only in 1.8% of cases multiple passes were necessary (noting that a low multiple pass rate was observed when applying ultrasound guidance), but in another study[55], two needle passes were required in 20% of the patients and 3 needle passes in 0.2% of the cases.

From our point of view, after a long experience in performing PLB, we consider that the visual inspection of the hepatic fragment obtained by LB represents the guarantee that enough histological material was obtained. If we are unhappy with the size of the specimen, we perform another hepatic pass in the same session, rather than make a new biopsy later.

The experience of the operator

There are controversial results regarding this issue. In one study, Gilmore *et al*[55] showed that the rate of complications in PLB was 3.2% if the operator had performed less than 20 biopsies and only 1.1% if the operator had performed more than 100 biopsies. In the study of Chevalier's group[56] the operator's experience did not influence either the final histological diagnosis or the degree of pain suffered by the patients.

The safety of PLB

In a very large multicentric retrospective study concerning 98 445 liver biopsies, Poynard *et al*[57] showed that the LB was followed by severe adverse events in 3.1‰ of the cases and by mortality in 0.3‰ of the cases. In another large study the mortality rate from fatal hemorrhage after PLB was 0.11%[58]. In the well-known retrospective study performed by Piccinino *et al*[48] on 68 276 PLB, death was infrequent (0.09/1000 biopsies). The rate of major complications after PLB ranges from 0.09% to 2.3%[40], while in a French study, severe complications appeared in 0.57% of cases[38].

Another important question is: when did the post biopsy complications appear? From the retrospective multicentric study of Piccinino[48] we found that 61% of the complications appeared in the first 2 hours after the biopsy, 82% in the first 10 hours and 96% in the first 24 hours after biopsy. Some studies showed that the rate of complications is similar in out or inpatients[58,59].

CONCLUSION

Percutaneous echo-guided liver biopsy is a safe method for the diagnosis of chronic diffuse liver diseases (cost-effective in comparison with blind biopsy) and the rate of complications seems to be related to the experience of the physician and the type of the needle used (the Menghini type needle seems to be safer).

Perhaps the use of non-invasive markers will be used in the future. For now, liver biopsy is useful and necessary for the evaluation of chronic hepatopathies, despite the fact that it is not a perfect test.

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REVIEW

Updating magnetic resonance imaging of small bowel: Imaging protocols and clinical indications

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Abstract

High soft tissue contrast resolution, acquisition of multi-planar images and the possibility to obtain functional information make magnetic resonance an interesting imaging technique to evaluate the small bowel disease. The absence of ionizing radiation is an important feature of magnetic resonance imaging (MRI) examinations because inflammatory diseases such as Crohn's disease (CD) are studied most frequently, which are prevalent among children and young adults. MRI, using modern equipment and a rigorous technical approach, can offer detailed morphologic information and functional data on the small bowel. This article discusses the MRI protocols for small bowel and the MR imaging findings of small bowel diseases, such as CD and small bowel neoplasms.

Key words: Magnetic resonance imaging; Small bowel; Crohn's; Neoplasm

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INTRODUCTION

Magnetic resonance imaging (MRI) of the small bowel has been an unexplored field of application for years. Since 1998 the number of the publications has started to increase[1-6]. The reason was not lack of interest, but the technical inadequacy of the MR scanners to perform motion-free examinations. With the development of hardware (gradients, multi-channel coils) and software (fast and ultrafast sequences), which enabled breath-held studies, freezing voluntary (respiratory) and involuntary (peristaltic) motion artifacts, it opened the access to modern abdominal MRI.

High soft tissue contrast resolution, acquisition of multi-planar images and the possibility to obtain functional information make MR an interesting imaging technique to evaluate the small bowel disease. The absence of ionizing radiation is an important feature of MRI examinations because inflammatory diseases such as Crohn's disease (CD) are studied most frequently, which are prevalent among children and young adults[7-9].

The major advantage of MRI, compared with conventional barium radiographic studies, is direct visualization of small bowel wall. This feature dramatically changes the image interpretation process. Radiologists must shift their attention from analysis of mucosal profile and lumen caliber to direct evaluation of bowel wall thickness and parietal inflammatory changes.

IMAGING PROTOCOLS

Small bowel distension

Bowel distension is a most important requisite for any method of the small bowel. A collapsed bowel loop can hide lesions or simulate pathologic wall thickening. The presence of the lesion that generates small bowel obstruction creates a natural distention of lumen and the possibility of examining the patient without any preparation[6,10-11]. In contrast, the relative collapse of bowel loops under standard conditions has led researchers to study a variety of methods of luminal distension.

There are two main approaches for MRI of the small bowel: (1) study following oral administration of contrast material; and (2) study with distension of lumen obtained with contrast material that is introduced through a naso-jejunal tube (MR enteroclysis).

Oral contrast agents for small bowel MRI

Oral contrast agents can be classified into positive, negative and biphasic categories according to their action on the signal intensity of bowel lumen.

A positive agent is a paramagnetic substance that produces a high signal intensity on T1-weighted sequences. It reduces T1 relaxation time without, or only minimally, influencing T2 relaxation time. Because of the water content of the contrast solution, it also results in high signal intensity on T2-weighted images. Positive contrast agents include paramagnetic substances, such as gadolinium chelates, ferrous and manganic ions and manganese ions[12-16]. The use of positive oral contrast agents has been abandoned almost completely because a hyperintense lumen does not enable a clear differentiation with inflammatory parietal enhancement.

A negative agent is a substance that produces a low signal intensity on T1- and T2-weighted sequences. These substances induce local inhomogeneity in the magnetic field that affects T1 and T2 relaxation time. T2 effects predominate and are caused by spin dephasing with a consequent loss of signal intensity. Negative contrast agents include perfluorooctyl bromide[17], iron oxides[15,18], and oral magnetic particles[14,15]. Barium sulfate, if used at high concentrations, can be considered a negative contrast agent[19]. Negative contrast agents are more favorable if hyperintense signal of the bowel wall and the surrounding fat tissue signs of acute inflammation have to be detected on T2-weighted sequences[15]. However, magnetic susceptibility on gradient echo sequences may alter image quality on breath-held T1-weighted images.

The term "biphasic" recently was introduced to define those substances that show different signal intensities depending on different sequences[20]. The first group (hyperintense signal on T1-weighted images and hypointense signal on T2-weighted images) included manganese and substances that contain manganese, and gadolinium chelates, which can act as biphasic contrast agents if administered at high concentrations[15]. The second group (hypointense signal on T1-weighted images and hyperintense signal on T2-weighted images) included water, hyperosmolar and isosmolar watery solutions, and barium sulfate[15]. Although water is the safest and cheapest agent, it has the limitation of intestinal absorption, which compromises an adequate distension of distal ileum in many patients[21]. To obviate this problem, hyperosmolar solutions, such as mannitol-based solutions, have been used. Mannitol reduces water absorption and distends the distal ileal loops well. Major drawbacks are undesirable side effects, such as diarrhea, meteorism and abdominal cramps[21-25].

In an attempt to reduce undesirable side effects and to obtain better distension of the distal ileum, some new oral mixtures, such as Polyethylene glycol, a water solution combined with low concentration sorbitol and locus bean gum (LBG), were used as the oral contrast agents[19,26,27]. They are all hyperosmolar. Some of them can reduce the side effect and ensure optimal intestinal distension with appropriate concentration and reasonable transit time.

Magnetic resonance enteroclysis

MR enteroclysis (MRE) is an emerging technique for the evaluation of small intestinal diseases. Administration of an iso-osmotic water solution through a nasojejunal catheter can guarantee adequate luminal distention, and in combination with ultrafast sequences, such as single shot TSE, true FISP, HASTE and 3D FLASH, resulting in excellent anatomic demonstration of the small intestine. MR fluoroscopy can be performed during MRE examination to monitor the filling process and might be useful in studying low-grade stenosis or motility related disorders. MRE is a very promising technique for the detection and characterization of involved small bowel segments in patients with Crohn's disease while its diagnostic performance in disclosing lumen narrowing and extramural manifestations and complications of the disease is outstanding. Initial experience shows that MRE is very efficient in the diagnosis of small bowel tumors and can be used in the evaluation of small bowel obstruction[20,28,29].

Sequences

Fast sequences that are able to acquire T1- and T2-weighted images within a single breath-hold are essential requisites for MRI evaluation of small bowel. In T2-weighted images, several studies[1,5,6,28,29] support the validity of single-shot sequences, including half-Fourier single-shot turbo spin-echo (HASTE) and single-shot fast spin-echo. Because these sequences, based on the half-Fourier reconstruction technique, have extremely fast acquisition time (approximately 1 second per image), they are able to freeze motion artifacts. Single-shot sequences differ from each other depending on echo time (TE). Using long TE (e.g. about 600 m) can obtain selective images of fluids with cancellation of surrounding organs (similar to magnetic resonance cholangiopancreatography). Using shorter TE (60-90 m) can obtain simultaneous evaluation of fluids, bowel wall and surrounding structures. The use of fat saturation pulses is a useful complement to the acquisition of T2-weighted sequences[2,13,14,29-31]. Fat saturation causes an increase in contrast between bowel wall and the surrounding fat tissue. This can help assess the bowel wall inflammation and identify the inflammatory changes in peritoneal fat tissue.

The "balanced" or "hybrid" gradient-echo sequence has been introduced in clinical practice. This sequence, known as true fast imaging with steady-state precession (true-FISP), presents with an intermediate contrast between T1- and T2-weighted images[29,31-33]. Shorter repetition times (TR) are used (< 3 m) and the acquisition time is short. The true FISP sequence provides motion-free, high-resolution images similar to T2-weighted images of the intestine, mesentery and

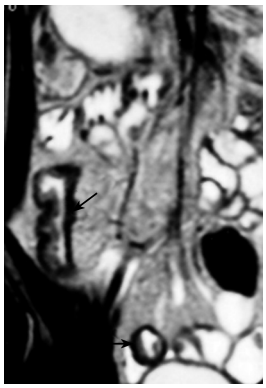

Figure 1 A 36-year-old man with Crohn's disease, the small bowel thickness exceeds 4-5 mm on T2W image, and stratified appearance (so-called "target" or "double halo" appearance) can be seen.

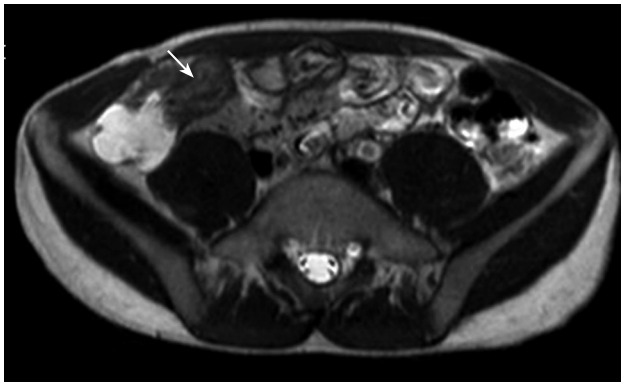

Figure 2 A 25-year-old man with Crohn's disease and inflammation of ileocecal junction. T2W image shows "double halo" appearance (arrows) of thickened (8 mm) bowel wall.

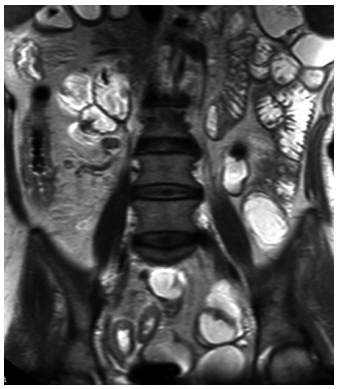

Figure 3 A 42-year-old man with Crohn's disease, T2W image shows skip lesions of ileum with thickened (7 mm) bowel wall.

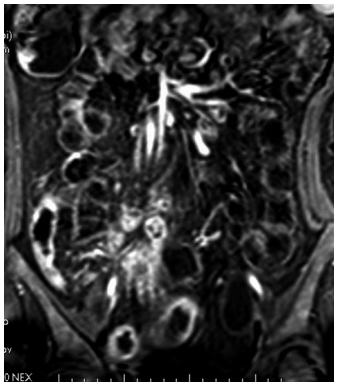

Figure 4 A 36-year-old woman with Crohn's disease, the bowel wall of the involved segment has a homogeneous enhancement at CE-T1W image. And the "comb sign" also can be seen.

vasculature in 1.5 s. However, this sequence is prone to susceptibility artifacts from intraluminal air and from "black boundary" artifact due to the chemical shift phenomenon, which may obscure the subtle bowel wall thickening. The black boundary artifact can be eliminated with use of fat suppression.

T1-weighted images are obtained with fast spoiled-gradient-echo sequences, using 2-D of 3-D acquisition. The acquisition time ranges from 15 to 20 seconds. T1-weighted sequences generally are used following intravenous injection of contrast material to evaluate enhancement, a useful parameter to assess disease activity, especially the inflammatory activity. T1-weighted images also benefit from the use of fat saturation. The gadolinium-enhanced fat-suppressed spoiled gradient-echo sequence provides T1-weighted images with excellent visualization of the enhanced bowel wall, which contrasts well with the low-signal-intensity mesenteric fat and negative intraluminal contrast material[34,35].

The latest technical development to speed up the acquisition process is parallel imaging, based on simultaneous acquisition of spatial harmonics, or sensitivity encoding techniques. Marked improvement in spatial resolution can be achieved in shorter acquisition times[36]. Parallel imaging can reduce the number of phase encodings to be acquired per TR. Consequently, the spatial resolution can be increased while maintaining an acquisition time that is compatible with a single breath-hold, or the number of scans to be acquired, which allows a larger volume coverage. Alternatively, the acquisition time can be reduced drastically. The drawback in the use of parallel imaging is the reduction of signal-to-noise ratio (SNR) and the need to perform a calibration of equipment immediately before image acquisition[37].

CLINICAL INDICATIONS

Inflammatory bowel disease (CD) (Figures 1-4)

Chronic inflammatory disease, and in particular, CD, represents the most common application of MRI of small bowel[1-5,8,9,15,28-35,38].

Imaging findings

With MRI, both inflammatory changes of the bowel wall and extramural complications of Crohn's disease can be assessed. The non-invasiveness of this technique, as well as its lack of ionizing radiation, has prompted many radiologists to perform systematic studies of MRI for evaluation of Crohn's disease.

In patients having proved suspected CD, cross-sectional images, including CT and MRI, should be analyzed specifically for the presence and character of a pathologically altered bowel segment (wall thickness, pattern of attenuation, degree of enhancement, length of involvement), stenosis and prestenotic dilatation, skip lesions, fistulas, abscess, fibrofatty proliferation, increased vascularity of the vasa recta (comb sign), mesenteric adenopathy, and other extraintestinal disease involvement.

The normal small bowel wall thickness is between 1 mm and 3 mm when the lumen is well distended. Any portion of the bowel wall that exceeds 4-5 mm is

considered abnormal[9,12,17,19,26,29,39,40]. An adequate intestinal distension is mandatory because collapsed loops or spastic intestinal segments may mimic wall thickening. Most optimal distension is obtained with MR enteroclysis with instillation of contrast medium after nasojejunal intubation under fluoroscopic guidance. Although many authors reporting on MR enteroclysis administer antiperistaltic drugs to reduce motion artifacts, reflex atony is induced by high flow rates, theoretically allowing images (almost) free of motion artifacts[41,42]. Drawbacks are that this technique is uncomfortable for patients and exposes them to a considerable dose of ionizing radiation of up to 8 mSv during intubation[43]. To avoid such disadvantages, MRI has been performed by many researchers using oral contrast media. Many contrast media have been proposed, but no oral contrast medium has yet been accepted universally as optimal for use[2,3,7,14-19,22-26].

Small bowel wall thickening is a sensitive, but not pathognomonic, sign of CD. It is observed in several other intestinal diseases, such as ischemic disorders and infections.

Although superficial mucosal lesions are missed easily as a result of inadequate spatial resolution, MR imaging can detect early inflammatory changes of the bowel wall, based on enhancement after intravenous injection of contrast medium. The bowel wall of the involved segment may have a homogeneous or stratified appearance at MR imaging after enhancement. The homogeneous enhancement is diffuse and transmural with no recognition of different bowel layers. The stratified appearance (so-called "target" or "double halo" appearance) is related to alternating layers of higher or lower attenuation or signal intensity. The stratified appearance also can be seen on T2-weighted imaging. "Target" or "double halo" appearance is often seen in active lesions, particularly after the intravenous administration of contrast medium, and related to submucosal edema. The intensity of enhancement correlates with the degree of inflammatory lesion activity. Inactive disease is characterized by no abnormalities or bowel wall thickening with relative low signal intensity representing fibrosis with limited, homogeneous contrast enhancement. Absence of stratification on T2-weighted images with stratified enhancement on T1-weighted images is often due to fibrosis, which is a typical long-standing CD[29,33,35,44]. This sign (stratified enhancement) also can be seen on MSCT (multi-slice CT) images[45,46].

Increased vascularity of the vasa recta (comb sign) is a sign of active inflammation. It arises from the combination of vascular engorgement of vasa recta and fibro-fatty proliferation and is demonstrated as multiple tubular, tortuous opacities on mesenteric side of ileum, aligned as the teeth of a comb[29]. "Comb sign" is frequently seen on enhanced MSCT images[46,47]. Abscess and phlegmon can occur in the small bowel mesentery, abdominal wall, or psoas muscle or around the anus. Abscesses and phlegmon are well demonstrated at fat-saturated T2-weighted MR imaging and can be distinguished reliably, which aids in management planning. Fistulas and sinus tracts are also depicted, however, the reported sensitivity of MR imaging for depicting sinus tracts is 50%-75% when a conventional enteroclysis study is used as a reference[32,39,45]. Mesenteric lymphadenopathy ranging from 3 to 8 mm in size is depicted at MR imaging with a true fast imaging with steady state precession (FISP) or T2-weighted turbo spin-echo sequence[32,41]. If these sequences are not available, axial T1- or T2-weighted spin-echo imaging should be added. When lymph nodes are larger than 10 mm, lymphoma and carcinoma must be excluded.

Assessment of disease activity

Imaging techniques form a very important part of the evaluation of CD. However, several clinical scoring systems have been developed as well to assess disease activity and response to therapy, especially in trials. The Crohn's disease activity index (CDAI) is currently the gold standard for clinical evaluation of disease activity[47,48]. This index is relatively subjective since an important part of the total score is derived from items that reflect the patient's perception of disease (general well being and "intensity of abdominal pain"). However, in many studies, this index has been used as gold standard for disease activity since it is a validated and extensively used clinical index. Scores ranging from 0 to approximately 600 with values below 150 are considered as remission and values over 150 as active disease. MR imaging was used to evaluate disease activity[9,30-35,38,41,44,48,49]. Based on different experiences, contrast-enhanced(CE) fat-suppressed T1-weighted images offer the best correlation between MR findings and CDAI, although a correlation that used fat-suppressed T2-weighted images is also demonstrated. MRI can clearly distinct pathologic from normal bowel wall in CD, as it detects significant variations in bowel wall thickness with clinical improvement and is able to reflect pathologic inflammatory changes at the bowel wall based on variations in the CE. In most patients with active disease, abnormal bowel identified on MR imaging was isointense or slightly hypointense to the psoas muscles on T1-weighted imaging. On T2-weighted imaging, the abnormal bowel segments were usually isointense or slightly hyperintense compared with the psoas muscle. MR imaging can correctly identify active disease, the enhancement pattern of abnormal bowel is diffuse and layered. The layered pattern is seen only in patients with active disease. Consequently, this technique is reliably applicable to the follow-up of patients with CD. MRI is able to detect significant variations in bowel wall thickness and contrast enhancement (CE), reflecting favorable clinical response to medical treatment of CD's relapse[30,34,49].

NEOPLASMS (Figure 5)

Benign masses

Benign and malignant small intestinal tumors are uncommon. Adenomas, leiomyomas and lipomas constitute the three most common primary benign small intestinal tumors[50]. In general, benign tumors occur less commonly in the duodenum and increase in frequency

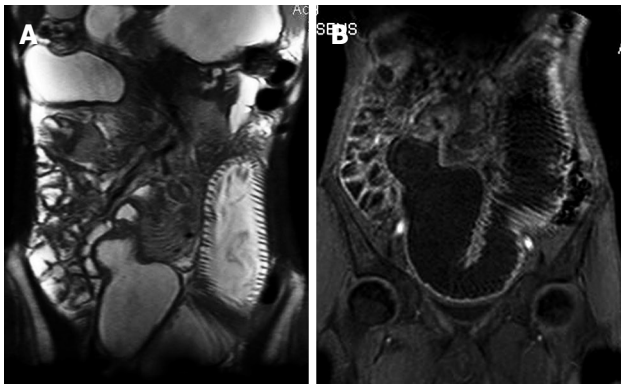

Figure 5 **A**: A 68-year-old man with adenocarcinomas, T2W image shows the tumor with similar signal intensity, the proximate jejunum dilating conspicuously; **B**: The same patient, tumor shows heterogeneous enhancement greater than adjacent bowel on gadolinium-enhanced image.

in the ileum. The term "polyp" is a clinical term for any tumorous mass that projects above the surrounding normal mucosa. Hamartomatous, hyperplastic and inflammatory polyps are benign, non-neoplastic lesions and adenomatous polyps are true neoplastic tumors containing dysplastic epithelium and are precursors of carcinoma. Polyps are infrequently symptomatic and are usually incidental findings at autopsy. Current convention is that leiomyomas should be classified as gastrointestinal stromal tumors (GIST), and benignancy can never be determined with absolute certainty. Small bowel GIST accounts for 25% of these tumors. As in the stomach, these may be large and ulcerating.

Malignant masses

Adenocarcinomas account for 50% of all small bowel malignancies, but only account for less than 1% of all gastrointestinal malignancies[51]. The most common site for small bowel adenocarcinoma is the duodenum. This tumor frequently occurs in close proximity to the ampulla and as a result may cause obstructive jaundice[52]. Adenocarcinoma and metastases can be seen rarely in the jejunum.

Most primary gastrointestinal non-Hodgkin lymphomas are of B-cell type, and appear to arise from B cells of mucosa-associated lymphoid tissue (MALT). In the small intestine, the terminal ileum is the most common site affected, which may reflect the relatively greater amount of lymphoid tissue present in this segment compared with the duodenum and jejunum.

Carcinoids are the most common primary neoplasm of the small bowel. They are well-differentiated neuroendocrine neoplasms that occur primarily in the distal ileum. Men and women are affected with equal frequency. Most patients present with tumor-related symptoms of bleeding and bowel obstruction or intussusception. Ileal carcinoids are regional mesenteric metastases and vascular sclerosis. The primary tumor may be quite small with the accompanying lymphadenopathy and desmoplastic reaction in the root of the mesentery presenting as the only visible manifestation of disease. Liver metastases are responsible for the "carcinoid syndrome", which is characterized by vasomotor instability, intestinal hypomotility and bronchoconstriction[53].

Imaging findings

Tumors had similar signal intensity to normal small bowel on precontrast images. Tumors showed heterogeneous enhancement greater than adjacent bowel on gadolinium-enhanced images. Tumor local extent was best shown on precontrast-spoiled gradient-echo images and postgadolinium T1-weighted fat-suppressed images. Image quality was most consistent on breath-hold images. Precontrast breath-hold T1-weighted spoiled gradient-echo images and gadolinium-enhanced fat suppressed images demonstrate tumor extent most reliably. The accuracy of the technique in cases of non-occlusive tumors of the lumen is not known, given the lack of large case series[28,54].

CONCLUSION

MR imaging, using modern equipment and a rigorous technical approach, can offer detailed morphologic information and functional data on the small bowel. The optimal study technique is debatable, although the oral administration of contrast material as a first-line approach is less expensive, faster, easier to perform and better tolerated by patients. MR enteroclysis might be reserved for selected cases as a second-line study.

The major clinical indication is the evaluation of patients who have suspected or known CD. The absence of ionizing radiation, in view of the young age of most of the patients and the frequency of the examinations, is an important advantage over other techniques (radiography and CT enteroclysis).

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BASIC RESEARCH

Effect of honey on bacterial translocation and intestinal morphology in obstructive jaundice

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Abstract

AIM: To evaluate the effects of honey on bacterial translocation and intestinal villus histopathology in experimental obstructive jaundice.

METHODS: Thirty Wistar-Albino rats were randomly divided into three groups each including 10 animals: group Ⅰ, sham-operated; group Ⅱ, ligation and section of the common bile duct (BDL); group Ⅲ, bile duct ligation followed by oral supplementation of honey (BDL + honey) 10 g/kg per day. Liver, blood, spleen, mesenteric lymph nodes, and ileal samples were taken for microbiological, light and transmission electrone microscopic examination.

RESULTS: Although the number of villi per centimeter and the height of the mucosa were higher in sham group, there was no statistically significant difference between sham and BDL + honey groups ($P > 0.05$). On the other hand, there was a statistically significant difference between BDL group and other groups ($P < 0.05$). The electron microscopic changes were also different between these groups. Sham and honey groups had similar incidence of bacterial translocation ($P > 0.05$). BDL group had significantly higher rates of bacterial translocation as compared with sham and honey groups. Bacterial translocation was predominantly detected in mesenteric lymph nodes.

CONCLUSION: Supplementation of honey in presence of obstructive jaundice ameliorates bacterial translocation and improves ileal morphology.

Key words: Honey; Obstructive jaundice; Intestinal villus atrophy; Bacterial translocation

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INTRODUCTION

Obstructive jaundice is a common clinical entity complicated by intestinal failure and endotoxemia, leading to high postoperative morbidity and mortality rates. The gastrointestinal tract performs a variety of functions in digestion, selective absorption, and secretion. However, its barrier function, which prevents spread of intraluminal bacteria and endotoxins to the organs and tissues, plays a key role[1-2]. Intestinal barrier failure is associated with an increased incidence of bacteria and toxin translocation from the intestinal lumen to the systemic circulation, causing systemic infection and multiple organ failure in the critically ill or injured patient[3]. Gut barrier failure may result from one or more of the three basic pathophysiologic conditions; disruption of the normal ecologic balance of the indigenous gut microflora, impaired host immune defenses, and physical disruption of the gut mucosal barrier[4].

Bacterial translocation is the passage of bacteria or endotoxins from the gastrointestinal tract to extraintestinal sites, such as mesenteric lymph nodes, liver, spleen, and/or bloodstream. In a normal, healthy individual, gut-originated bacteremia and sepsis do not occur because the host has multiple defense mechanisms to prevent the bacteria and their products from crossing the mucosal barrier and spreading to systemic tissues. Under certain experimental and clinical circumstances, this intestinal barrier function becomes overwhelmed or impaired, resulting in bacterial translocation[4]. Current advances in the pathophysiology of intestinal failure in obstructive jaundice have showed that the breakage of gut barrier is multifactorial, involving disruption of the immunologic, biological, mechanical, and biochemical barrier[1].

Honey is a supersaturated sugar solution produced by honey bees from nectar of different plants. It has a long tradition of use for wound healing since ancient times. Honey has bactericidal, bacteriostatic, antifungal, antiviral, scolicidal, antioxidant, antitumoral, and anti-inflammatory effects[5-12].

In this study, we investigated the effects of honey on bacterial translocation and intestinal morphology in experimental obstructive jaundice.

MATERIALS AND METHODS

Animals

Thirty Wistar-Albino male rats, weighing 250 ± 25 g, were housed under constant temperature (21 ± 2℃) individually in wire cages with 12 h light-dark cycle. Twelve hours before anesthesia, animals were deprived of food, but had free access to water 2 h before anesthesia. No enteral or parenteral antibiotics were administered at any time. The rats that died during the experiment were excluded from the experiment and no new rat was included. The procedures in this experimental study were performed in accordance with the National Guidelines for The Use and Care of Laboratory Animals and approved by Animal Ethics Committee of Ankara Research and Training Hospital.

Study groups

Rats were randomly divided into three groups each including 10 animals: group Ⅰ, sham-operated; group Ⅱ, ligation and section of the common bile duct (BDL); group Ⅲ, BDL followed by oral supplementation of honey 10 g/kg per day (Balparmak LTD, Istanbul, Turkey), once a day, with nasogastric tube (7 Gauge feeding tube) that was inserted daily and taken off after honey supplementation. Animals were sacrificed by high-dose diethyl ether inhalation on postoperative day 7. Liver, blood, spleen, mesenteric lymph nodes, and ileal samples were taken for microbiological, light and TEM (transmission electron microscopic) examination.

There isn't a standard dose for honey in experimental studies. The dose used in previous studies ranges between 0.078 g/kg to 5 g honey/rat per day[12-15]. We gave 10 g/kg per day to each rat.

Operative procedure

Animals were anesthetized by intramuscular injection of 30 mg/kg ketamine hydrochloride (Ketalar®; Parke-Davis, Istanbul, Turkey) and 5 mg/kg xylasine (Rompun®, Bayer, Istanbul, Turkey). Midline laparotomy was performed under sterile conditions. In the sham-operated group (group Ⅰ) the common bile duct (CBD) was freed from the surrounding soft tissue and was manipulated without ligation and transection. In group Ⅱ and Ⅲ, CBDs of the rats were identified, double ligated with 5-0 silk, and sectioned between the ligatures. The same surgeon performed all procedures. The abdominal incisions were closed in two layers with continuous 3-0 silk sutures. Animals were allowed to feed after the operation.

Microbiological and biochemical examination

The mesenteric lymph nodes (MNLs), spleen and liver were chopped with sterile instruments under aseptic conditions. Then the tissue samples were weighed and placed in tubes containing 1.5 mL broth (thioglycolate, Oxoid, UK) and homogenized. After that 0.01 mL tissue samples were inoculated on blood agar (Oxoid, UK) and Levine Eosine Methylene Blue (EMB) agar (Oxoid, UK). Plates were incubated at 37℃ for examination of bacterial growth. The growth of bacteria in quantitative culture was observed at 24 h and 48 h.

Blood samples taken from inferior vena cava of rats were inoculated on the medium of aerobic and anaerobic blood culture. The aerobic and anaerobic blood cultures were observed by incubation in BACTEC 9240 blood culture system (Becton Dickinson, USA) at 37℃ for seven days. Samples taken from the blood culture bottle giving positive alarm were subcultured by inoculating on blood agar and EMB agar. The subcultures were inoculated at 37℃ under aerobic and anaerobic condition and examined at 24 h and 48 h.

Total bilirubin, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels were measured as parameters indicative of hepatic function by an autoanalyser (Olympus AU640, Japan).

Histopathological examination

For light microscope analyses, tissue samples from the terminal ileum were obtained from all animals. In order to avoid mucosal suffering, the intestinal lumen was carefully cannulated and gently washed with normal saline solution before the sampling. The ileal samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 μm by Leica RM 2125 RT, and stained with hematoxylin and eosin (HE) for routine light microscopic examination. Histopathological examinations were performed by a pathologist who was blinded to the study design and photographs were taken with Nikon Eclipse E 600. The number of villi per centimeter (V/cm) and the total mucosal thickness were assessed in all groups. The mucosal thickness was measured in a minimum of 20 well-preserved villi in each randomly selected sample from each tissue block.

For TEM (transmission electron microscopic)

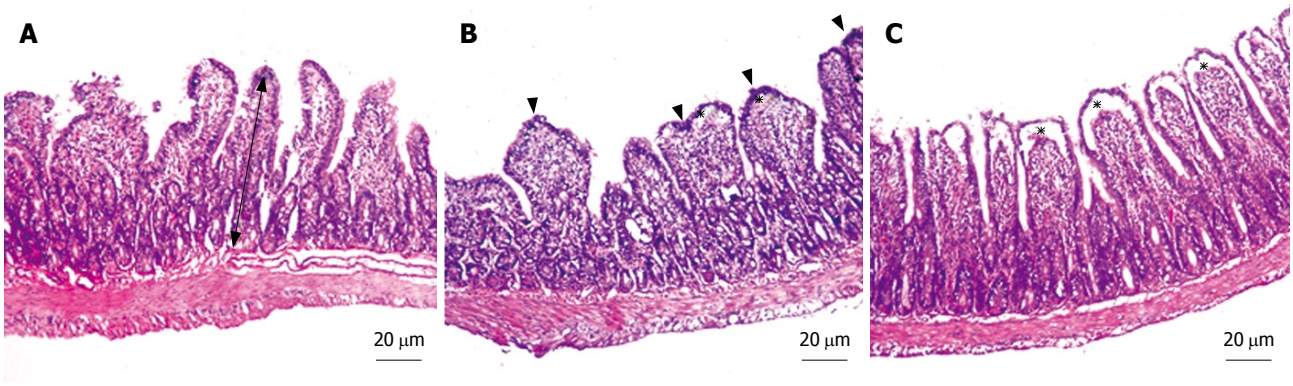

Figure 1 The micrographs of light microscope stained with haematoxylin and eosin. Micrograph (**A**) illustrates the typical structure of villi; (**B**) blunting of the villi (arrow head) and subepithelial edema (asterisk); (**C**) existing subepithelial edema (asterisk) and the total mucosal thickness (arrow).

analyses, samples were fixed with phosphate buffered (pH 7.3) 2.5% glutaraldehyde and 2% PFA mixture solution for 2 h at room temperature. They were washed with phosphate buffered saline solution (PBS, pH 7.3) and were fixed with 1% osmium tetraoxide for 2 h as secondary fixation. After washing, they were embedded in Araldite 6005 and were cut with Leica EM FCS (Wien, Austria) ultramicrotome. One μm semi-thin sections were stained with Toluidin blue-Azur Ⅱ to select the region of interest for the following procedures. Sixty to 70 nm thin sections were stained with uranil acetate and lead citrate. They were examined and photographed using a LEO 906 E TEM (80 Kv, Oberkochen, Germany). The pathologist was blinded about the groups.

Statistical analysis

Differences between the numbers of positive cultures of the groups were evaluated by chi-square test. Scores of total mucosal thickness and number of villi per centimeter were presented as mean ± SD and compared by One-Way ANOVA or Kruskal-Wallis variance analysis. If the *P* values of the variance analyses were statistically significant, differences between groups were analyzed with the Mann-Whitney *U* test. Statistical analyses were performed with the Statistical Package for the Social Sciences (SPSS), version 13.0 for Windows (SPSS Inc., Chicago, USA). $P < 0.05$ was considered to be statistically significant.

RESULTS

General

All rats were sacrificed on postoperative day 7. Two rats from group Ⅱ (BDL group) and one from group Ⅲ (BDL + honey group), totally 3 rats, died during the early postoperative period probably due to anesthesia. The liver function tests and bilirubin levels were normal in sham group and high in BDL and BDL + honey groups.

Intestinal morphology

In all specimens of the sham group, the histological features showed regular appearance of ileal tissue. When we evaluated the specimens systematically, including assessment of villous architecture, surface and crypt epithelia, lamina propria constituents and submucosal structures, no alteration was found in sham group (Figure 1A). The specimens of the BDL group presented villous blunting associated with reduced mucosal thickness. We identified subepithelial edema mostly located at the tip of the villi, but also extended throughout the villus, with epithelial layer moderately lifted from the lamina propria. We observed that the crypts were generally preserved. The number of villi per centimeter (V/cm) (villus density) was decreased in BDL group (Figure 1B). In group Ⅲ, the subepithelial edema still existed, but villous blunting was not evident. Farther, the crypts generally appeared to be preserved (Figure 1C). Although the number of villi per centimeter and the height of the mucosa were higher in sham group, there was no statistically significant difference between sham and BDL + honey groups ($P > 0.05$). On the other hand, there was a statistically significant difference between BDL group and other groups ($P < 0.05$). Mean number of villi per centimeter and mean mucosal height of the groups are given in Table 1.

Table 1 Mean number of villi per cm and mean height of mucosa (μm)

Groups	Mean number of villi per cm	Mean height of mucosa
Sham (Group Ⅰ)	84.40 ± 3.75[b]	640.02 ± 43.72
BDL (Group Ⅱ)	73.01 ± 2.83	567.50 ± 34.54
BDL + Honey (Group Ⅲ)	81.33 ± 3.46[b]	625.56 ± 38.77

[b]$P < 0.01$ *vs* Ⅱ, *P* values for mean height of mucosa: 0.002, Ⅰ *vs* Ⅱ; 0.005, Ⅱ *vs* Ⅲ.

The ultrastructure of intestinal epithelial junctional complexes was observed by electron microscopy. In the sham group, enterocytes were tightly bound to the luminal surface by junctional complexes. Zonulae occludens, zonulae adherentes and maculae adherentes appeared normal in the sham group. The luminal surface was covered with microvilli (Figure 2A). When we evaluated the BDL group, we

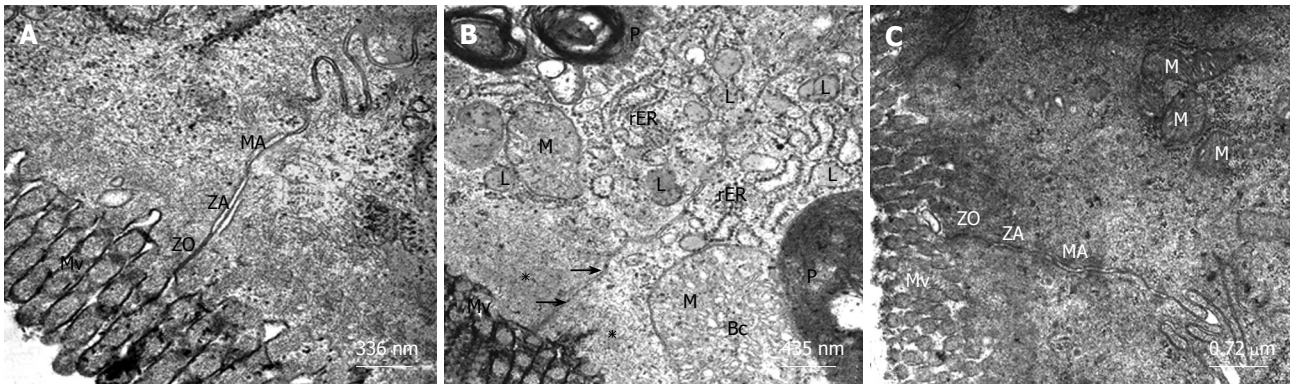

Figure 2 These transmission electron microscope (TEM) micrographs illustrate the main ultrastructural features of enterocytes, the absorptive cells of the ileum. Micrograph (**A**) shows the regular structure of microvilli (Mv) and the three components of junctional complex at the luminal end of the lateral plasma membrane, zonula occludens (ZO), zonula adherens (ZA) and macula adherens (MA); Micrograph (**B**) shows the disintegration of the zonula occludens and disordered structure of junctional complexes (arrows). The lipid droplets (L) and phagosomes (P) in the cytoplasm, rough endoplasmic reticulum (rER), swollen mitochondria (M) with ballooned cristae (Bc) and apical surface edema (asterisk) viewed; Micrograph (**C**) illustrates the regular structure of microvilli (Mv) and junctional complexes (ZO, ZA, MA).

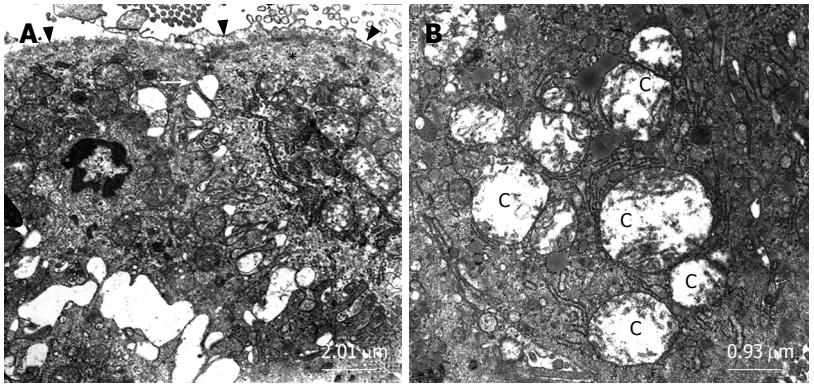

Figure 3 Micrograph (**A**) shows the disintegration of the zonula occludens (arrow), apical surface edema (asterisk) and the desquamation of the epithelial tissue (arrow head); Micrograph (**B**) illustrates the swollen mitochondria with cavitations of matrix (C).

observed desquamated epithelial tissue, cytoplasmic vacuoles, phagosomes and disrupted structure of the tight junction between epithelial cells possibly due to apical surface edema. Zonulae occludens located within the plasma membranes of adjacent epithelial cells diverged (Figures 2B and 3A) and the mitochondria were swollen with electrolucent matrix and ballooned cristae. Markedly swollen mitochondria with peripherally placed, disoriented and disintegrating cristae and cavitations of the matrix were also observed in BDL group (Figures 2B and 3B). The structure of the microvilli and mitochondria were regular in the BDL + honey group. The junctional complexes had normal appearance (Figure 2C).

Bacterial translocation

The rates of bacterial translocation (BT) in all groups are summarized in Table 2. Sham and BDL + honey groups had similar incidence of BT. BDL group had significantly higher rates of BT as compared with sham and BDL + honey groups. Only BT to spleen was not significantly different between the BDL and BDL + honey groups. BT was predominantly detected in MLNs.

The most commonly isolated bacteria was *Escherichia coli*. The other isolated microorganisms were *Enterococcus spp.*, *Staphylococcus spp.*, *Proteus spp.*, *Staphylococcus aureus* and *Enterobacter cloacae*.

Table 2 Bacterial translocation rates of the groups

Groups	Liver	Spleen	MLNs	Blood
Sham (Group Ⅰ)	0/10 (0.0%)	0/10 (0.0%)	1/10 (10.0%)	0/10 (0.0%)
BDL (Group Ⅱ)	6/8 (75.0%)	4/8 (50.0%)	7/8 (87.5%)	4/8 (50.0%)
BDL + Honey (Group Ⅲ)	1/9 (11.1%)	2/9 (22.2%)	2/9 (22.2%)	0/9 (0.0%)
P values				
Ⅰ *vs* Ⅱ	0.002	0.023	0.002	0.023
Ⅱ *vs* Ⅲ	0.013	> 0.05	0.012	0.029
Ⅰ *vs* Ⅲ	> 0.05	> 0.05	> 0.05	> 0.05

DISCUSSION

Bacterial translocation is the migration of bacteria or bacterial products from the intestinal lumen to mesenteric lymph nodes or other extraintestinal organs and sites. In addition to nutrient absorption, the gut functions as a barrier to prevent the spread of intraluminal bacteria and endotoxin to systemic organs and tissues[4,16,17].

Bile inhibits bacterial overgrowth, has a trophic effect on the intestinal mucosa, decreases epithelial internalization of enteric bacteria, exerts detergent actions with anti-adherence effects, and binds endotoxins. Therefore, the absence of bile in the intestine facilitates BT and enhances endotoxin-induced BT[16]. Obstructive jaundice is almost universally believed to promote bacterial translocation. Absence of bile from the lumen

of gut is also associated with a quantitative increase in small intestinal microflora[18].

Translocation from the intestine is most commonly detected by measuring the presence of viable bacteria in the tissues. This can reflect not only the integrity of the intrinsic barrier function of the mucosa but also the numbers and types of microbes in the lumen and the ability of the host to kill the bacteria that translocate[19].

Honey is a supersaturated sugar solution produced by honey bees from the nectar of plants. Some of the components of the honey are added by the bees during the maturation process or are derived from the plants[20]. The antimicrobial properties of honey are well documented[5-9]. The antibacterial activity of honey lies partially in its high osmolarity due to its high sugar content, and in its acidity due mostly to the presence of gluconic acid. Although hydrogen peroxide is thought to be the main antibacterial factor in honey, the presence of non-peroxide activity was also notable. This activity is usually attributed to the presence of organic components such as syringic acid, methyl syringate, pinocembrin, pinobanksin, caffeic acid, ferulic acid, vanillic acid, cinnamic acid, and benzoic acid[21].

The physicochemical properties of honey not only contribute to its antibacterial properties but also to its wound healing capabilities. The anti-inflammatory action of honey has been investigated, but no definite mechanism has been identified. Honey provides glucose supply for leucocytes. It also provides substrate for glycolysis, which is the major mechanism for energy production in the macrophages. Honey may modulate the activation state of immunocompetent cells (e.g. monocytes) within the wound. These data suggest that honey may have a number of effects on the molecular mechanisms of wound healing[20].

As we mentioned before, the physical barrier function of the mucosa appears to have primary importance for preventing or limiting bacterial translocation, especially in a host with a normal gut flora, whereas the immune system appears to serve a secondary or supportive role to the intestinal mucosal barrier[4]. In our study, mean number of villi per centimeter, mean mucosal height, and electron microscopic changes of the honey group were significantly better than the data observed in the BDL group. In the sham and BDL + honey groups, enterocytes were tightly bound to the luminal surface by junctional complexes. Zonulae occludens, zonulae adherentes and maculae adherentes appeared normal in these groups. The luminal surface was covered with microvilli. When we evaluated the BDL group, we observed desquamated epithelial tissue, cytoplasmic vacuoles, phagosomes and disrupted structure of the tight junction between epithelial cells possibly due to apical surface edema. Zonulae occludens located within the plasma membranes of adjacent epithelial cells were diverging and the mitochondria were swollen with electrolucent matrix and ballooned cristae. Reduced bacterial translocation rates in honey group could be explained by decreased atrophy of intestinal mucosal villi and somewhat regular structure of enterocytes and microvilli. We concluded that wound healing properties and cytoprotective effects of honey might be the reason of the decreased atrophy of intestinal mucosal villi. On the other hand, antimicrobial effects of honey on enteric bacteria could also decrease the overgrowth of these bacteria and reduce bacterial translocation.

Assimakopoulos *et al*[22] investigated the oxidative alterations in the intestinal mucosa of patients with obstructive jaundice and found that obstructive jaundice in humans induced intestinal oxidative stress, which might be a key factor contributing to intestinal barrier failure and the development of septic complications in this patient population. In another study, these authors showed that intestinal mucosal atrophy in obstructive jaundice was based on inhibition of proliferation and promotion of apoptotic death of enterocytes, and reactive oxygen species might be responsible for this effect[23].

The antioxidant properties of honey have been well-documented in recent studies[24-27]. Schramm *et al*[25] found that phenolic antioxidants from processed honey were bioavailable, and these antioxidants increased antioxidant activity of plasma. Gheldof *et al*[11] also showed that the *in vivo* serum antioxidant capacity increased significantly following consumption of buckwheat honey in human. These studies showed that the antioxidant effect of honey was not only local, but also a systemic effect. According to the results of studies about antioxidative effects of honey and intestinal oxidative stress in obstructive jaundice, we concluded that the protective effect of honey on intestinal villi and mucosal structure might be attributable to antioxidative effects of honey in our study. Since we investigated only the effects of honey on bacterial translocation and intestinal villus atrophy, not the mechanism of this effect, we did not evaluate oxidative stress parameters. These parameters should be analyzed in further studies that investigate the mechanism of this effect of honey.

Since a normal functioning immune system is another important factor for adequate gut barrier function[4], honey may also reduce bacterial translocation by its modulatory effects on immunocompetent cells[28-29].

In this study, we demonstrated that honey reduced bacterial translocation rates and protected intestinal villus structure in experimental obstructive jaundice model. These effects of honey might be attributable to its antibacterial, antioxidant, anti-inflammatory, and immunomodulatory activities. Further studies are needed for evaluation of the exact mechanism of this effect. After the results of these studies, honey might be used for preventing harmful effects of obstructive jaundice in clinical settings.

COMMENTS

Background

Spontaneous bacterial infection and septicemia due to increased bacterial translocation in patients with obstrictive jaundice result in significant morbidity and mortality.

Research frontiers

The present study investigated the effects of honey on bacterial translocation

and intestinal morphology in experimental obstructive jaundice.

Innovations and breakthroughs

Obstructive jaundice is a common clinical entity complicated by intestinal failure and endotoxemia, leading to high postoperative morbidity and mortality. Our experience from the present study shows that honey can be used safely in this situation.

Applications

This study demonstrated that honey reduced bacterial translocation rates and protected intestinal villus structure in experimental obstructive jaundice model. Honey might be used for preventing harmful effects of obstructive jaundice in clinical settings.

Peer review

The rationale behind this study is that the authors have previously shown that honey has reduced bacterial translocation rates and protected intestinal villus structure in experimental obstructive jaundice.

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RAPID COMMUNICATION

Treatment responses in Asians and Caucasians with chronic hepatitis C infection

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Abstract

AIM: To conduct a multicentre retrospective review of virological response rates in Asians infected with genotype 1 chronic hepatitis C (CHC) treated with combination interferon and ribavirin and then to compare their responses to that among Caucasians.

METHODS: Asian patients infected with genotype 1 CHC treated at 4 Australian centres between 2001 to 2005 were identified through hospital databases. Baseline demographic characteristics, biochemical, virological and histological data and details of treatment were collected. Sustained virological responses (SVR) in this cohort were then compared to that in Caucasian subjects, matched by genotype, age, gender and the stage of hepatic fibrosis.

RESULTS: A total of 108 Asians with genotype 1 CHC were identified. The end of treatment response (ETR) for the cohort was 79% while the SVR was 67%. Due to the relatively advanced age of the Asian cohort, only sixty-four subjects could be matched with Caucasians. The ETR among matched Asians and Caucasians was 81% and 56% respectively (P = 0.003), while the SVR rates were 73% and 36% (P < 0.001) respectively. This difference remained significant after adjusting for other predictive variables.

CONCLUSION: Genotype 1 CHC in Asian subjects is associated with higher rates of virological response compared to that in Caucasians.

Key words: Hepatitis C; Treatment; Asians; Retrospective studies; Comparative study; Interferon; Ribavirin; Statistical data analysis

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INTRODUCTION

Chronic hepatitis C (CHC) virus infection is the leading cause of chronic liver disease worldwide. The prevalence of hepatitis C virus (HCV) infection in western countries including Australia and the United States approximates 1%[1-2], while it is more common in most Asian countries[3-4]. Combination therapy with pegylated interferon and ribavirin given for 24 wk or 48 wk remains the most effective antiviral treatment, achieving sustained virological response (SVR) rates ranging from 50% to 80%[5-9].

Various factors have been identified that influence response rates, including HCV genotype, body mass index and co-existent liver disease. Ethnicity was recently noted to impact on treatment responses. Studies conducted in African Americans suggest that these individuals have lower SVR rates when compared to Caucasians, even after adjusting for confounders that could potentially influence treatment response rates[10-13]. More recently, comparative studies between Asians and Caucasians have suggested a higher SVR to antiviral therapy among Asians[14-16].

In Asians, HCV prevalence rates are approximately

6%[3]. Often subjects acquire the infection at a younger age and are therefore at increased risk of developing advanced liver disease and hepatocellular carcinoma. Despite this, Asians seem to be under-represented in clinical trials evaluating SVRs, with the largest available study comprising only 52 individuals[16]. In addition, there has been no head-to-head comparative study evaluating responses to antiviral therapy between Asians and Caucasians. The aims of the present study were therefore to: (1) assess the overall SVR rates in Asians infected with genotype 1 CHC receiving combination antiviral therapy; and (2) to undertake a case-control study comparing SVR rates in Asians compared to that in Caucasians matched by infecting virus genotype, age, gender and the extent of hepatic fibrosis.

MATERIALS AND METHODS

Clinical databases of HCV infected patients who received combination interferon and ribavirin therapy between 2001 to 2005 at four Australian centres were reviewed and all Asian patients identified. Individual patient files were retrieved and their clinical status confirmed. Asian and Caucasian patients over the age of 18 years who received antiviral therapy for genotype 1 CHC were subsequently identified.

Exclusion criteria included patients who were HBsAg-positive, co-infection with HIV, liver transplant recipients or those receiving dialysis for chronic renal failure. Patient demographic characteristics, baseline biochemical, virological and histological data prior to commencing anti-viral therapy were recorded. Those with bridging fibrosis or cirrhosis were considered to have advanced liver disease. While baseline viral loads of individual patients were recorded, multiple different assays and units were used at the various study centres over time, making this data not reportable, or comparable. For alcohol intake, we defined significant intake as either documented daily consumption of more than 30 grams, or medical record documentation that alcoholism was an issue. Details of antiviral therapy were recorded. These included a history of previous anti-viral treatment, treatment regimen (interferon and ribavirin or pegylated interferon and ribavirin), adverse effects on therapy and any treatment dose reductions or interruptions due to either adverse effects or non-compliance. As a standard of care, all patients with HCV genotype 1 were scheduled for 48 wk of treatment.

The primary end point of this study was the proportion of patients achieving an SVR, defined as a documented non-detectable HCV RNA at least 24 wk after treatment. The end of treatment response (ETR) was defined as non-detectable HCV RNA at the end of treatment. Patients who received at least one dose of interferon but did not complete 48 wk of treatment for any reason were defined as non-responders. Asians and Caucasians infected with HCV genotype 1 were then matched by three criteria: age (within 5 years), gender and the extent of hepatic fibrosis, and their ETR and SVR rates were compared.

Table 1 Demographic characteristics, treatment details and treatment responses of 108 HCV genotype 1-infected Asian patients

Variables	Frequency
Countries of origin	
Vietnam	43
China	34
Cambodia	19
Korea	5
Burma	3
Others	4
Gender (%)	
Male	69%
Age (yr, range)	51 (22–76)
Median weight (kg, range)	64 (33-104)
Median ALT (IU/mL, range)	94 (11-558)
Extend of fibrosis (%)	
Bridging fibrosis or cirrhosis	21%
Treatment regimen (%)	
Pegylated interferon + Ribavirin	89%
Interferon + Ribavirin	11%
Dose reduction of either drug	33%
Dose interruption of either drug	11%
Treatment responses (%)	
End of treatment virological response	77%
SVR	67%

All statistical analyses were performed by SAS software v11 (SAS Institute Inc., Cary, NC). Continuous variables are reported as median (range). Comparison of baseline demographics was performed by the paired Student *t*-test, Mann-Whitney test or χ^2 test as appropriate. Univariate analysis was performed with SVR as the dependent variable.

RESULTS

Baseline features of the Asian cohort

A total of 108 HCV genotype 1 infected Asian patients were identified. Their demographic characteristics, treatment details and treatment outcomes are shown in Table 1. The majority of patients were born in Vietnam, China or Cambodia; 69% were male. The cohort had a median age of 51 (22-76) and a median body weight of 64 kg (33-104 kg). The baseline alanine aminotransaminase (ALT) was 94 IU/mL (11-558 IU/mL). 21% of those who had liver biopsies had histological evidence of bridging fibrosis or cirrhosis. Eighty-nine percent received pegylated interferon and ribavirin while the remainder received standard interferon and ribavirin. Dose reduction was required in 33%, while 11% required dose interruption, for either adverse effects or non-compliance.

End of treatment and SVR rates in Asians

An ETR occurred in 77% of the Asian cohort while 67% achieved an SVR. Factors influencing SVR rates including treatment regimen, fibrosis stage, age, gender and weight were examined by univariate analysis (Table 2). None of these factors were found to be predictive of an SVR in the Asian cohort. In particular, we did not observe a difference in ETR or SVR between those who received pegylated interferon versus standard interferon.

Table 2 Univariate analysis of 108 HCV genotype 1-infected Asian patients: Predictors of SVR

Variables	OR (95% CI)	*P*-value
Gender (male)	0.77 (0.32-1.85)	0.55
Age (yr)	1.02 (0.99-1.06)	0.20
Weight (kg)	0.98 (0.95-1.06)	0.41
Pegylated interferon *vs* standard interferon	1.00 (0.28-3.57)	0.99
Treatment naïve	0.49 (0.22-1.12)	0.09
Dose reduction not required	1.45 (0.65-3.34)	0.39
Dose interruption not required	1.50 (0.44-5.10)	0.52
Absence of bridging fibrosis or cirrhosis	1.57 (0.61-4.07)	0.35

Table 3 Comparison of the demographic characteristics and treatment details of the matched Asian and Caucasian patients

Variables	Asians	Caucasians	*P*-value
Age (yr, range)	47 (28-64)	46 (30-61)	Matched
Gender	70% male	70% male	Matched
Weight (kg)			
< 75	82%	42%	< 0.01
> 75	18%	58%	
Alcohol intake			
Minimal intake	86%	71%	0.12
Significant intake	14%	29%	
Liver fibrosis			
Minimal injury	48 (75%)	48 (75%)	Matched
Bridging fibrosis/Cirrhosis	16 (25%)	16 (25%)	
Treatment naïve	80%	91%	NS
Peginterferon + Ribavirin	83%	88%	NS
Dose modification	30%	28%	NS
Dose interruption	8%	10%	NS

Baseline features of the matched cohort

Due to the more advanced age and extent of hepatic fibrosis among Asians, we were only able to match 64 Asian subjects with Caucasians by the set criteria. Comparison of the baseline demographics between the two groups is shown in Table 3. Only 18% of Asian patients weighed more than 75 kg while 58% of their Caucasian counterparts weighed more than 75 kg ($P < 0.01$). Alcohol intake was less in Asian subjects with 14% of Asians and 29% of Caucasians reporting a significant alcohol intake history ($P = 0.12$). There was no difference between the two groups in terms of previous therapy, type of treatment received or in the extent of dose reduction or interruptions from treatment adverse effects or non-compliance.

End of treatment and SVR rates in the matched cohort

Comparison of the ETR and SVR between the 64 Asian patients and the matched Caucasian cohort is illustrated in Figure 1. Compared to Caucasians, Asian subjects had a significantly better ETR, 81% *vs* 56% ($P = 0.003$) and SVR, 73% *vs* 36% ($P < 0.001$). On univariate analysis (Table 4), Asian ethnicity was the most predictive factor for a SVR. Other factors that were associated with an SVR in this cohort were body weight < 75 kg, and minimal alcohol intake. Due to the large number of variables and the limited number of patients, we were unable to perform a multiple logistic regression analysis with SVR as the dependent variable. Hence the effect of ethnicity on SVR was only adjusted for individual variables that were not matched. In this analysis (Table 5), Asian ethnicity remained a significant predictor of SVR after allowing for other variables that were not matched.

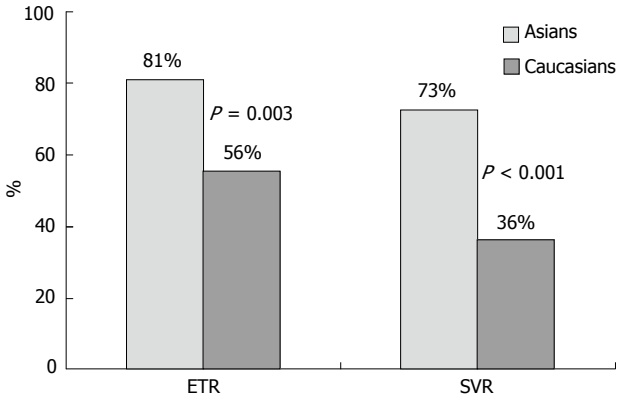

Figure 1 Comparison of ETR and SVR rates between HCV genotype 1-infected Asians and Caucasians matched for age, gender and fibrosis stage.

Table 4 Univariate analysis of predictors of SVR on matched HCV genotype 1-infected Asians and Caucasians

Variables	OR (95% CI)	*P*-value
Ethnicity (Asians)	4.92 (2.32-10.50)	< 0.001
Gender (male)	0.83 (0.39-1.79)	0.64
Age (per year)	1.04 (1.00-1.96)	0.061
Weight (< 75 kg)	2.59 (1.20-5.61)	0.016
Minimal alcohol intake	3.20 (1.17-8.73)	0.023
Peginterferon *vs* standard interferon	1.10 (0.42-2.92)	0.84
Treatment naïve	0.77 (0.29-2.04)	0.60
Dose reduction not required	1.04 (0.48-2.23)	0.93
Dose interruption not required	2.31 (0.64-8.33)	0.20
Absence of bridging fibrosis or cirrhosis	1.80 (0.80-4.04)	0.15

Table 5 Effects of ethnicity on SVR after adjusting for individual unmatched variables

	Adjusted OR (95% CI)	*P*-value
Weight (< 75 kg)	4.64 (1.97-10.94)	< 0.001
Minimal alcohol intake	2.74 (0.97-7.75)	0.057
Pegylated interferon *vs* standard interferon	5.01 (2.35-10.71)	< 0.001
Treatment naïve	5.01 (2.32-10.81)	< 0.001
Dose reduction not required	4.94 (2.32-10.50)	< 0.001
Dose interruption not required	4.84 (2.26-10.38)	< 0.001

DISCUSSION

In this retrospective study, we confirmed that in CHC, ethnicity is an important variable influencing response to antiviral therapy. Our study of Asians infected with HCV genotype 1 has permitted several important observations to be made. Firstly, the overall SVR among Asians approached 70%. Secondly, Asians with genotype 1 CHC were more likely to respond favourably to antiviral therapy compared to matched Caucasians. Finally, excess alcohol intake and increased body weight adversely affected treatment outcomes.

The observation of a favourable response rate among Asians is in accordance with other reports[14-16]. Importantly, the effect of ethnicity on SVR rates remained significant after adjusting for other confounders including age, gender, treatment regimen and the extent of hepatic fibrosis. The present study also observed that lower body weight and minimal alcohol intake were predictive of an SVR.

Body mass index[17-18], insulin resistance[19-21] and hepatic steatosis[22-24] are now known to play a major role in the pathogenesis of HCV infection. Insulin resistance in genotype 1 CHC is most likely related to host factors, in particular obesity, rather than virological factors, and is associated with reduced treatment response rates[25]. It was therefore interesting to observe in our study that the matched Asian cohort overall had lower body weights than Caucasians. Similar findings were reported in the studies by Hepburn *et al*[15] and Missiha *et al*[16]. Although in these previous studies, as well as in the current report, the effect of ethnicity remained significant after allowing for body weight, central adiposity or underlying insulin resistance were not measured. This is a limitation of the retrospective nature of this study. There are no published data on the impact of hepatic steatosis on CHC infection among Asians. It is therefore important to examine these factors in future studies as they might potentially explain the reasons why Asians having better treatment responses.

Not surprisingly, we observed that Asian subjects consumed less alcohol, possibly a reflection of cultural influences. Our study was the first to adjust for this variable. We noted that the effect of ethnicity on SVR was modified after allowing for alcohol consumption and did not reach statistical significance ($P = 0.057$). Although the difference in SVR was not significant (probably related to patient numbers), this observation highlights the importance of taking alcohol intake into account in studies comparing response rates stratified for ethnicity[26-27].

The biological basis for the difference in SVR rates between Asians and Caucasians has not been examined previously. Studies into the effects of ethnicity on CHC treatment has to date focussed entirely on African Americans. It was found that African Americans had different class Ⅱ human-leukocyte antigen alleles from Caucasians[28], which could have accounted for their worse SVR. Further, viral kinetics studies have shown that African Americans exhibit significantly lower interferon effectiveness and achieve a lower reduction in HCV RNA in the first 24 h of treatment[29]. It was also noted that African Americans had different pre-treatment cytokine profiles[30]. In addition, while they mounted a more robust HCV-specific CD4 Th1 proliferative response, it did not translate into a higher rate of IFN-gamma production, potentially secondary to their dysfunctional nature, which was associated with a failure of interferon therapy[31-32]. The significance of these studies is that the impact of ethnicity, on treatment response is more likely to be related to host factors, particularly to genetic differences in immune regulation rather than environmental factors.

Our study suffered the usual limitations of retrospective observational reports. In particular, we now know that a proportion of Asians who were initially genotyped by INNO-LiPA as 1b, were in fact genotypes 7, 8 or 9 if direct sequencing of the core region is performed[14]. Direct core sequencing was not performed in our study. It is arguable, however, if inclusion of genotypes 7, 8 or 9 would have altered the better SVR rates achieved by the Asian cohort since the original article noted that SVR rates were identical among Asians infected with genotypes 7, 8 or 9 and those infected with genotype 1 HCV infection[14]. Similar findings were noted in another study where it was shown that there was no difference in response rates between genotype 1b infected Asians and the rest of the genotype 1 infected Asians[15]. We therefore believe that the difference in SVR rates noted in our study was more likely related to ethnicity than a bias from a potentially small group of patients who might have been mistyped. It is clear however that prospective trials on larger patient cohorts including all genotypes are needed to clarify this issue.

The other limitation of this type of retrospective comparative study, as pointed out by both Hepburn *et al*[15] and Missiha *et al*[16], is that it failed to recognise the wide genetic heterogeneity and different environmental factors that might exist within the same ethnic group. While we defined Asians as those who migrated from East Asia including China, Japan, Korea and South East Asia, and of parents of those origin, we do not, and could not, know if they essentially represent the same group of patients genetically.

In conclusion, Asians infected with HCV genotype 1 achieved a higher SVR rate when compared to a cohort of matched Caucasians. Future studies should focus on confirming our observations in large prospective cohorts and on characterizing the immunogenetic basis for these observations.

COMMENTS

Background

Treatment with interferon and ribavirin combination therapy remains currently the most effective treatment for chronic hepatitis C (CHC) virus infection, but treatment response can only be achieved in 50% to 80% of patients. Various factors including genotype, viral load, extent of liver injury on liver biopsy, age and gender of patients were known to impact on treatment responses. Identification of these factors aids selection of patients for treatment and determination of duration of therapy.

Research frontiers

Recent studies on African Americans suggested that ethnicity might also impact on treatment responses. Data from studies on one ethnic group may not be extrapolated to other ethnic groups, and different ethnic groups may require different treatment regimens.

Innovations and breakthroughs

This study found that Asian patients infected with hepatitis C genotype 1 had better treatment responses than Caucasian patients, even after adjusting for other predictive factors.

Applications

The implication of the results of this study is twofold. Firstly, it prompts further basic scientific research into difference in immune response to CHC among different ethnic groups to better understand the pathogenesis of CHC. Secondly, it suggests that clinically, different treatment regimen should be studied and compared among different ethnic groups.

Peer review

The results provide sufficient evidence, which allow authors to make firm conclusion that ethnicity is an important factor variable influencing response to antiviral therapy in patients with CHC. The further studies to confirm the results of this observational study for patients from different ethnic backgrounds are needed. The references are appropriate, relevant and updated.

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RAPID COMMUNICATION

Hypermethylation and aberrant expression of secreted fizzled-related protein genes in pancreatic cancer

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Abstract

AIM: To determine the methylation status and aberrant expression of some secreted frizzled-related protein (SFRP) genes in pancreatic cancer and explore their role in pancreatic carcinogenesis.

METHODS: Methylation status and expression of SFRP genes were detected by methylation-specific PCR (MSPCR) and reverse-transcription PCR (RT-PCR) respectively.

RESULTS: The frequencies of methylation for SFRP genes 1, 2, 4, 5 were 70%, 48.3%, 60% and 76.7% in pancreatic cancer samples, and 21.7%, 20%, 10% and 36.7% in matched cancer adjacent normal tissue samples, respectively (χ^2 = 28.23, P < 0.0001 for SFRP gene 1; χ^2 = 10.71, P = 0.001 for SFRP gene 2; χ^2 = 32.97, P < 0.0001 for SFRP gene 4; χ^2 = 19.55, P < 0.0001 for SFRP gene 5). Expression loss of SFRP genes 1, 2, 4 and 5 was found in 65%, 40%, 55% and 71.7% of 60 pancreatic cancer samples, and 25%, 15%, 18.3% and 31.7% of matched cancer adjacent normal tissue samples, respectively (χ^2 = 19.39, P < 0.0001 for SFRP gene 1; χ^2 = 9.40, P = 0.002 for SFRP gene 2; χ^2 = 17.37, P < 0.0001 for SFRP gene 4; χ^2 = 19.22, P < 0.0001 for SFRP gene 5). SFRP gene 1 was methylated but not expressed in PC-3 and PANC-1, SFRP gene 2 was methylated but not expressed in PANC-1 and CFPAC-1, SFRP gene 4 was methylated but not expressed in PC-3, and SFRP gene 5 was methylated but not expressed in CFPAC-1.

CONCLUSION: Hypermethylation and aberrant expression of SFRP genes are common in pancreatic cancer, which may be involved in pancreatic carcinogenesis.

Key words: Hypermethylation; Secreted frizzled-related protein; Pancreatic cancer

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INTRODUCTION

Secreted frizzled-related proteins (SFRPs) are a group of negative regulators of the Wnt signaling pathway[1-3]. These proteins contain a cysteine-rich domain (CRD) which shares a sequence similarity of 30%-50% with Wnt receptor frizzled proteins. Through the CRD, SFRPs can antagonize Wnt signaling by interacting with Wnt ligand. As the Wnt signaling pathway plays an important role in cell proliferation, differentiation and apoptosis in adult tissues, aberrant activation of the Wnt pathway caused by down-regulation of SFRPs may induce tumorigenesis. It was recently reported that some members of the SFRP family are down-regulated by hypermethylation in a series of human cancers[4-9].

The prognosis of pancreatic cancer, one of the most malignant tumors, is usually very poor. The pathogenesis of pancreatic cancer is still not very clear. It has been found that hypermethylation and subsequent expression loss of some tumor suppressor genes and tumor-related genes, such as p16[10], RASSF1A[11], SOCS-1[12], and hMLH1[13] occur frequently in pancreatic cancer.

This study was designed to determine the methylation status and aberrant expression of some members of the SFRP family in pancreatic cancer and explore their role in pancreatic carcinogenesis.

MATERIALS AND METHODS

Cell lines, cancer and matched adjacent tissue samples

Human pancreatic cancer cell lines PC-3, PANC-1 and CFPAC-1 (from KEYGEN, Nanjing, China) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 μg/mL penicillin, and 100 μg/mL streptomycin, at 37℃ in a humid incubator containing 50 mL/L CO_2. Pancreatic cancer and matched adjacent tissue samples were obtained from patients who underwent operation at the Second Affiliated Hospital of China Medical University. The samples were frozen in liquid nitrogen immediately after surgery. Haematoxylin and eosin staining was used to assure that cancer samples were consisted mostly of tumor cells with no tumor cells in the tumor adjacent tissue samples.

DNA and RNA extraction

DNA was extracted by a standard phenol/chloroform extraction and ethanol precipitation procedure. RNA was isolated using Tri reagent (Takara, Dalian, China) according to its manufacturer's instructions.

Reverse transcription-PCR (RT-PCR)

RT-PCR was performed using a RNA PCR 3.0 kit (Takara, Dalian, China). cDNA was synthesized from 1 μg RNA using a random 9 primer and AMV reverse transcriptase. One cycle was performed at 30℃ for 10 min, at 42℃ for 25 min, at 99℃ for 5 min, and at 5℃ for 5 min. The primer sequences used in PCR are described elsewhere[7]. PCR was performed for one cycle at 94℃ for 2 min, followed by 30 cycles at 94℃ for 30 s, at 60℃ for 30 s and at 72℃ for 2 min.

Methylation-specific PCR (MSPCR)

Methylation of SFRP1 was detected with a MSPCR kit (GENMED, Shanghai, China) according to its manufacturer's instructions. The primer sequences are described elsewhere[7]. MSPCR was performed for one cycle at 95℃ for 5 min, followed by 35 cycles at 95℃ for 30 s, at 60℃ for 30 s and at 72℃ for 30 s.

Statistical analysis

Methylation and expression of SFRP1 in primary pancreatic cancer and its adjacent tissue samples were compared by chi-square test. $P < 0.05$ was considered statistically significant.

RESULTS

Hypermethylation and expression of SFRPs in pancreatic cancer cell lines

The methylation status of SFRPs was detected by MSPCR. SFRP1 was methylated in PC-3 and PANC-1, SFRP2 in PANC-1 and CFPAC-1, SFRP4 in PC-3 and SFRP5 was methylated in CFPAC-1, respectively (Figure 1). The mRNA expression of SFRPs was determined by RT-PCR. No expression of SFRP1, SFRP2, SFRP4 and SFRP5 was found in PC-3 and PANC-1, PANC-1 and CFPAC-1, PC-3 and in CFPAC-1, respectively (Figure 2).

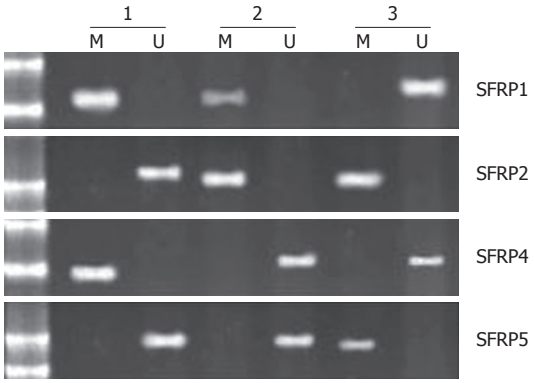

Figure 1 Hypermethylation of SFRP genes in pancreatic cancer cell lines detected by MSPCR. 1: PC-3; 2: PANC-1; 3: CFPAC-1; M: Methylated; U: Unmethylated.

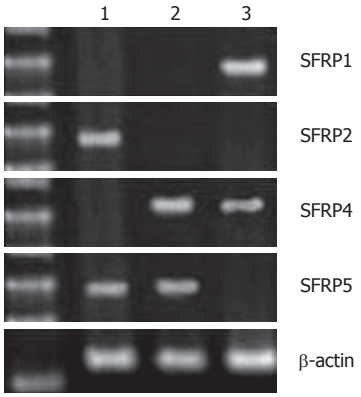

Figure 2 Expression of SFRPs in pancreatic cancer cell lines detected by RT-PCR. 1: PC-3; 2: PANC-1; 3: CFPAC-1.

The expression loss of SFRPs was correlated with the methylation status.

Hypermethylation and expression of SFRPs in pancreatic cancer and its adjacent tissue samples

Hypermethylation of SFRP1, SFRP2, SFRP4 and SFRP5 was detected in 42 (70%), 29 (48.3%), 36 (60%) and 46 (76.7%) of 60 pancreatic cancer samples, and 13 (21.7%), 12 (20%), 6 (10%) and 22 (36.7%) of its adjacent tissue samples, respectively. The hypermethylation of each SFRP gene differed significantly in cancer and its adjacent tissue samples ($\chi^2 = 28.23$, $P < 0.0001$ for SFRP1; $\chi^2 = 10.71$, $P = 0.001$ for SFRP 2; $\chi^2 = 32.97$, $P < 0.0001$ for SFRP 4; $\chi^2 = 19.55$, $P < 0.0001$ for SFRP 5; Table 1). Expression loss of SFRP1, SFRP2, SFRP4 and SFRP5 was found in 39 (65%), 24 (40%), 33 (55%) and 43 (71.7%) of 60 pancreatic cancer samples, and 15 (25%), 9 (15%), 11 (18.3%) and 19(31.7%) of its adjacent tissue samples, respectively. The expression loss of each SFRP gene differed significantly in cancer and its adjacent tissue samples ($\chi^2 = 19.39$, $P < 0.0001$ for SFRP1; $\chi^2 = 9.40$, $P = 0.002$ for SFRP2; $\chi^2 = 17.37$, $P < 0.0001$ for SFRP4; $\chi^2 = 19.22$, $P < 0.0001$ for SFRP5; Table 2).

DISCUSSION

The Wnt signaling pathway plays an important role not only in development of cancer but also in cell proliferation, differentiation and apoptosis in adult tissues.

Table 1 Hypermethylation of SFRPs in pancreatic cancer and its adjacent tissue samples

	n	SFRR1	SFRR2	SFRP4	SFRP5
Pancreatic cancer samples	60	42	29	36	46
Adjacent tissue samples	60	13	12	6	22
χ^2		28.23	10.71	32.97	19.55
P		< 0.0001	0.001	< 0.0001	< 0.0001

Table 2 Expression loss of SFRPs in pancreatic cancer and its adjacent tissue samples

	n	SFRR1	SFRR2	SFRP4	SFRP5
Pancreatic cancer samples	60	39	24	33	43
Adjacent tissue samples	60	15	9	11	19
χ^2		19.39	9.40	17.37	19.22
P		< 0.0001	0.002	< 0.0001	< 0.0001

Aberrant activation of Wnt signaling in tumorigenesis has been reported frequently, and some members of the Wnt family are over-expressed in breast cancer, gastrointestinal cancer and prostate cancer[14-16]. Down-regulation of the Wnt inhibitors DKKs and SFRPs also occurs frequently in human cancers[17,18]. Most of these reports show that expression loss of these inhibitors is mainly caused by promoter hypermethylation, an important epigenetic gene silencing mechanism.

Aberrant Wnt signals are also involved in pancreatic cancer. It was reported that activated mutation of β-catenin on exon 3, a downstream component in the Wnt signaling pathway, plays an important role in pancreatic tumorigenesis. This kind of mutation leads to excessive accumulation of β-catenin and aberrant activation of the Wnt pathway[19-23]. Over-expression of many members of the Wnt family, such as Wnt1[24], Wnt5a[25], Wnt5b[25], Wnt7a[26], Wnt10b[27] in pancreatic cancer, has been reported in recent years, further suggesting that the Wnt pathway plays a role in the pathogenesis of pancreatic cancer. It has recently been shown that epigenetic inactivation of Wnt inhibitory factor 1 by hypermethylation occurs frequently in pancreatic cancer[28].

The pathogenesis of pancreatic cancer, a very malignant carcinoma, has been poorly understood. In this study, we analyzed the hypermethylation and expression of SFRPs in pancreatic cancer and explored their role in pancreatic carcinogenesis, showing that hypermethylation and expression loss of SFRPs occur frequently in pancreatic cancer. The frequencies of hypermethylation and expression loss of SFRPs in pancreatic cancer samples were significantly higher than those in its adjacent normal tissue samples, suggesting that hypermethyaltion and subsequent expression loss of SFRPs occur early and play an important role in the pathogenesis of pancreatic cancer.

As we know, Wnt signaling can be divided into canonical Wnt/β-catenin pathway and non-canonical pathway which includes the planar cell polarity pathway and the Wnt/Ca^{2+} pathway. As we did not measure the level of β-catenin, we could not determine whether the pathway through which SFRP1 expression loss is involved in the pancreatic carcinogenesis. Further study is needed to elucidate its mechanism.

COMMENTS

Background

Secreted frizzled-related proteins (SFRPs) are a group of negative regulators of the Wnt signaling pathway. Aberrant activation of the Wnt pathway caused by down-regulation of SFRPs may induce tumorigenesis.

Research frontiers

The pathogenesis of pancreatic cancer, a very malignant carcinoma, is poorly understood. In this study, we found that hypermethylation status and expression of SFRPs played an important role in pancreatic carcinogenesis.

Innovations and breakthroughs

In this study, we analyzed the hypermethylation status and expression of SFRPs in pancreatic cancer and explored their role in pancreatic carcinogenesis.

Applications

Our study suggested that hypermethyaltion and subsequent expression loss of SFRPs play an important role in pancreatic carcinogenesis. For this reason, demethylated agents may be used to treat cancer in clinical practice.

Peer review

In this study, the authors reported that the expression loss of some SFRP genes caused by hypermethylation was common in pancreatic cancer, which may play an important role in pancreatic carcinogenesis. This paper is original and informative.

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RAPID COMMUNICATION

Pancreaticoduodenectomy for advanced gastric cancer with pancreaticoduodenal region involvement

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Author contributions: Wang XB performed the operation and wrote the paper; Yang LT analyzed the data; Zhang ZW followed up the patients; Guo JM and Cheng XD performed the operation.
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Abstract

AIM: To characterize the factors of the improved survival following combined pancreaticoduodenectomy (PD) and gastrectomy for the treatment of advanced gastric cancer with pancreaticoduodenal region involvement.

METHODS: From 1995 to 2004, 53 patients with primary gastric cancer were diagnosed with synchronous (n = 44) or metachronous (n = 9) pancreaticoduodenal region involvement. Of these, 17 patients (32%) underwent total gastrectomy (TG) or distal subtotal gastrectomy (SG) combined with PD simultaneously. The preoperative demographic, clinical information, clinicopathologic features and the surgical results of these 17 patients were considered as factors influencing survival and were analyzed by the Kaplan-Meier method with log-rank comparison.

RESULTS: The actual 1- and 3-year survival rates of these 17 patients after resection were 77% and 34%, respectively, and three patients survived for more than 5 years after surgery. The tumor-free resection margin (P = 0.0174) and a well-differentiated histologic type (P = 0.0011) were significant prognostic factors on univariate analysis. No mortality occurred within one mo after operation, postoperative weight loss of different degree was present in all the patients with TG and 12 cases had other complications. There were 9 (53%) cases of recurrence in 5-48 mo after operation. The survival rate in the palliative and explorative group was significantly (P = 0.0064) lower than in the combined PD group.

CONCLUSION: Judicious use of en bloc PD and gastrectomy and strictly preventing postoperative complications may improve the long-term survival for advanced gastric cancer patients with pancreaticoduodenal region involvement. Well-differentiated histology and negative resection margin are the most important predictors of long survival.

Key words: Pancreaticoduodenectomy; Gastric cancer; Gastrectomy; Predictive factor; Patients

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INTRODUCTION

Because of earlier diagnosis, more accurate staging and safer operations, outcomes after treatment of gastric cancer are being improved, especially for early gastric cancer the results have become satisfactory[1]. And in the treatment of advanced carcinoma of the stomach, gastrectomy with extensive lymph node dissection has been reported to acquire a substantial improvement in survival[2-4]. In spite of these advances, there were still some problems as for areas of resection for advanced gastric cancer involving local organs. Arguments against enlarged resection are based on the observed increase in the morbidity and mortality rates, with little objective benefit in survival[5]. And some surgeons still consider the invasion of adjacent organs by the carcinoma of stomach as a sign of incurable disease. But others believe patients with T4 gastric cancer will benefit from extended en bloc surgical resection[6-8].

For the anatomic reason, pancreaticoduodenal region involved in advanced gastric cancer was not scarce. But few articles about its surgical treatment were available. The aim of this study was to report our experience in undergoing pancreaticoduodenectomy

(PD) with gastrectomy for advanced gastric cancer with pancreaticoduodenal region involvement.

MATERIALS AND METHODS

Patients

From January 1995 to January 2004, 916 patients with gastric carcinoma underwent surgical treatment in the Department of Hepatobiliary-Pancreatic-Gastric Surgery, Zhejiang Cancer Hospital. Of the 916 cases, 44 were found to have synchronous pancreaticoduodenal region involved and 9 metachronous pancreaticoduodenal region invaded or involved. Among the 53 patients, palliative gastrectomy was performed in 6 patients, bypass through exploration performed in 14, explorations in 7 and surgical treatment was given up in 9 because of additional organs metastasis or poor physical state. And 17 patients who underwent PD with gastrectomy were selected for this study, including 11 men and 6 women with a mean age of 56 years (range from 38 to 71 years). Overall radical resectability was 32.1% (17/53) for the 53 patients, 32% (14/44) for synchronous metastases, and 33.3% (3/9) for metachronous lesions. Follow-up period ranged from 2 to 72 mo (median 38 mo).

Methods

PD was indicated for patients with visibly synchronous pancreaticoduodenal region involved lesions who did not have peritoneal dissemination or any other distant metastasis, or for patients with metachronous pancreaticoduodenal region involved who did not have any other recurrent lesions. Three patients who had a desmoplastic reaction at the site of presumed tumor invasion were not included in this study. Confirmation of cancerous invasion of pancreaticoduodenal region was established histologically in operation. In addition to PD, TG was done in 11 (64.4%) and SG in 6 (35.6%) patients, depending on the location of the primary gastric lesion. En bloc surgical resection and D2 lymphadenectomy were performed as the standard radical gastrectomy for these 17 patients. As for reconstruction of digestive tract, binding pancreaticojejunostomy[9] and Roux-en-Y anastomosis were adopted for all the cases. In this group, two patients underwent right hemicolectomy and one patient underwent cholangiocystectomy additionally at the same time. All patients were treated with postoperative adjuvant chemotherapy using the same chemotherapy regimens (ELF regimens: etoposide + leucovorin + fluorouracil)[10,11].

The preoperative demographic and clinical information was obtained from the patient records: age, gender, interval between gastrectomy and PD, surgical procedure and recurrence. The number and size of the tumors, extent of lymph node metastasis and surgical margin of the tumors were also recorded. The pathologic diagnosis and classification of the tumors were performed by a minimum of two pathologists.

Data analysis

All data were treated with statistic software kit of SPSS 12.0. Parameters influencing survival were compared using the Kaplan-Meier method with log-rank comparison. $P < 0.05$ was considered significant differences.

Table 1 Characteristics and their prognostic significance for PD group

Factors	Number of patients	P
Age (yr)		0.1405
< 56	7	
≥ 56	10	
Gender		0.4412
Male	11	
Female	6	
Metastases		0.2010
Synchronous	14	
Metachronous	3	
Tumor size		0.9837
< 4 cm	8	
≥ 4 cm	9	
Histologic differentiation		0.0011
Well	4	
Moderate or poor	13	
Gastric carcinoma depth of invasion		0.0610
Borrmann Ⅲ	6	
Borrmann Ⅳ	11	0.0516
Lymph node metastasis		
Positive	10	
Negative	7	0.7948
Gastrectomy pattern		
Total gastrectomy	11	
Subtotal gastrectomy	6	0.0174
Resection margins		
Positive	5	
Negative	12	0.1486
Combined other organs		
No	14	
Yes	3	

RESULTS

No patient died during the initial hospital stay or within 1 mo after surgery. The median diameter of the tumors was 4.0 cm (range 2.8-9.5 cm). Only four patients had well differentiated tumors and the other 13 patients had moderately or poorly differentiated tumors. According to Borrmanm Type, six patients were Borrmann Ⅲ and 11 were Borrmann Ⅳ. Ten patients had positive lymph node metastasis and seven had negative lymph node metastasis. Resection margins in five patients were tumor-positive and 12 were tumor-free. The actual 1- and 3-year survival rates after PD with gastrectomy were 77% and 34%, respectively. The results of the analysis of the prognostic factors are given in Table 1. Tumor-free (negative) resection margin ($P = 0.0174$) was significant determinants for a favorable prognosis after PD. In terms of pathologic features, a well-differentiated type of metastases ($P = 0.0011$) was a significant prognostic factor. Factors associated with the primary lesion and surgical procedures were not significant prognostic determinants. Cancer recurred in 11 (59%) of the 17 patients between 5 mo and 48 mo after PD resection. The site of initial recurrence after PD and gastrectomy was the gastric and pancreaticoduodenal

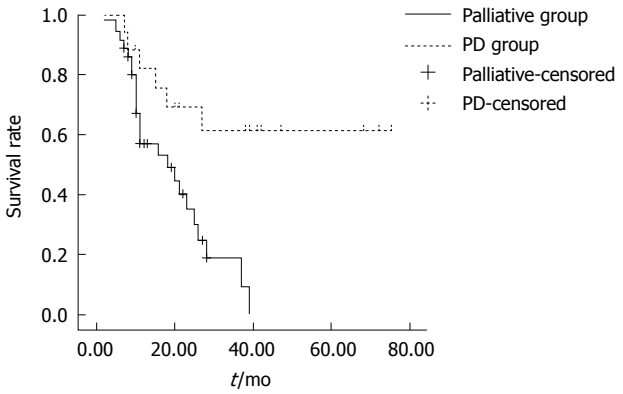

Figure 1 Comparison of survival between patients with palliative surgery and PD. Significant difference was found between the two groups (P = 0.0064).

bed in 6 patients, the liver in 3, and the retroperitoneal lymph nodes in 2, which were diagnosed by image methods (CT, MRI or US). Three patients survived more than 5 years after PD. No mortality occurred in one mo after operation, postoperative weight loss of different degree was present in all the patients with TG. Twelve had other complications among the 17 patients, including intra-abdominal abscesses 5 (41.7%) and anastomotic leak 3 (gastrointestinal leak 1 and bile leak 2) (25%), pneumonia 2 (16.7%), returned esophagitis 1 (8.3%) and acute renal failure 1 (8.3%). All the complications were cured by operative or conservative treatment.

Of the 36 patients with pancreaticoduodenal region involvement who did not undergo radical resection, the actual 1- and 3-year survival rates were 41.7%(15/36) and 5.6%(2/36), respectively. The survival rate of these 36 patients (palliative group) was significantly (P = 0.0064) lower than that of PD group (Figure 1).

DISCUSSION

Surgery remains the only method of treatment that offers the potential for cure of gastric cancer[12]. But gastric cancer is usually diagnosed at an advanced stage because of its vague and nonspecific symptoms. And poor survival will be followed by late stage, especially when tumors invade the serosa or other organs. There is a report that with serosal involvement alone, less than 30% of the patients are living five years after surgery, and with involvement of both serosa and lymph nodes, the 5-year survival is less than 15%[13]. At the same time, opinions about extended surgical resections for advanced gastric cancer remains controversial. Takeuchi *et al*[12] retrospectively studied 65 patients without distant metastasis who underwent TG with pancreaticosplenectomy (PS) and 98 patients without distant metastasis who underwent TG alone (the TG alone group) by essentially the same technique, and concluded that combined PS with TG should never be performed as the standard surgical procedure for every stage of gastric cancer, especially stage Ⅱ. But we think that in his report the PS was performed with TG to facilitate dissection of the lymph nodes around the splenic artery and splenic hilus, but not performed to resect the involved lesions of spleen or pancreas tail.

On the other hand, some authors agreed to extend gastric resection in patients with adjacent organs involved. In the report by Kodama *et al*[14], 77 patients with carcinoma of the stomach directly invading adjacent organs or structures were analyzed retrospectively to investigate the efficacy of en bloc resection. Forty-one patients underwent gastrectomy combined with resection of one or more invaded organs (combined resection group), while the other 36 patients underwent gastrectomy with palliative abrasion between the primary tumor and the invasion site (non-combined resection group). The results demonstrated that the five-year survival rate was 23% in the combined resection group and 0% in the non-combined resection group.They thought that an en bloc combined resection would be worth trying. Iriyama *et al*[15] reported the highest 5-year survival rate of 46% after extended gastric resection with adjacent organs in patients with T4 gastric cancer. And Korenaga *et al*[16] reported that the 5-year survival was 36.7% for those who underwent radical resection of adjacent organs and 17.4% for those who underwent palliative resection of adjacent involved organs. Ozaki *et al*[17] and others[16] have found that an aggressive approach to resection of the stomach with the body and tail of the pancreas or PD and right hemicolectomy can lead to an acceptable 5-year survival rate of 29%. Yonemura *et al*[18] have performed 26 SG with PD in combination with right hemicolectomy without any operative mortality and a 5-year survival of 33% even for patients with N3 metastases (e.g. retropancreatic nodes or superior mesenteric nodes). Cho *et al*[19] reported their 15-year experience of extended gastrectomy for advanced gastric cancer. The median survival time of the positive margin group was 34 mo. The negative margin group had a significantly longer median survival of 69 mo (P = 0.025). When both groups of patients were stratified according to nodal stage, a positive resection margin determined a worse prognosis only in patients with node-negative disease (174 mo *vs* 37 mo, P = 0.0001). In patients with nodal metastasis, the median survival time was similar in both groups. Their results suggested that a positive microscopic margin was associated with a worse outcome in patients with node-negative disease. Therefore, a more aggressive treatment, such as reoperation, was needed in node-negative patients with a positive microscopic disease. Our results have shown that three patients survived more than five years in radical resection group (PD group) and no patient survived more than five years in the palliative group. And the actual survival of these two groups is statistically different. Although factors associated with the primary lesion, patient demographic data and surgical procedures were not significant prognostic determinants, but negative resection margin was very important for the higher survival rate. And when pancreaticoduodenal region is involved by advanced gastric cancer, combined PD and gastrectomy will bring the chance of tumor-free resection margin. And curative (R0) resection improves prognosis[19,20]. PD with gastrectomy will benefit the lymphadenectomy of No. 7, 8, 9 and 11. Sometimes, it

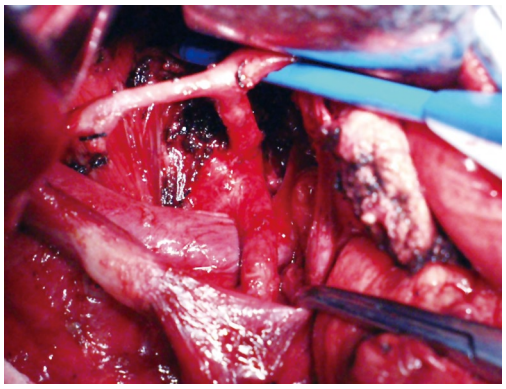

Figure 2 Lymphadenectomy of lymph nodes of No. 7, 8, 9, 11 and 16.

is helpful for lymphadenectomy of No. 16 (Figure 2), though lymph nodes of the No.16 were not resected routinely in our group. We proposed the indication of PD with gastrectomy as follows: (1) head of pancreas was invaded by gastric cancer, (2) metastasis of lymph nodes of No.6 and head of pancreas was infiltrated, (3) duodenum below pylorus 2 cm was invaded by gastric cancer, and (4) the inferior segment of common bile duct was invaded by gastric cancer. Occasionally, the above condition was not found by preoperative examination. In our group, three cases were not found till during operations. So it is necessary to check the head of pancreas, the inferior segment of common bile duct and the superior segment of duodenum below pylorus during operations when tumor was near the pancreaticoduodenal region. As for the pattern of gastrectomy, we adopted TG or SG according to the different locations of the cancer within the stomach, its pattern of growth, and the level of local spread. Subtotal gastrectomies were reserved for exophytic and small infiltrative tumors located in the lower third of the stomach. Total gastrectomy was used for tumors located in the middle and upper third of the stomach, or tumors with an infiltrative growth pattern. Our results manifested that tumor-free resection margins will benefit the survival. And we emphasize that frozen-histologic examination during operation should benefit both the definition of resection margins and the definition of tumor invasion on pancreaticoduodenal region. Large inflammatory perigasrtic lymph nodes or desmoplastic reaction around gastric cancer can be inaccurately presumed to be tumor invasion and adopt extended gastric resection mistakenly. Three patients who had desmoplastic reaction at the site of presumed tumor invasion were found in our hospital during operation, the pancreas and duodenums were reserved. And no inflammatory perigasrtic lymph nodes or desmoplastic reaction around gastric cancer were found during postoperative histologic examination in our data.

Upon decision of extended resection, as the procedure of PD and gastrectomy was relatively complicated and status of most patients was poor, a high complication rate could not be ignored. The complication rates of additional organ resection with gastrectomy have been consistently reported to be higher when compared with the patients undergoing gastrectomy alone[21-23]. And the increasing overall complications and infectious complications have been found to be factors for the decrease in the survival of the patients. So, reinforcing perioperative management is a key point to prevent postoperative complications. Our data demonstrate that the overall complication rate was very high, and postoperative weight loss of different degree was present in all the patients with TG. Judicious use of additional PD with gastrectomy is also important for reducing postoperative complications. If a patient could not tolerate a prolonged operation, we would take simple operative method such as bypass operation. And simple gastroenterostomy or esophagojejunostomy should be adopted to reduce postoperative complications and postoperative nutritional support would improve the state of weight loss.

We adopted total parenteral nutrition (TPN) at the early stage after TG to spur positive nitrogen balance and reduce weight loss, and after an interval we adopted suitable enteral nutrition to reduce complications. A leak or fistula from the pancreatic anastomosis is the leading cause of morbidity and mortality after PD, but no pancreatioenteral anastomosis leak occurred in our study, as we adopted binding pancreaticojejunostomy[9], by which 3 cm of the serosa-muscular sheath of the jejunum was bound to the pancreatic remnant and could effectively prevent the development of pancreatic leak or fistula. It is a safe, simple and efficient technique.

Our study confirmed that patients with advanced gastric cancer could benefit from aggressive en bloc surgical resection and should not render unresectable when pancreaticoduodenal region was found invaded or involved. With careful patient selection, gastrectomy with PD can be performed with acceptable morbidity and minimum mortality. Well histologic differentiation and negative resection margin are the most powerful determinants of survival following an extended resection. The survival rate in the palliative and explorative group was significantly (P = 0.0064) lower than in the combined PD group, partially because that their baseline conditions were different. And in this article, we did not analyze the survival rates of patients with TG or SG , because the number of the patients was too large to follow up. We have no complete data of these patients. So we can not compare the survival rates between patients with TG or SG and those with TG or SG combined with PD. It is the default of this study.

COMMENTS

Background

For advanced gastric carcinoma, gastrectomy with extensive lymph node dissection has been reported to acquire a substantial improvement in survival. But there are still some problems as for areas of resection for advanced gastric cancer involved local organs. For the anatomic reason, pancreaticoduodenal region involved in advanced gastric cancer was not scarce. But few articles about its surgical treatment have been reported. This study was to report the authors' experience in undergoing pancreaticoduodenectomy (PD) and gastrectomy for advanced gastric cancer with pancreaticoduodenal region involved.

Research frontiers

The focus of controversy for the advanced gastric cancer involved organ

is either performing the extensive resection or giving up surgical resection. Arguments against enlarged resection are based on the observed increase in the morbidity and mortality rates, with little objective benefit in survival. And some surgeons still consider the invasion of adjacent organs by the carcinoma of stomach as a sign of incurable disease. But others believe patients with T4 gastric cancer will benefit from extended en bloc surgical resection.

Innovations and breakthroughs

This study confirmed that patients with advanced gastric cancer could benefit from aggressive en bloc surgical resection, which should not be rendered unresectable when pancreaticoduodenal region was found invaded or involved. Negative resection margin is an important factor for the patients with extended resection.

Applications

For the patients with advanced gastric cancer with pancreaticoduodenal region invaded, PD and extensive resection should be performed if the status of the patients permits.

Peer review

This retrospective study assessed the performance of PD for advanced gastric cancer with pancreaticoduodenal region involvement, and observed that the 1-year and 3-year survival rates after total gastrectomy (TG) or distal subtotal gastrectomy (SG) combined with PD were 77% and 34%, which were significantly higher than in the patients with palliative treatment. It was also found that histological differentiation ad negative resection margin were most important predictors.

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CASE REPORT

Acute small bowel obstruction caused by endometriosis: A case report and review of the literature

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Abstract

Gastrointestinal involvement of endometriosis has been found in 3%-37% of menstruating women and exclusive localization on the ileum is very rare (1%-7%). Endometriosis of the distal ileum is an infrequent cause of intestinal obstruction, ranging from 7% to 23% of all cases with intestinal involvement. We report a case in which endometrial infiltration of the small bowel caused acute obstruction requiring emergency surgery, in a woman whose symptoms were not related to menses. Histology of the resected specimen showed that endometriosis was mainly prevalent in the muscularis propria and submucosa and that the mucosa was not ulcerated but had inflammation and glandular alteration. Endometrial lymph node involvement, with a cystic glandular pattern was also detected.

Key words: Endometriosis; Small bowel; Ileum; Obstruction; Abdominal pain; Intestinal resection

Peer reviewers: Kazuma Fujimoto, Professor, Department of Internal Medicine, Saga Medical School, Nabeshima, Saga, Saga 849-8501, Japan; Amado S Peña, Professor, Department of Pathology, Immunogenetics, VU University Medical Centre, De Boelelaan 1117, PO Box 7057, Amsterdam 1007 MB, The Netherlands

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INTRODUCTION

Endometriosis is characterized by the presence of functional endometrial tissue consisting of glands and/or stroma located outside the uterus[1]. It is a painful chronic disease occurring in 5%-15% of menstruating women[1-3].

The reported prevalence of endometriosis is 1%-20% in asymptomatic women, 10%-25% in infertile patients and 60%-70% in women with chronic pelvic pain[4-5].

Endometriosis can be divided into intra- and extra-peritoneal sites. In decreasing order of frequency, the intra-peritoneal locations are ovaries (30%), uterosacral and large ligaments (18%-24%), fallopian tubes (20%), pelvic peritoneum, pouch of Douglas, and gastrointestinal (GI) tract. Extra-peritoneal locations include cervical portio (0.5%), vagina and rectovaginal septum, round ligament and inguinal hernia sac (0.3%-0.6%), navel (1%), abdominal scars after gynaecological surgery (1.5%) and caesarian section (0.5%). Endometriosis rarely affects extra-abdominal organs such as the lungs, urinary system, skin and the central nervous system[6-9].

GI involvement of endometriosis has been found in 3%-37% of women, most commonly in the sigmoid colon, rectum and terminal ileum[10-13].

We report a case in which endometrial infiltration of the small bowel caused acute obstruction, requiring emergency surgery. Diagnosis of ileal endometriosis was made by pathological examination of the resected specimen.

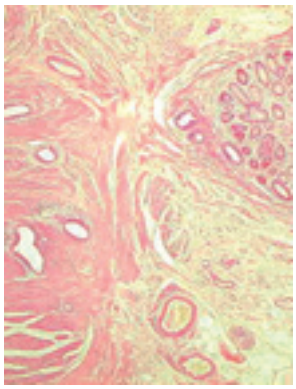

Figure 1 Histology of ileal wall showing endometrial tissue in the muscular layer, with foci of mucosal involvement.

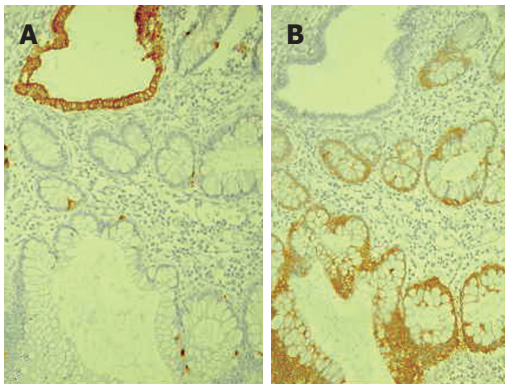

Figure 2 Histopathology showing CK20 immunostaining of intestinal epithelium (**A**) and CK 7 immunostaining of endometrioid glands (**B**).

CASE REPORT

A 44-year old woman was referred to our unit because of diffuse abdominal pain, associated with diarrhoea alternating with constipation.

The patient began complaining of mild abdominal discomfort nine months earlier. Episodes of pain relapsed irregularly, lasted a few hours and were not related to menses. She had two normal labors at the age of 30 and 32 years, regular menses and no history of dyspareunia. No other symptoms were present. Her past medical history was unremarkable.

Three months earlier, evaluation by her primary care physician revealed normal blood tests, and an abdominal ultrasound examination was unremarkable. Faecal analysis was negative for both parasites and occult blood. Antispasmodic drugs were administered, but the patient experienced a progressive worsening of the cramping abdominal pain and the onset of constipation.

Physical examination showed mild diffuse abdominal tenderness. No abdominal masses or enlarged lymph nodes were noted. Auscultation detected an increase of bowel sounds and peristaltic rushes. Colonoscopy and abdominal computed tomography (CT) scan were planned. However, 48 h later, the patient required emergency admission for small bowel occlusion. Abdominal X-ray examination showed dilated loops of the small intestine with no air in the colon, and CT scan revealed an irregular mass involving the ileum with dilation of the small intestine. A colonoscopy was performed to refine the diagnosis. The colon was normal, but the ileum, at about 5 cm from the ileocecal valve, showed a tight extrinsic compression with intact mucosa.

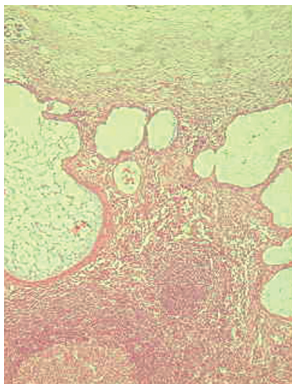

Figure 3 Endometriosis involving lymph nodes with a cystic glandular pattern.

Surgery was immediately carried out and an obstructing mass 5 cm in length involving the distal ileum was detected, with diffuse dilation of the small intestine. A right hemicolectomy with resection of 25 cm of ileum was performed. There was no evidence of macroscopic lesions in other abdominal and pelvic organs. The post-operative course was uneventful and the patient left the hospital 9 d later.

Histology of the resected specimen showed endometriosis involving the ileum and causing a stricture. The bowel wall was infiltrated, but the mucosa was not ulcerated. Endometriosis was mainly prevalent in the muscularis propria and submucosa. The mucosal involvement showed inflammation and glandular alteration (Figure 1). Immunocytochemistry with cytokeratin (CK) of different molecular weight (CK7 and CK20) was performed. Endometrioid glands and the intestinal epithelium were positive for CK7 and CK20, respectively (Figure 2). Endometrial lymph node involvement, with a cystic glandular pattern, was also detected (Figure 3).

DISCUSSION

Endometriosis is a common disease of unknown etiology. Many theories have been proposed to explain this condition. The most widely accepted is Sampson's retrograde menstruation theory: during menstruation, endometrial tissue refluxes through the fallopian tubes, implanting and growing on the serosal surface of abdominal and pelvic organs[14-15].

Alternatively, extrauterine growth of endometriotic tissue could occur as a result of metaplastic transformation of pluripotential peritoneal mesothelium (Minh's theory)[16].

Another theory implies the migration of cells through the lymphatic system or via hematogenous spread[17]. Donnez *et al*[18] hypothesized that endometrial nodules may develop from metaplasia of mullerian remnants. In some cases, they could result from iatrogenic displacement of the decidua during a caesarean section[19].

The "neurologic hypothesis" is a new concept in the pathogenesis of endometriosis: the lesions seem

to infiltrate the large bowel wall along the nerves, at a distance from the primary lesion[20]. However, other factors, immunological, genetic and familial, could be involved in the pathogenesis of this disease[21-23].

Endometriosis usually becomes apparent in the reproductive years when the lesions are stimulated by ovarian hormones. Forty percent of the patients present symptoms in a cyclic manner, which are usually related with menses[24]. In our patient, symptoms relapsed irregularly and were not related with menses.

At present, superficial endometriosis is considered a normal phenomenon in women at the childbearing age, whereas deep infiltrative endometriosis (DIE) and endometrial ovarian cysts are the severe and painful manifestations of the condition[25]. DIE occurs in 30%-40% of the patients with endometriosis[26].

Pelvic pain, infertility and dyspareunia are the characteristic symptoms of the disease[25], but the clinical presentation is often non-specific.

Extra-pelvic endometriosis affects the GI tract of 5% of women with this condition[27]. The rectosigmoid is the most common site for intestinal endometriosis, accounting for 70% of all cases, while small bowel involvement, usually confined to the distal ileum, is less frequent (1%-7%) and exclusive localization on the ileum is very rare (1%-7%)[13]. Different incidence rates of endometriosis at different sites may be due to the fact that endometriosis is often an incidental finding at surgery[9].

In a review of 1000 women who underwent laparotomy for gynecological symptoms, Jubanyik *et al*[28] described 181 (18%) cases of GI endometriosis, but only one patient had small bowel involvement. Melody *et al*[29] reported distal ileum involvement in 35 out of 36 patients. In a radiologic study, Scarmato *et al*[30] detected endometriosis of the terminal and mid-ileum in four patients and one patient, respectively. Endometriosis in the jejunum[31] and proximal ileum has also been documented[32]. Anaf *et al*[20], considering bowel endometriosis an "infiltration or invasion phenomenon", found that there is a histological continuity between the superficial and underlying deep lesions of the large bowel wall, suggesting that lesions originating from the serosa progressively invade the muscularis propria. The mucosa is rarely involved as it is poorly innervated. Pelvic, pericolonic and para-aortic lymph node involvement of endometriosis has also been reported, often coexisting with endometriosis of the bowel wall[33]. Lymph node involvement may be a consequence of lymphatic dissemination from endometrial foci in the intestinal wall[34-35].

Symptoms are initially cyclical but may become permanent when the lesions progress.

It is difficult to establish a preoperative diagnosis of GI endometriosis, because GI tract symptoms can mimic a wide spectrum of diseases, including irritable bowel syndrome, infectious diseases, ischemic enteritis/colitis, inflammatory bowel disease and neoplasm[10,30,36-37]. GI endometriosis patients present with relapsing bouts of abdominal pain, abdominal distention, tenesmus, constipation and diarrhoea[9]. Rectal bleeding and pain during defecation may also occur[38].

Endometriosis infiltrating the muscularis propria may lead to localized fibrosis in the bowel wall, strictures, and small or large bowel obstruction[9-10].

The true incidence of endometriosis causing bowel obstruction is unknown[11], although complete obstruction of the bowel lumen occurs in less than 1% of cases[39].

Endometriosis of the distal ileum is an infrequent cause of intestinal obstruction, ranging from 7% to 23% of all cases with intestinal involvement[31,33,40-41].

The incidence of intestinal resection for bowel obstruction is 0.7% among patients undergone surgical treatment for abdominopelvic endometriosis[39].

In our case, as in others previously reported in the literature, it was impossible to establish a timely and accurate preoperative diagnosis for the vagueness of symptoms, similar to other cases of bowel obstruction.

However, endometriosis of the small bowel should be suspected in young, nulliparous patients with abdominal pain, in conjunction with signs of obstruction[12]. Mussa *et al*[42] reported a case of small bowel endometriosis with intestinal obstruction, protein-losing enteropathy and anasarca. Wong *et al*[43] described a case of endometriosis of the small bowel mimicking pancreatitis.

Rarely, intestinal endometriosis may occur with perforation[44-45]. Malignancy has been reported in 0.7%-1% of patients and 78.7% of the cases occur in the ovary[46]. The colorectum is involved in only 5% of patients[47].

The differential histologic diagnosis of endometrioid adenocarcinoma (AC) and colonic AC is difficult because colonic AC has a significant mucosal component, while endometrioid AC usually involves the outer layers of the colon[45,47-48]. Immunohistochemical staining for CK7 and CK20 seems to be useful in differentiating colonic and endometrioid AC[49-50]. Approximately, 75%-95% of primary colonic AC cases have a CK7-negative and CK20-positive phenotype, whereas 80%-100% of endometrial AC cases have a CK7-positive and CK20-negative phenotype[50]. Although endoscopic diagnosis of colonic endometriosis has been reported[51], the mucosa is usually normal or shows minimal mucosal abnormalities[45,47-48], friability[52], extrinsic process or fibrosed stenoses[53].

Rectal bleeding may be caused by mucosal injury during the passage of stools through a stenosed colon with the intramural endometriotic tissue increased at the time of menses if it occurs. Colonic mucosa heals rapidly and no signs are detectable at endoscopy[54].

Endoscopic biopsies usually yield insufficient tissue for a definitive pathologic diagnosis as endometriosis involves the deep layers of the bowel wall. Endometriosis can induce mucosal changes without any specific pattern, which mimic findings of other diseases such as inflammatory bowel disease, ischemic colitis or neoplasm[55].

Radiologically, lesions of endometriosis are either of

constricting and polypoid type or both[54]. On barium studies, radiographic findings caused by implants in the ileum are similar to those in the colon. Rectosigmoid or cecal endometriosis on double contrast barium enema studies is seen as an extrinsic mass with spiculation and tethering of folds[30,56].

The diagnosis of endometriosis may be suspected on the basis of the clinical history[30]. Less than 50% of patients have concurrent pelvic endometriosis[57].

CT is not the primary imaging modality for evaluation of bowel endometriosis, although it can occasionally demonstrate a stenosing rectosigmoid mass[58].

Multislice CT (MSCT) has a great potential for detecting alterations in the intestinal wall, especially if it is combined with enteroclysis (MSCTe). Biscaldi *et al*[59] carried out a study on 98 women with symptoms suggestive of colorectal endometriosis and MSCTe identified 94.8% of bowel endometriotic nodules.

Magnetic resonance imaging (MRI) has a high sensitivity (77%-93%) in the diagnosis of bowel endometriosis[60-61].

The depth of rectal wall infiltration by endometriosis is poorly defined by MRI. A combination of MRI and rectal endoscopic ultrasonography (EUS) has recently been proposed[62]. When retroperitoneal infiltration is present, it is mandatory to know if the bowel wall is involved in order to identify patients requiring bowel resection.

Both rectal EUS sensitivity and negative predictive value range from 92% to 100%. The specificity and positive predictive value are rather poor, which are 66% and 64%, 83% and 94%, respectively, as reported in two different studies[63-64].

There is a great interest in the use of serum markers to diagnose endometriosis, but they are not sufficiently accurate for use in clinical practice[65].

Cancer antigen CA-125 has been used to monitor the progress of endometriosis[66]. CA19-9 has a lower sensitivity than CA-125, and cytokine interleukin-6 may be more sensitive and specific than CA-125.

Surgery is the choice of treatment for intestinal endometriosis in most cases. For the accidental finding without symptoms of obstruction, hormone therapy with danazol or gonadotrophin-releasing hormone (GnRH) analogs may be considered[9]. Surgical treatment should be indicated for women with pain, bleeding, changes in bowel habits and intestinal obstruction[51]. In the small bowel, the treatment of endometriosis is surgical resection of the involved bowel, while medical therapy is only a temporary treatment[12].

Intestinal endometriosis may be active in the peri- and post- menopausal years and even surgery may be necessary for these patients[54].

In this paper, we report an unusual presentation of endometriosis characterized by abrupt onset of small bowel occlusion. The present report points out that endometriosis remains a challenging condition for clinicians, especially, as in our case, when the symptoms are not related to menses. Intestinal endometriosis should be considered in patients with epigastric, abdominal and/or pelvic pain, in conjunction with signs of obstruction.

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CASE REPORT

Simultaneous laparoscopy-assisted low anterior resection and distal gastrectomy for synchronous carcinoma of rectum and stomach

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Abstract

Laparoscopic resection of rectal cancer or gastric cancer has been advocated for the benefits of a reduced morbidity, a shorter treatment time, and similar outcomes. However, simultaneous laparoscopy-assisted low anterior resection and distal gastrectomy for synchronous carcinoma of rectum and stomach are rarely documented in literature. Endoscopic examination revealed a synchronous carcinoma of rectum and stomach in a 55-year-old male patient with rectal bleeding and epigastric discomfort. He underwent a simultaneous laparoscopy-assisted low anterior resection and distal gastrectomy with regional lymph nodes dissected. The operation time was 270 min and the estimated blood loss was 120 mL. The patient required parenteral analgesia for less than 24 h. Flatus was passed on postoperative day 3, and a solid diet was resumed on postoperative day 7. He was discharged on postoperative day 13. With the advances in laparoscopic technology and experience, simultaneous resection is an attractive alternative to a synchronous gastrointestinal cancer.

Key words: Laparoscopy; Gastric cancer; Rectal cancer; Simultaneous resection; Synchronous carcinoma

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INTRODUCTION

Rectal and gastric cancers are sometimes diagnosed simultaneously as a synchronous carcinoma of rectum and stomach in a single patient. An enlarged open procedure combining both rectal resection and gastrectomy with a curable intention is normally performed for such cases, but a long operation time and severe surgical trauma often result in a reluctant recovery.

Laparoscopic resection is feasible and safe for both rectal and gastric cancers nowadays. It has been widely advocated for the benefits of a reduced morbidity and a shorter hospitalization time without sacrificing the oncological outcome. As this novel technique permits a multiple segmental resection, simultaneous laparoscopy-assisted low anterior resection and distal gastrectomy for a single patient have become attractive to a synchronous carcinoma of rectum and stomach. We present such a case.

CASE REPORT

A 55-year-old male patient presented with rectal bleeding and epigastric discomfort with alternation in bowl habit, mucus, and weight loss for 6 mo. Colonoscopy revealed a circumferential mass 8 cm from the anal verge, which was confirmed to be a rectal adenocarcinoma by biopsy. Meanwhile, gastroscopy revealed a 3 cm × 3 cm ulcerative lesion located at the lesser curvature near the

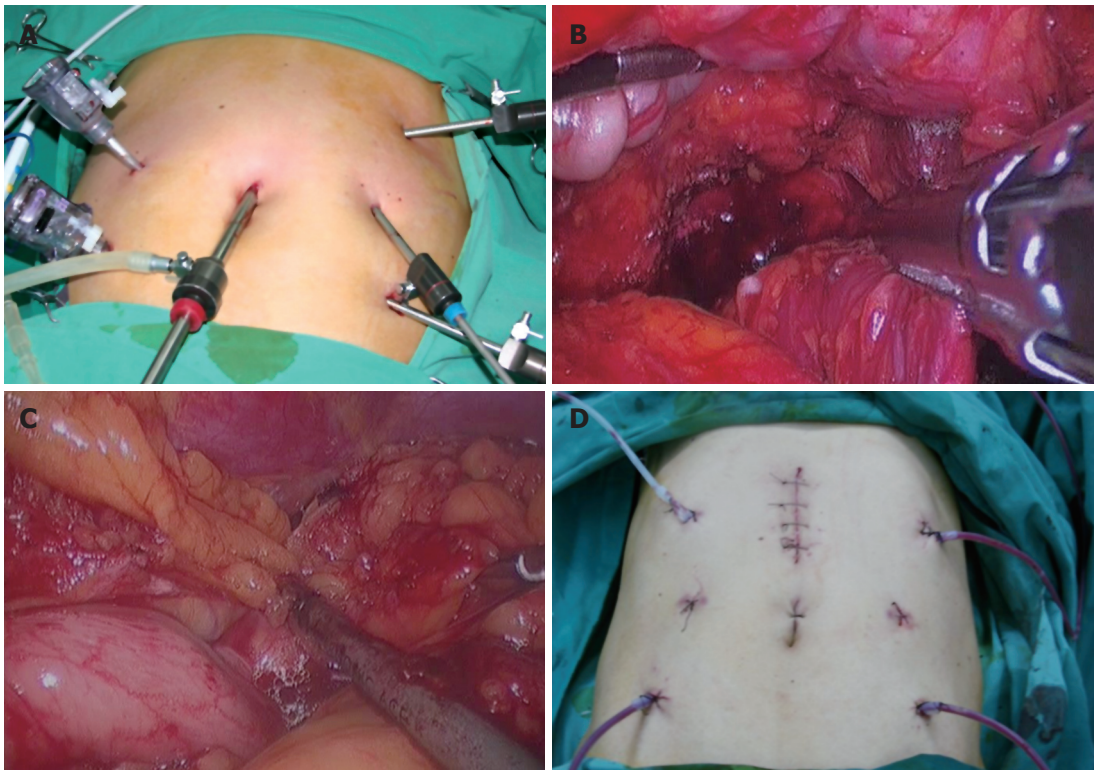

Figure 1 **A**: Position of the working ports; **B**: Transection of the rectum with a linear stapler; **C**: Dissection of short gastric vessels with harmonic scalpel; **D**: Placement of drainage tubes.

pylorus, which was confirmed to be a signet-ring cell adenocarcinoma by biopsy. Computed tomography (CT) of the abdomen and thorax demonstrated no significant metastatic lesions. Various treatment strategies were discussed and the patient underwent simultaneous laparoscopy-assisted low anterior resection and distal gastrectomy (LADG) and D2 lymphadenectomy.

The operation was performed under general anesthesia. The patient was first at a Lloyd-Davis position to accomplish the low anterior resection, and then changed into a Trendelenberg position to complete the distal gastrectomy. We used the Veress method to establish pneumoperitoneum and maintain the intra-abdominal pressure at around 15 mmHg. Trocars for low anterior resection were placed. In brief, an umbilical port (10 mm) was used for a video scope (usually a 30-degree scope), and two ports were created for working in the right and left low quadrants of abdomen, with the lower port in the right quadrant for main working (12 mm) and the rest three for assistance (5 mm). When gastrectomy was performed, another 5 mm assistant port was made in the left subcostal region (Figure 1A). The main surgeon stood on the right of the patient and then between the split legs of the patient after completion of the low anterior resection.

The right peritoneum was dissected along the iliac vessels with a harmonic scalpel, then the sigmoid colon and rectum as well as their mesenteries were mobilized down to the pelvic floor. The lymphovascular pedicle was ligated at the radical site of the inferior mesenteric vessel with a polymer plastic clip, while the distal rectum was transected intracorporeally with a laparoscopic linear stapler (Figure 1B). The first part of the operation was completed within 75 min.

The gastrocolic ligament was divided from the hepatic flexure towards the splenic flexure for gastric mobilization, while the gastroepiploic vessels and short gastric vessels were ligated simultaneously (Figure 1C). The gastrohepatic and duodenohepatic ligaments were then divided to allow ligation of the right and left gastric vessels. After completion of the whole procedure, the proximal stomach was entirely mobilized with an adequate perigastric lymphadenectomy. This part of the operation was accomplished within 2 h and 30 min, and the estimated blood loss was 120 mL.

A 5 cm median epigastric incision was made and protected with a plastic bag to ensure the transection of the two specimens. The bowels were then anastomosed intra-corporeally with a double stapler. The first portion of the duodenum and stomach body was transected with the gastrointestinal continuity performed in a Billroth-I fashion. Four protective drainage tubes were placed near the two anastomotic junctions (Figure 1D). The total operation time was 4 h and 30 min. The patient received parenteral analgesia for less than one day. Flatus was passed on postoperative day 3, the patent resumed a liquid diet on postoperative day 5 and a solid diet on postoperative day 7. No clinically significant complication occurred postoperatively and all the drainage tubes were pulled out consecutively. The patient was discharged 13 d after the operation. Pathologic examination of the rectum showed a moderately differentiated adenocarcinoma invading the subserosal tissue without breaching the serosa. The distal margin measured 2 cm, and no involvement of tumor was found in the mesenteric or paracolic lymph nodes. Pathological examination of the

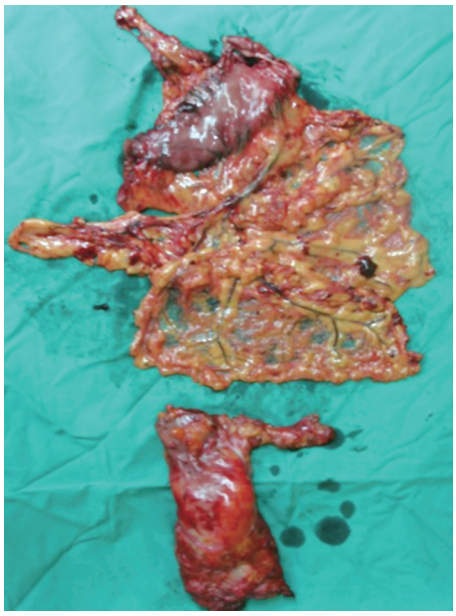

Figure 2 Specimens obtained at distal gastrectomy, regional lymphadenectomy and low anterior resection from their anatomical sites.

stomach showed a poorly differentiated adenocarcinoma invading the muscular layer, involvement of tumor was observed in 8 of the 20 lymph nodes (Figure 2).

DISCUSSION

Synchronous gastrointestinal cancers are normally treated with conventional surgical approaches. In brief, a long medial incision from the xiphoid to the pubic symphysis is made to ensure the upper-abdominal and pelvic procedures, with a dissection of regional lymph nodes. These maneuvers might cause surgical traumas, and result in a slow postoperative recovery, with a negative influence on the prognosis of tumors. Laparoscopic technique provides a simultaneous resection of the rectal and gastric lesions and minimizes the surgical influence on human body. For the rectal procedure, the amplifying effect of laparoscopy helps the surgeons to identify and protect the ureter and automatic nerve plexus in a narrow pelvic cavity in spite of a bulky tumor and thickened mesentery. A 30-degree camera offers a multi-angle image, which greatly assists the surgeon to complete a TME approach as well as the intra-corporeal transection and anastomosis of the bowels[1]. For the gastric procedure, advanced laparoscopic instruments and materials, such as harmonic scalpel, multi-sized titanium or polymer clips significantly reduce the intra-operative hemorrhage. A broad view of the intra-peritoneal cavity under laparoscope facilitates the multi-plane manipulation of gastrectomy, especially during the dissection of the upper branches of perigastric vessels like short gastric vessels[2]. We transected the two specimens and reconstructed the upper gastrointestinal duct extra-corporeally with a manual method, which could obviously reduce the operation time and cost. To summarize, laparoscopic colorectal and gastric surgery is feasible and safe in clinical trials with the benefit of avoiding a second operation, thus reducing morbidity, and shortening the hospital stay without sacrificing the oncological outcome[3,4]. As some other radical surgeries for synchronous gastrointestinal cancers have also been reported, simultaneous laparoscopic approach obviously reduces the morbidity and recurrence rate[5,6].

To the best of our knowledge, this is the first report of simultaneous laparoscopy-assisted low anterior resection and distal gastrectomy for synchronous carcinoma of the rectum and stomach. A good recovery of the patient reveals that this technique is advantageous over the conventional approaches in treating gastrointestinal malignancies and broadens the indications for laparoscopic approaches. This minimally invasive technique may have a bright future.

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LETTERS TO THE EDITOR

Hepatic encephalopathy in patients with liver cirrhosis: Is there a role of malnutrition?

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Abstract

Hepatic encephalopathy (HE) is a common complication in patients with liver cirrhosis but its pathogenesis remains incompletely understood. Malnutrition is commonly encountered in patients with liver cirrhosis and it has been reported to affect the quality of life of this group of patients. Experimental studies suggest that low energy intake and poor nutritional status may facilitate the development of HE but there are scarce data on the potential role of malnutrition in HE in patients with liver cirrhosis. Two recently published studies have evaluated the potential role of malnutrition in the development of HE in cirrhotic patients with conflicting results. In this letter to the editor we briefly present the results of the two studies as well as potential reasons for the conflicting results reported.

Key words: Hepatic encephalopathy; Liver cirrhosis; Malnutrition

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TO THE EDITOR

The pathogenesis of hepatic encephalopathy (HE), a common complication of liver cirrhosis, remains incompletely understood but it is probably multifactorial in most cases[1]. Malnutrition is also commonly encountered in patients with cirrhosis and it has been reported to have an effect on health related quality of life[2]. Although experimental studies suggest that low energy intake and poor nutritional status may facilitate the development of HE[3], there are scarce data on the potential role of malnutrition in HE in patients with liver cirrhosis.

Recently Soros *et al*[4] performed a study investigating the potential role of malnutrition and hypermetabolism in HE in which 223 patients with non-alcoholic cirrhosis were enrolled. They were evaluated for the presence of HE according to the West Haven criteria and for malnutrition by means of body mass index (BMI), anthropometric measurements, and bioelectrical impedance analysis. Energy metabolism was also assessed by means of indirect calorimetry. Eighty-five (38%) out of 223 patients had no clinically evident HE, 123/223 (55%) had HE grade 1 and 15/223 (7%) had HE grade 2 or 3. Neither metabolic variables or BMI nor fat free mass or muscle mass differed significantly in patients with HE grade 1-3 from those without HE. In multivariate analysis none of these parameters was found to be independently related to HE. The authors concluded that malnutrition or catabolism does not seem to be independent risk factors for the presence of HE in patients with liver cirrhosis[4].

Recently, we performed a prospective study evaluating HE in 128 patients with liver cirrhosis of various etiologies[5]. HE was evaluated by means of the West Haven criteria and two psychometric tests (number connection test A and B). HE was defined as overt HE according to the West Haven criteria and/or number connection test A and/or B > 3 standard deviations of the general population. Nutritional status was evaluated with BMI and anthropometric measurement as well as estimation of recent weight change. Malnutrition was defined as anthropometric measurement below the 5th

percentile according to standard values for the general population and/or BMI < 20 kg/m^2 and/or weight loss ⩾ 5%-10% in the previous 3-6 mo. The presence of diabetes mellitus was also assessed with fasting glucose measurement. Forty percent of our patients were malnourished, 26% had diabetes, and 34% had HE. Patients with malnutrition suffered more frequently from HE compared to those without malnutrition (46% *vs* 27%, $P = 0.031$) , but there was no difference in age, etiology, or severity of liver cirrhosis. In multivariate analysis, the time needed to perform number connection test A was independently correlated to age, severity of cirrhosis expressed as the Child-Pugh score, diabetes and malnutrition[5]. This is in agreement with a previous study showing that diabetes mellitus is associated with HE in patients with hepatitis C cirrhosis[6].

In the paper of Soros *et al*[4], they did not report how many patients had diabetes mellitus. However, the risk of diabetes mellitus has been reported to be increased in patients with cirrhosis due to hepatitis C[7] and the majority of patients enrolled in the study of Soros *et al*[4] (56%) had viral cirrhosis[4]. It is therefore unknown whether the patients with HE had a higher proportion of diabetes compared with the patients without HE. This might have had an effect on the median BMI in the two groups as diabetes is more prevalent in patients with increased BMI, thus accounting for the lack of difference in median BMI between patients with HE and those without HE[4]. In fact, the BMI of patients with HE ranged from 14.5 to 36.3 kg/m^2 as compared to 17.5-28.4 kg/m^2 in those without HE[4]. Furthermore, in our study, recent weight change was included in the definition of malnutrition[4] whereas in the study of Sörös *et al* no definition of malnutrition was provided[4]. Interestingly we found that although patients with and without low fat or muscle mass did not differ in number connection A performance times, a recent weight loss was related to longer performance times [81 s (51) *vs* 54 s (32), $P = 0.001$][5]. It is therefore conceivable that deterioration in nutritional status, rather than nutritional status itself, may be of great importance for cognitive dysfunction in patients with liver cirrhosis. Finally, another factor that may, at least in part, explain the differences between the results of the two studies[4,5] is that we also included patients with minimal HE in our analyses[5] whereas as Soros *et al*[4] included only patients with clinically overt HE in their study.

In conclusion, methodological differences regarding the definitions of HE and malnutrition as well as the assessment of the role of diabetes mellitus in cognitive dysfunction may explain the differences in the results of the two studies[4,5]. As both studies had limitations mentioned by their authors[4,5] and the pathophysiology of HE is complex, it is clear that further studies are warranted to fully delineate the potential role of malnutrition in cognitive dysfunction in patients with liver cirrhosis.

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Meetings

Events Calendar 2008-2009

FALK SYMPOSIA 2008
January 24-25, Frankfurt, Germany
Falk Workshop: Perspectives in Liver
Transplantation

International Gastroenterological Congresses 2008
February 14-16, Paris, France
EASL-AASLD-APASL-ALEH-IASL Conference Hepatitis B and C virus resistance to antiviral therapies
www.easl.ch/hepatitis-conference

February 14-17, Berlin, Germany
8th International Conference on New Trends in Immunosuppression and Immunotherapy
www.kenes.com/immuno

February 28, Lyon, France
3rd Congress of ECCO - the European Crohn's and Colitis Organisation
Inflammatory Bowel Diseases 2008
www.ecco-ibd.eu

February 29, Québec, Canada
Canadian Association of Gastroenterology
E-mail: general@cag-acg.org

March 10-13, Birmingham, UK
British Society of Gastroenterology Annual Meeting
E-mail: BSG@mailbox.ulcc.ac.uk

March 14-15, HangZhou, China
Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea
Asian Pacific Association for the Study of the Liver
18th Conference of APASL: New Horizons in Hepatology
www.apaslseoul2008.org

March 29-April 1, Shanghai, China
Shanghai-Hong Kong International Liver Congress
www.livercongress.org

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco
OESO 9th World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation-Management of Adeno-carcinomas
E-mail: robert.giuli@oeso.org

April 9-12, Los Angeles, USA
SAGES 2008 Annual Meeting - part of Surgical Spring Week
www.sages.org/08program/html/

April 18-22, Buenos Aires, Argentina
9th World Congress of the International Hepato-Pancreato Biliary Association
Association for the Study of the Liver
www.ca-ihpba.com.ar

April 23-27, Milan, Italy
43rd Annual Meeting of the European Association for the Study of the Liver
www.easl.ch

May 2-3, Budapest, Hungary
Falk Symposium 164: Intestinal Disorders

May 18-21, San Diego, California, USA
Digestive Disease Week 2008

May 21-22, California, USA
ASGE Annual Postgraduate Course
Endoscopic Practice 2008: At the Interface of Evidence and Expert Opinion
E-mail: education@asge.org

June 4-7, Helsinki, Finland
The 39th Nordic Meeting of Gastroenterology
www.congrex.com/ngc2008

June 5-8, Sitges (Barcelona), Spain
Semana de las Enfermedades Digestivas
E-mail: sepd@sepd.es

June 6-8, Prague, Czech Republic
3rd Annual European Meeting: Perspectives in Inflammatory Bowel Diseases
E-mail: meetings@imedex.com

June 10-13, Istanbul, Turkey
ESGAR 2008 19th Annual Meeting and Postgraduate Course
E-mail: fca@netvisao.pt

June 11-13, Stockholm, Sweden
16th International Congress of the European Association for Endoscopic Surgery
E-mail: info@eaes-eur.org

June 13-14, Amsterdam, Netherlands
Falk Symposium 165: XX International Bile Acid Meeting. B ile Acid Biology and Therapeutic Actions

June 13-14, Prague, Czech Republic
Central and Eastern European Conference on Colorectal "Cancer" Screening, Prevention and Management
E-mail: idca2008@guarant.cz

June 25-28, Barcelona, Spain
10th World Congress on Gastrointestinal Cancer
Imedex and ESMO
E-mail: meetings@imedex.com

June 25-28, Lodz, Poland
Joint Meeting of the European Pancreatic Club (EPC)
and the International Association of Pancreatology (IAP)
E-mail: office@epc-iap2008.org
www.e-p-c.org
www.pancreatology.org

June 26-28, Bratislava, Slovakia
5th Central European Gastroenterology Meeting
www.ceurgem2008.cz

July 9-12, Paris, France
ILTS 14th Annual International Congress
www.ilts.org

September 10-13, Budapest, Hungary
11th World Congress of the International Society for Diseases of the Esophagus
E-mail: isde@isde.net

September 13-16, New Delhi, India
Asia Pacific Digestive Week
E-mail: apdw@apdw2008.net

III FALK GASTRO-CONFERENCE
September 17, Mainz, Germany
Falk Workshop: Strategies of Cancer Prevention in Gastroenterology

September 18-19, Mainz, Germany
Falk Symposium 166:
GI Endoscopy - Standards & Innovations

September 18-20, Prague, Czech Republic
Prague Hepatology Meeting 2008
www.czech-hepatology.cz/phm2008

September 20-21, Mainz, Germany
Falk Symposium 167:
Liver Under Constant Attack - From Fat to Viruses

September 24-27, Nantes, France
Third Annual Meeting
European Society of Coloproctology
www.escp.eu.com

October 8-11, Istanbul, Turkey
18th World Congress of the International Association of Surgeons,
Gastroenterologists and Oncologists
E-mail: orkun.sahin@serenas.com.tr

October 18-22, Vienna, Austria
16th United European Gastroenterology Week
www.negf.org
www.acv.at

October 22-25, Minnesota, USA
Anstralian Gastroenterology Week 2008
E-mail: gesa@gesa.org.au

October 22-25, Brisbane, Australia
71st Annual Colon and Rectal Surgery Conference
E-mail: info@colonrectalcourse.org

October 31-November 4, Moscone West Convention Center, San Francisco, CA
59th AASLD Annual Meeting and Postgraduate Course
The Liver Meeting
Information: www.aasld.org

November 6-9, Lucerne, Switzerland
Neurogastroenterology & Motility Joint International Meeting 2008
E-mail: ngm2008@mci-group.com
www.ngm2008.com

November 12, Santiago de Chile, Chile
Falk Workshop: Digestive Diseases: State of the Art and Daily Practice

November 28-29, Cairo, Egypt
1st Hepatology and Gastroenterology Post Graduate Course
www.egyptgastrohep.com

December 7-9, Seoul, Korea
6th International Meeting
Hepatocellular Carcinoma: Eastern and Western Experiences
E-mail: sglee@amc.seoul.kr

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E-mail: symposia@falkfoundation.de
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N.O.T.E.S
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Laparoscopic Digestive Surgery

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Interventional GI Endoscopy Techniques
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E-mail: bsg@mailbox.ulcc.ac.uk

May 17-20, Denver, Colorado, USA
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In press

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]

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]

No author given

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

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment

of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325]

Issue with no volume

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]

No volume or issue

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

Books

Personal author(s)

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

Chapter in a book (list all authors)

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

Author(s) and editor(s)

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

Conference proceedings

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

Conference paper

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)

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/eid.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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