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FRONTIER

Advances in traction methods for endoscopic submucosal dissection: What is the best traction method and traction direction?

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Abstract

Endoscopic submucosal dissection (ESD) has been developed as a treatment for superficial gastrointestinal neoplasms, which can achieve en bloc resection regardless of the lesion size. However, ESD is technically difficult because endoscopists cannot bring their hand into the gastrointestinal tract, unlike surgeons in regular surgery. It is difficult to obtain sufficient tension in the dissection plane and a good field of vision. Therefore, ESD is associated with a long procedure time and a high risk of adverse events in comparison with endoscopic mucosal resection. Traction methods have been developed to provide sufficient tension for the dissection plane and a good field of vision during the ESD procedure. However, traction direction is limited in most traction methods, resulting in insufficient effect in some cases. Although traction direction is considered important, there have been few investigations of its effect. In the first half of this review, important traction methods are discussed, including traction direction. In second half, appropriate traction methods for each organ are considered. Other important considerations for traction method, such as ability to adjust traction strength, interference between traction device and endoscope, and the need for specialized devices are also discussed.

Key Words: Endoscopic submucosal dissection; Traction method; Countertraction; Traction direction; Vertical traction

Core Tip: Endoscopic submucosal dissection is associated with a long procedure time and adverse events (*e.g.*, perforation) due to technical difficulty—the absence of tension for the dissection plane and poor field of vision. Traction methods allow efficient dissection and a good field of vision. Although many traction methods have

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been developed, traction direction is limited in most. Each traction method has advantages and disadvantages. It is important to select an appropriate traction method to obtain proper traction direction, depending on lesion location. We discuss the characteristics of different traction methods and their effects depending on traction direction.

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INTRODUCTION

Endoscopic submucosal dissection (ESD) allows en bloc resection, regardless of lesion size, where endoscopic mucosal resection (EMR) is considered impossible, enabling accurate pathological assessment and a lower recurrence[1]. However, ESD is still a challenging therapy due to technical difficulty, which results in long procedure time and high perforation rate[2]. Surgeons may use the nondominant hand to provide traction for the lesion while they dissect using the dominant hand. By contrast, endoscopists cannot use their nondominant hand to provide traction for the lesion during ESD because they cannot put their hand into the gastrointestinal tract—it is like cutting a steak using only a knife, with no fork. It is important to obtain traction during ESD because this enables two important effects: creating a visual field by turning over the mucosal flap, and facilitating dissection by providing tension for the dissection plane. A basic hood attached to the endoscope can be used to obtain traction. However, this is occasionally insufficient.

The clip-with-line method (Figure 1), which may be the first traction method ever used, was reported in 2002[3,4]. Many other methods have since been developed. Although the clip-with-line method is simple and low cost, its traction direction is limited to the direction in which the line is pulled. In a multicenter prospective randomized controlled trial comparing the conventional and the clip-with-line methods, the clip-with-line method did not demonstrate a reduction in procedure time for gastric ESD[5] but did for esophageal ESD[6]. These results suggest the efficacy of the traction method is different depending on traction direction, because traction direction in the clip-with-line method is limited to the direction toward the endoscope in esophageal ESD, while it changes depending on the lesion location in gastric ESD.

Unlike the clip-with-line method, several other traction methods can provide traction in any direction. These include the internal traction method, which uses an spring-and-loop with clip (S-O clip; Zeon Medical, Tokyo, Japan)[7-10] (Figure 2). We reported a single-center prospective randomized controlled trial comparing the conventional and the S-O clip-assisted methods in gastric ESD, which demonstrated that the S-O clip-assisted method reduced the median gastric ESD procedure time (29.1 min *vs* 52.6 min; $P = 0.005$)[11]. In this study, a direction vertical to the gastric wall was selected for the S-O clip-assisted method, using its multidirectional traction function.

These outcomes suggest that traction direction is the important factor for traction-assisted ESD. However, little study has been done to explore the influence of traction direction during the procedure. Each traction method has characteristics other than traction direction, and it is necessary to understand the characteristics in order to use the methods effectively. The purpose of this article is to review the characteristic of traction methods. Then follows a discussion of appropriate traction methods for each organ, based on the results of clinical trials.

BASICS OF TRACTION

Definition of traction

In published literature of ESD, the terms traction and countertraction are used interchangeably; the unclear distinction results in a potential for confusion[12]. In this

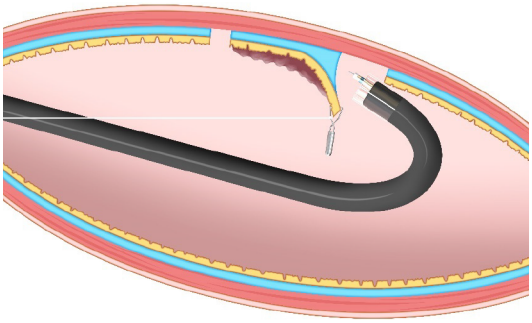

Figure 1 Clip-with-line method. This method provides traction for the lesion by pulling the line. The traction direction is limited to the direction in which the line is pulled.

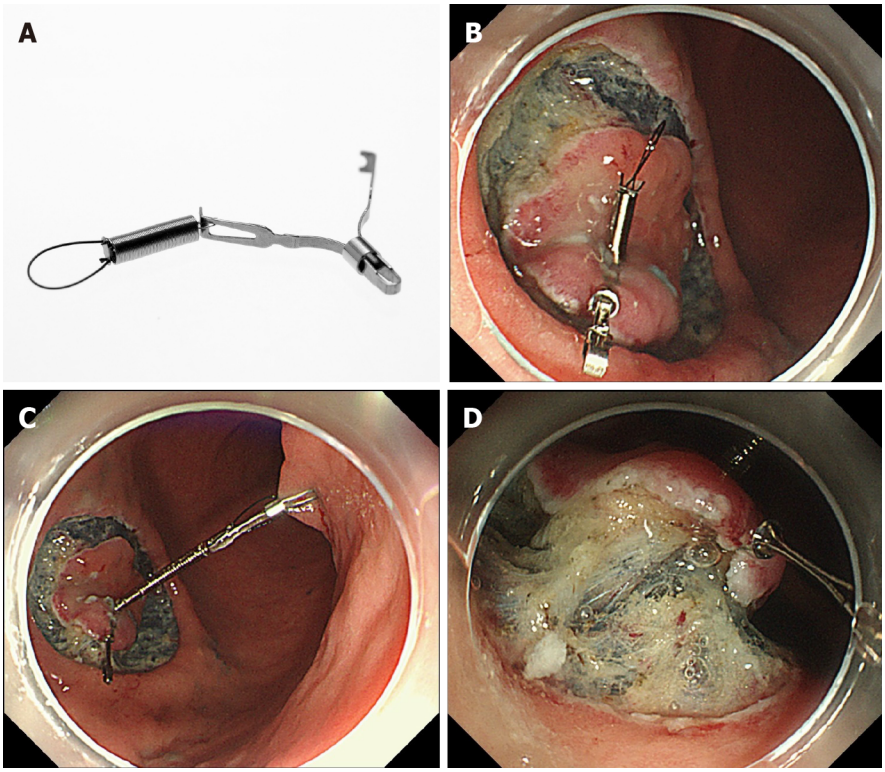

Figure 2 Internal traction method using the spring-and-loop with clip (Zeon Medical, Tokyo, Japan). A: The spring-and-loop with clip (S–O clip) has a 5-mm long spring and a 4-mm long loop at one side of the clip claws; B: The S–O clip is attached to the lesion; C: The regular clip anchors the loop of the S–O clip on the opposite side of the lesion; D: The extension of the spring provides traction on the lesion. Citation: Mitsuru Nagata. Internal traction method using a spring-and-loop with clip (S–O clip) allows countertraction in gastric endoscopic submucosal dissection. *Surg Endosc* 2020; 34(8): 3722–3733. Copyright © 2020 Mitsuru Nagata[10].

article, we do not use the term countertraction. We define traction as force acting on the target lesion.

Classification of traction direction

As a force, traction can be represented by a vector, characterized by size and direction. Traction direction can be divided into the following five categories, according to the relationship with the endoscope tip and the gastrointestinal wall: Proximal, diagonally proximal, vertical, diagonally distal, and distal (Figure 3). Of these five categories, vertical traction may be appropriate in any situation because it provides two important effects: Enabling visualization of the submucosa, by turning over the mucosa; and facilitating submucosal dissection by providing tension to submucosa (Figure 3A).

Proximal traction can provide sufficient tension to the submucosa. However, the mucosal flap falls toward the endoscope (Figure 3B). If the endoscope tip is not parallel to the gastrointestinal wall, it can be difficult to approach the submucosa, even

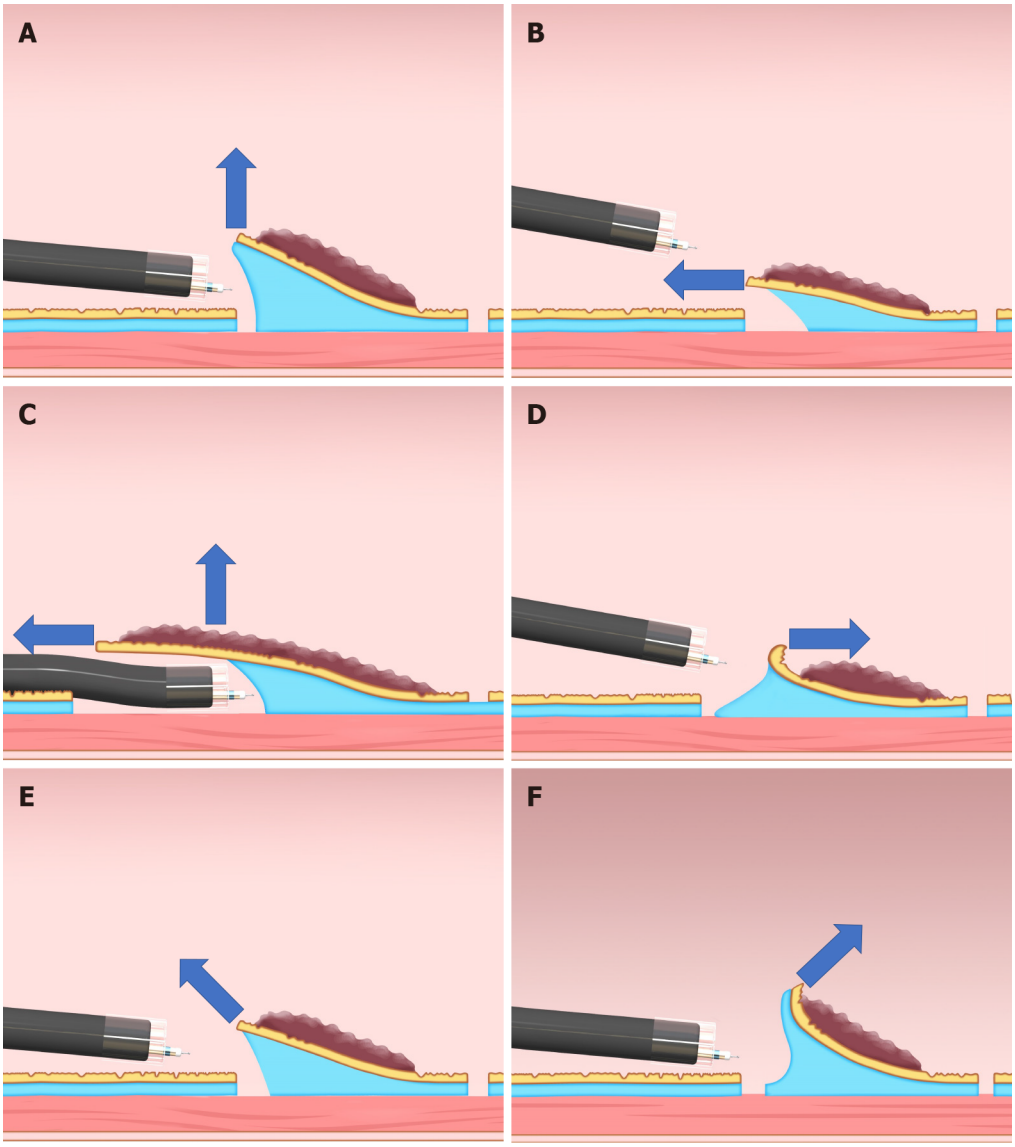

Figure 3 Classification of the traction direction. A: Vertical traction; B: Proximal traction; C: Proximal traction combined with hood traction; D: Distal traction; E: Diagonally proximal traction; F: Diagonally distal traction.

with proximal traction. If the endoscope tip is parallel to the gastrointestinal wall, it is easy to approach the submucosa, even if the mucosal flap falls down. Moreover, once the endoscope tip gets under the mucosal flap, proximal traction is combined with hood traction, resulting in diagonally proximal or vertical traction (Figure 3C). Proximal traction is suitable for situations where the endoscope tip can be placed parallel to the gastrointestinal wall, for example, in esophageal ESD.

Distal traction can cause the submucosal dissection plane to fall distally as submucosal dissection advances, resulting in submucosal thinning and subsequently, cutting the muscle layer or mucosa because of misrecognition of the layer (Figure 3D). Moreover, distal traction may decrease the effectiveness of the tension for the submucosal dissection plane, leading to inefficient dissection. Hence, distal traction may be the least useful approach for submucosal dissection in most cases.

Diagonally proximal traction (Figure 3E) and diagonally distal traction (Figure 3F) can be decomposed into horizontal and vertical vectors. The larger the horizontal component, the closer to the proximal or distal traction. The larger the vertical component, the closer to vertical traction.

MODALITY OF TRACTION

Traction can be roughly classified into hood traction, natural traction, and device-

assisted traction. Natural traction and device-assisted traction can be further subdivided.

Hood traction

A hood attached to the endoscope tip is used in ESD procedures primarily to secure the visual field. The hood also can be used to obtain traction; it can turn the mucosal flap and provide tension for the submucosa after the endoscope tip is inserted under the mucosa. The straight hood has a wide field of view but is sometimes difficult to get under the mucosa. A hood with a tapered tip may be effective in such a situation (Figure 4)[13,14]. However, in a situation where it is difficult to keep the endoscope tip under the mucosa, such as in severe submucosal fibrosis, substantial lesion movement (due to patient respiration), and vertical confrontation with the lesion, the hood alone is not effective.

Natural traction

Natural traction is defined as traction using natural power, such as gravity, mucosal tension, buoyancy, and water pressure. The advantage of natural traction is that it is easy to switch to other methods and there is no need for any special device.

Gravity: When the lesion is gravitationally upward, gravity keeps the mucosal flap open and provides tension for the submucosa (Figure 5). Changing the patient's posture to raise the lesion against gravity is the basic strategy for ESD. However, in esophageal, gastric, and duodenal ESD, the patient's posture is primarily the left lateral decubitus position, which is difficult to change. By contrast, it is easy to change the patient's posture in colorectal ESD (*e.g.*, left lateral, supine, right lateral, and prone positions). However, changing the patient's posture sometimes makes the ESD procedure complicated, for example, through poor maneuverability of the endoscope, a vertical approach to the lesion, and difficulty opening the lumen.

Mucosal tension, pocket creation method, and endoscopic submucosal tunnel dissection: When the mucosa around the lesion is incised circumferentially, the lesion loses tension from the surrounding mucosa and submucosa, making it difficult to get the endoscope tip under the mucosal flap. By leaving a part of the mucosa around the lesion, the remaining mucosa gives tension to the lesion. In conventional ESD, traction at the lesion can be maintained by using mucosal tension, as follows. A C-shaped or inverted C-shaped mucosal incision is made. Next, the submucosa under the lesion is dissected, while the remaining mucosa gives tension at the dissection plane. Finally, a circumferential mucosal incision is made and the remaining submucosa dissected.

The pocket creation method (PCM) and endoscopic submucosal tunnel dissection (ESTD) use the same principle, using mucosal tension for traction[15-19]. In PCM (Figure 6), an initial mucosal incision on the proximal side of the lesion is first performed, to make entry to the submucosa. Then, the submucosa under the lesion is dissected, followed by creation of a submucosal space. Finally, the mucosa and submucosa around the submucosal space is dissected to achieve en bloc resection. In ESTD, a mucosal incision on the distal side of the lesion is performed before creation of a submucosal space is completed, unlike the PCM procedure.

PCM and ESTD procedures have similar advantages, as follows. The endoscope inside the submucosal space provides tension for the dissection plane. The endoscope tip can take a parallel approach to the muscle layer. The submucosal space holds the endoscope, which achieves stabilization of the endoscope. Thus, PCM and ESTD may be particularly suitable for lesions that are located where maneuverability of the endoscope is poor. Moreover, minimal mucosal incision until completion of submucosal dissection may prevent leakage of the injected solution.

Buoyancy and water pressure: We have reported the usefulness of underwater ESD for buoyancy, easier use of water pressure from an endoscope that has a water supply function, clear visual field, and heat sink effect[20,21]. Buoyancy and water pressure can provide a traction function, where buoyancy acts opposite to gravitational pull.

In conventional ESD, when the lesion is located at the gravitationally lower side, the opening of the mucosal flap is obstructed by gravity. Moreover, the lesion is half-way submerged due to gravity, while the boundary between gas and liquid obstructs the visual field (Figure 7A and B). By switching from conventional ESD to underwater ESD, buoyancy aids opening of the mucosal flap and provides tension for the submucosa, while the visual field is unaffected by a gas-liquid boundary (Figure 7C and D). Water pressure from the endoscope (using its water supply function) also assists opening the mucosal flap. Whereas water pressure can temporarily deteriorate

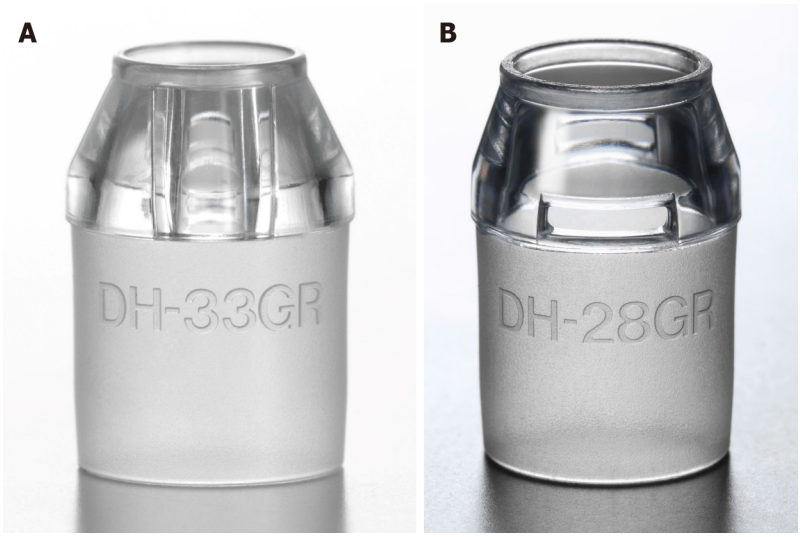

Figure 4 Small-caliber tip transparent hood (Fujifilm, Tokyo, Japan). A: DH-33GR [small-caliber tip transparent (ST) hood] with 7-mm tip opening diameter and 7-mm tip protruding length; B: DH-28GR (short ST hood) with 8-mm tip opening diameter and 7-mm tip protruding length.

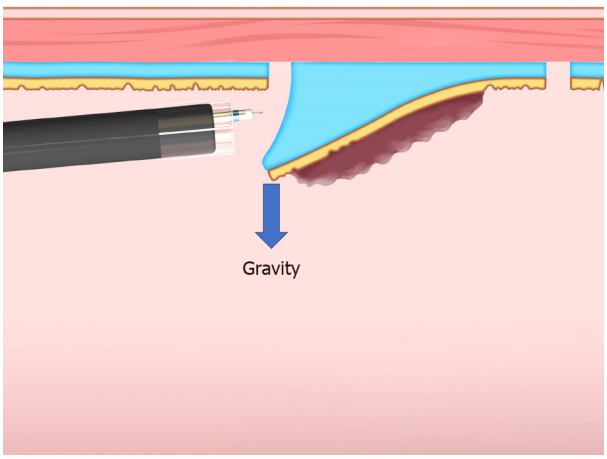

Figure 5 Gravity provides favorable traction when the lesion is located at the upper side of the gravitational force.

the visual field, due to splashing under gas insufflated conditions, it can be used without splashing in underwater conditions, allowing seamless submucosal dissection.

Saline solution is preferable for underwater ESD due to saline solution's higher specific gravity, compared with water, which provides a greater flotation effect.

Device-assisted traction method

A traction method using a device other than a hood can be defined as device-assisted. Device-assisted traction method implies traction in the narrow sense. They are broadly classified into external, internal, and other methods; each of these is further sub-classified (Table 1).

External traction methods: External traction can be defined as a method where the traction device acts from outside the body. Representative external traction method includes the clip-with-line method, pulley method, sheath traction method, external forceps method, double scope method, and magnetic anchor method.

Clip-with-line method: The clip-with-line method was reported by Oyama *et al*[3,4] in 2002 (Figure 1). It is performed as follows. After circumferential mucosal incision, the endoscope is withdrawn. The clip applicator is deployed into the accessory channel of the endoscope. The clip-with-line (a clip with a line tied to its arm) is attached to the clip applicator. The endoscope is inserted, then the clip-with-line is attached to the edge of the lesion. Using this procedure, the line comes out of the body without passing through the accessory channel of the endoscope, while pulling the line provides traction at the lesion.

Table 1 Classification of device-assisted traction method

	Traction direction	Control of traction force	Withdrawal of the endoscope	Recommended lesion location
External traction methods				
Clip-with-line method	One	Strengthen	Required	Esophagus, Greater curvature of the upper and middle third of the stomach, Colorectum
Pulley method	Any	Strengthen	Required	unclear because of fewer reports
Sheath traction method				
Clip-and-snare method	Two	Strengthen and weaken	Required	Stomach, Rectum
Endo Trac	Two	Strengthen and weaken	Required	Stomach, Rectum
External forceps method	Two	Strengthen and weaken	Required	Esophagus; Stomach except for cardia, lesser curvature or posterior wall of the upper gastric body; Rectum
Double scope method	Any	Strengthen and weaken	None	Stomach
Magnetic anchor method	Any	Strengthen and weaken	Required	Stomach, Colorectum
Internal traction method				
S–O clip	Any	Strengthen and weaken	None	Stomach, Colorectum
Ring thread	Any	Strengthen and weaken	None	Colorectum
Multiloop	Any	Strengthen and weaken	None	Colorectum
Double clip and rubber band	Any	Strengthen and weaken	None	Colorectum
Others				

The advantages of this method are its simplicity, low cost, and no requirement for a special device. The disadvantage is that traction direction is limited to the direction in which the line is pulled; therefore, submucosal dissection may be difficult, depending on traction direction. Although increasing traction force is possible, by pulling the line, it is difficult to weaken traction force. Moreover, friction between the endoscope and the line in the narrow space generates interference, which sometimes causes strong traction resulting in slip-off of the clip. In fact, the slip-off rate is reported to be 16.4% in esophageal ESD[6] and 13.2% in gastric ESD[5].

Pulley method: The pulley method is a modified clip-with-line method. By anchoring the line to the gastrointestinal wall, the direction of traction can be controlled in any direction (Figure 8). The pulley method can be classified into two types according to the pulley system used: Clip pulley[22] or suture pulley[23,24]. There are only a few reports on the pulley method, and its effectiveness needs to be verified.

Sheath traction method: In the sheath traction method, the line part of the clip-with-line method is replaced with a sheath. Since the sheath is harder than the line, it can provide not only pulling force but also pushing force to the lesion, thus allowing two traction directions (Figure 9). Sheath traction method includes the clip-and-snare method[25-29] and the Endo Trac[30,31] (TOP, Tokyo, Japan) (Figure 10A).

The clip-and-snare method requires only a polypectomy snare and a clip; therefore, this method may be performed anywhere. In the conventional clip-and-snare method, the snare is grasped with forceps and delivered to the lesion[25]. However, this procedure is sometimes difficult. The prelooping technique was developed to improve the delivery of the snare[26-29]. The prelooping technique for the clip-and-snare method is performed as follows. After circumferential mucosal incision, the endoscope is withdrawn to preloop the snare on the tip of the endoscope. Then, the endoscope is inserted to the lesion along with the snare sheath. The clip is attached to the edge of

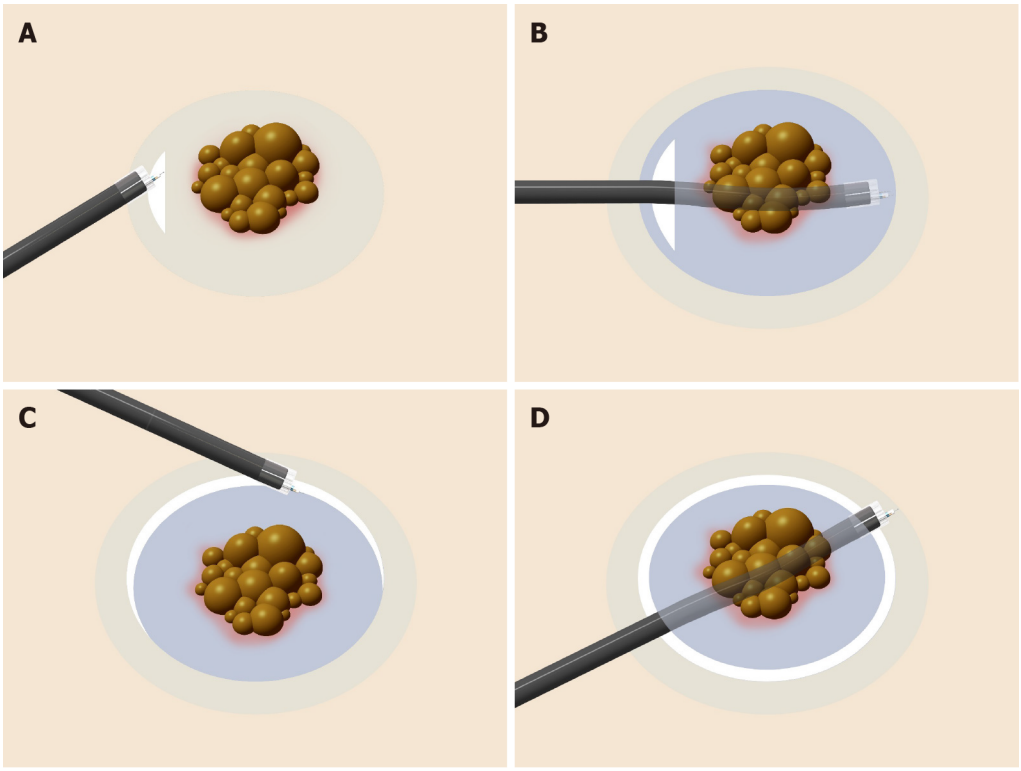

Figure 6 Procedure for the pocket creation method of endoscopic submucosal dissection. A: A minimal initial mucosal incision (approximately 20-mm in width) is made for entry into the submucosa; B: Submucosal dissection is performed, followed by creation of a submucosal pocket under the lesion; C: Additional mucosal incision of the gravitational lower side of the submucosal pocket; D: Opening around the submucosal pocket, then, en bloc resection is achieved.

the lesion. Then, the snare is loosened so that it can slide over the clip applicator toward the clip. Finally, the snare holds the clip, and the clip applicator is withdrawn.

Endo Trac is a product developed for the sheath traction method. Whereas interference between the sheath and the tip of the endoscope sometimes makes access to the submucosa difficult when the clip-and-snare method is used, the Endo Trac has a structure that can release the sheath from the lesion, to avoid interference between the sheath and the endoscope tip (Figure 10B).

The clip-and-snare method and the Endo Trac require withdrawal and reinsertion of the endoscope to set the traction system. Therefore, these methods are not suitable for colonic lesions, in which insertion of the endoscope is difficult and time-consuming. Moreover, interference between the sheath and the endoscope, due to friction, is possibly greater than with the clip-with-line method, due to the sheath being thicker than the line. In fact, it has been reported that even with a thin sheath (with a maximum diameter of 1.8 mm), interference with the endoscope can occur to some extent; the operator needs to move the endoscope carefully to avoid detachment of the snare from the clipped lesion[29].

External forceps method: The external forceps method is performed as follows[32-36]. After circumferential mucosal incision, the endoscope is withdrawn. The endoscope is reinserted with external forceps that are grasped by second forceps inserted through the accessory channel of the endoscope. External forceps grasp the edge of the lesion (Figure 11A) while the second forceps are withdrawn. The external forceps provide traction to two directions by pulling or pushing the lesion (Figure 11B). This method allows changing the traction point, by releasing and re-grasping the lesion.

However, this method has some disadvantages. It is difficult to deliver the external forceps, depending on lesion location, such as the cardia, lesser curvature or posterior wall of the upper gastric body, duodenum, and colon. Interference between the endoscope and the external forceps may be relatively strong compared with that of the clip-with-line and the sheath traction methods, because the forceps is thicker than the line and the sheath. Great care should be taken regarding potential damage to mucosa grasped by the external forceps, because of the strong traction.

Double scope method: The double scope method is performed by two experienced endoscopists with main and second endoscopes[37,38]. The second endoscope is inserted alongside the main endoscope. Then, the second endoscope deploys the forceps, through the accessory channel, and grasps the lesion to provide traction. A

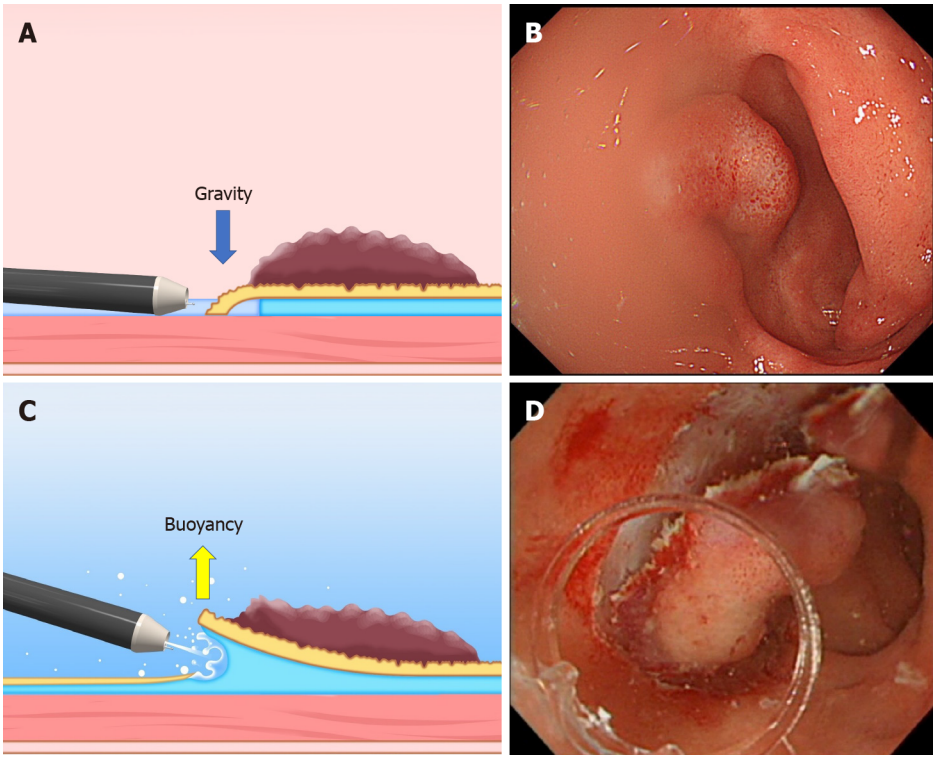

Figure 7 The difference between the conventional endoscopic submucosal dissection and the underwater endoscopic submucosal dissection. A and B: The conventional endoscopic submucosal dissection for the lesion is located at the gravitational lower side. Gravity obstructs the opening of the mucosal flap. Incomplete submersion deteriorates the visual field; C and D: The underwater condition aids the opening of the mucosal flap by buoyancy. Water pressure from the endoscope (using its water supply function) also assists in opening the mucosal flap. Complete submersion improves the visual field. Citation: Mitsuru Nagata. Underwater endoscopic submucosal dissection in saline solution using a bent-type knife for duodenal tumor. *VideoGIE* 2018; 3(12): 375–377. Copyright © 2018 American Society for Gastrointestinal Endoscopy. Published by Elsevier Inc[21].

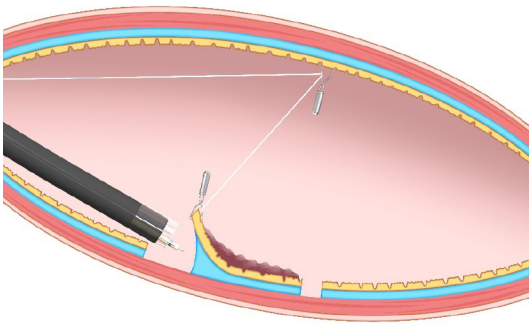

Figure 8 Pulley method. This method is the modified clip-with-line method, which can provide traction in any direction depending on the pulley site.

thin endoscope is recommended for the second endoscope, to avoid interference with the main endoscope.

This method has the great advantage that traction direction can be easily controlled by the second endoscope. Although the indication may be limited, as this method requires two experienced endoscopists and two endoscope systems, it may be a useful option for difficult cases, such as gastric cancers with ulcer scar[39]. This method has been reported to be useful in the treatment of superficial pharyngeal cancers[40] and gastric submucosal tumors[41].

Magnetic anchor method: The magnetic anchor method, as initially reported, used a large external electromagnet to provide traction, by moving an internal magnet attached to the lesion[42,43]. However, it was necessary to miniaturize the external electromagnet in clinical practice. Recently, use of neodymium rare earth magnets has allowed the external electromagnet to be minimized[44,45]; the feasibility of this method in clinical practice has been demonstrated in a prospective trial[46]. Although this method requires a special magnetic device and is not yet widespread, it is a promising method for the future due to the great advantage that it can provide

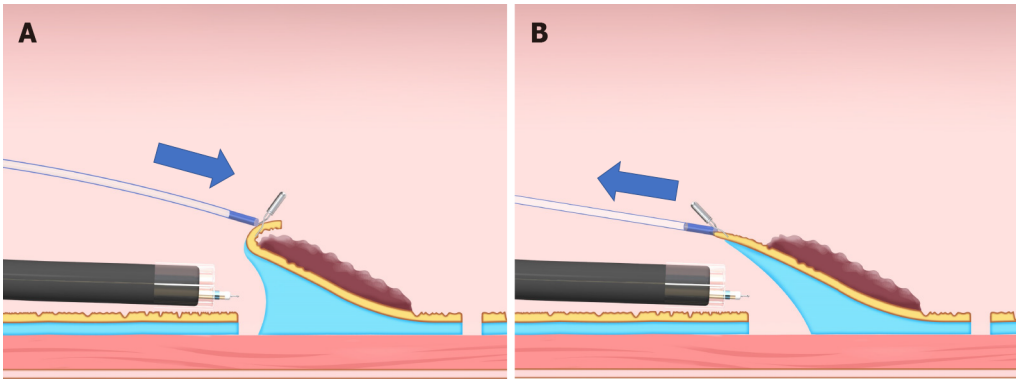

Figure 9 Sheath traction method. This method has two traction directions by pushing or pulling the sheath. A: Pushing the sheath; B: Pulling the sheath.

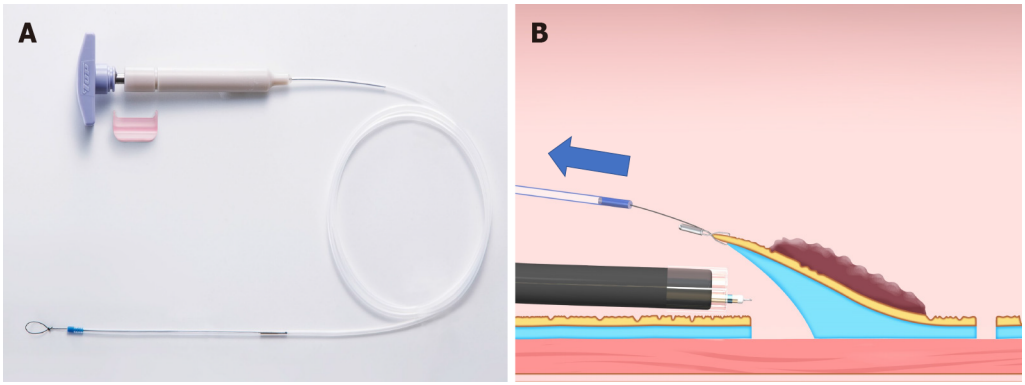

Figure 10 Endo Trac (TOP, Tokyo, Japan). A: This device can be used for the sheath traction method; B: This device has a structure that can release the sheath from the lesion to avoid interference between the sheath and the endoscope tip.

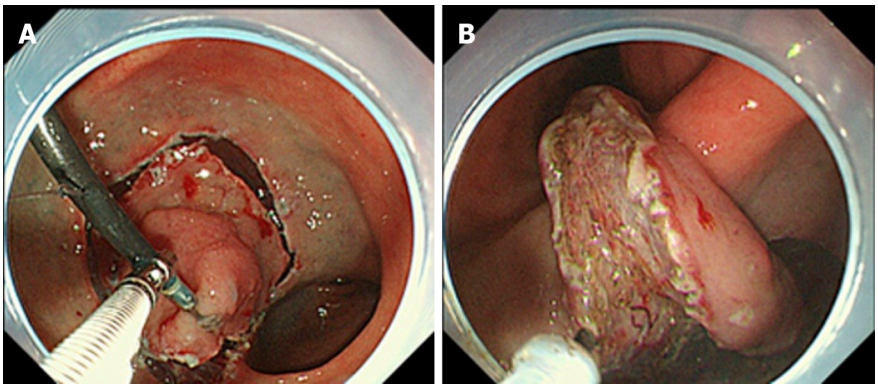

Figure 11 Endoscopic submucosal dissection using external forceps. A: External grasping forceps was anchored at the distal margin of the lesion in the lesser curvature of the antrum under the control of the endoscope and a second grasping forceps; B: With gentle oral traction applied with the external grasping forceps, the submucosal layer was dissected in retroversion from the aboral side. Citation: Imaeda H, Hosoe N, Kashiwagi K, Ohmori T, Yahagi N, Kanai T, Ogata H. Advanced endoscopic submucosal dissection with traction. *World J Gastrointest Endosc* 2014; 6(7): 286-295. Copyright © 2014 Baishideng Publishing Group Inc. Published by Baishideng Publishing Group Inc[35].

traction in any direction and control traction force. It should be noted that patients with a cardiac pacemaker or implantable cardioverter-defibrillator are not indicated for this method.

Internal traction methods: An internal traction method can be defined as a method in which the traction device acts only inside the gastrointestinal tract. Devices for internal traction include the S-O clip[7,8] (Zeon Medical, Tokyo, Japan) (Figure 2), ring thread [47], multiloop[48], double clip and rubber band[49], and clip band[50]. The principle for generating traction is the same in these devices, as follows. First, the clip with the

specific mechanism (*e.g.*, spring, thread, and band) for generating the traction is attached to the lesion (Figure 12A). Second, the regular clip anchors the tip of the mechanism to the gastrointestinal wall (Figure 12B). Finally, extension of the mechanism provides traction to the lesion (Figure 12C).

In these devices, the traction direction can be controlled in any direction by the anchor site. Traction force can also be controlled to some extent by inflating or collapsing the lumen. These devices are useful especially for colorectal ESD, as withdrawal of the endoscope is not required. The disadvantage is that a certain distance between the anchor clip and the clip attached to the lesion is required to generate the traction force. Therefore, these devices are usually utilized for gastric ESD [9-11] or colorectal ESD[47-50].

These devices are designed primarily for use with the forward endoscopic position, because there is a possibility that, in the retroflexed position, the endoscope may stretch the traction device, resulting in laceration of the mucosal flap or slip-off or breakage of the traction device. In gastric ESD, the retroflexed endoscopic position is as common as the forward position, due to the large lumen, unlike colorectal ESD. Therefore, we developed a modified method for attaching the S–O clip, to avoid stretching of the spring by the endoscope[9-11] (Figure 13). Although there are several devices for internal traction, the S–O clip may be the most appropriate in gastric ESD, as the S–O clip has a spring with higher elasticity than a thread or band. The elasticity of the spring can be easily adjusted for a large lumen, preventing laceration of the mucosal flap, slip-off, or breakage of the traction device. The S–O clip is sold only in Japan currently. However, it will be sold in future in Asian countries under the brand name "Countertraction CLIP".

The management of the anchor clip after traction is not standardized. Conventionally, the traction mechanism of the device (*e.g.*, thread, band) is cut to detach the resected specimen, while the anchor clip remains on the gastrointestinal wall. In colorectal ESD, the anchor clip may naturally drop by vermiculation. In contrast, vermiculation of the stomach is poor, except in the pars pylorica; there is a possibility of a permanent residual of the anchor clip after gastric ESD[51]. Therefore, we usually detach the anchor clip with forceps. So far, we have not experienced any adverse events from detaching the anchor clip (*e.g.*, perforation, post-ESD bleeding from the anchor site), probably because of the thicker stomach wall, compared with other organ of the gastrointestinal tract[10,11]. The safety of this management method for the anchor clip should be assessed in many gastric cases.

Others

Pocket creation method with traction device: The combination of the PCM and traction device (TD) for internal traction has been reported to facilitate better mucosal flap formation and opening of the submucosal pocket, compared with conventional PCM[52]. A retrospective study demonstrated that the median dissection speed in PCM with TD was significantly greater than in conventional ESD with TD (16.6 mm^2/min *vs* 12.2 mm^2/min; P = 0.003)[53]. Additional studies are needed to confirm whether TD has an additional effect in PCM, by comparing PCM alone against PCM with TD.

Clip flap method: The clip flap method has been reported as using a clip attached to the edge of the lesion to substitute for the mucosal flap until it is made (Figure 14)[54-56]. By using the clip flap method together with clip-based traction (*e.g.*, clip-with-line), the procedure of getting under the mucosal flap can be facilitated, especially when proximal traction makes the mucosal flap fall down (Figure 3B). Although a randomized controlled trial comparing the conventional method and clip flap method in gastric ESD demonstrated that the clip flap method had no advantage in efficacy and safety[57], this method may be effective when it is used along with other clip-based traction methods.

TRACTION METHODS ASSOCIATED WITH LESION SITE

Esophageal ESD

Representative traction methods that are reported to be effective in esophageal ESD include the clip-with-line method and ESTD. A multicenter randomized controlled trial demonstrated that the median ESD procedure time was significantly shorter with the clip-with-line method (n = 116) than with the conventional method (n = 117) (44.5

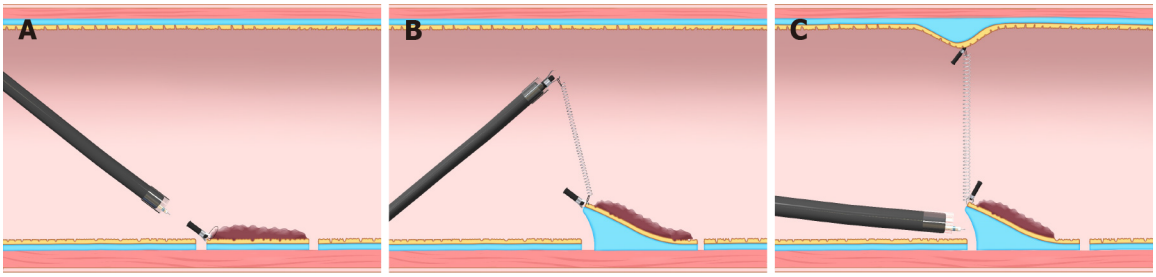

Figure 12 Internal traction method using the spring-and-loop with clip (Zeon Medical, Tokyo, Japan). A: The spring-and-loop with clip (S–O clip) is attached to the lesion; B: The regular clip anchors the loop part of the S–O clip on the gastrointestinal wall; C: The extension of the spring provides traction on the lesion. The traction direction can be controlled by the anchor site. Citation: Nagata M, Fujikawa T, Munakata H. Comparing a conventional and a spring-and-loop with clip traction method of endoscopic submucosal dissection for superficial gastric neoplasms: a randomized controlled trial (with videos). *Gastrointest Endosc* 2021; 93(5): 1097-1109. Copyright © 2021 American Society for Gastrointestinal Endoscopy. Published by Elsevier Inc[11].

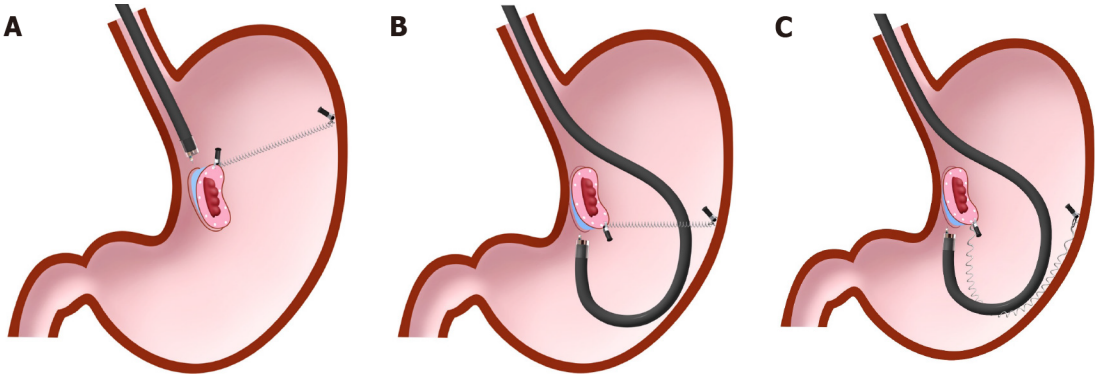

Figure 13 Spring-and-loop with clip-assisted gastric endoscopic submucosal dissection. A: Forward endoscopic position; B: Retroflexed endoscopic position; C: The endoscope has the possibility to stretch the spring, resulting in a loss of spring elasticity in the retroflexed endoscopic position. In this situation, the modified attachment method that we described in detail in the previous papers[9-11] is required to avoid this problem. Citation: Nagata M, Fujikawa T, Munakata H. Comparing a conventional and a spring-and-loop with clip traction method of endoscopic submucosal dissection for superficial gastric neoplasms: a randomized controlled trial (with videos). *Gastrointest Endosc* 2021; 93(5): 1097-1109. Copyright © 2021 American Society for Gastrointestinal Endoscopy. Published by Elsevier Inc[11].

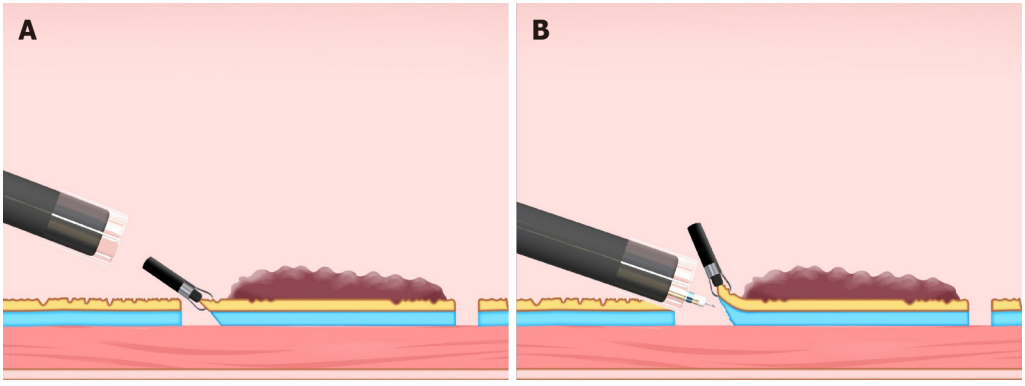

Figure 14 Clip flap method. A: The clip is attached to the edge of the lesion; B: The clip can be used as a substitute for the mucosal flap.

min *vs* 60.5 min; $P < 0.001$)[6]. Although traction using the clip-with-line method in esophageal ESD is limited to proximal traction, because the forward endoscopic position is predominantly used, due to the narrow cylindrical esophageal lumen, proximal traction may be effective, because the endoscope tip can approach parallel to the esophageal wall and can easily access the submucosa without vertical traction. After getting under the mucosal flap, hood traction and proximal traction using the clip-with-line method are combined, providing diagonally proximal or vertical traction to the submucosa (Figure 3C). Remarkably, the conventional method was changed to the clip-with-line method in six patients (5.2%) because of technical

difficulties. Moreover, five patients (4.3%) experienced perforation under the conventional method, whereas one patient (0.9%) could not complete the ESD procedure because of perforation. Conversely, no perforations were observed in the clip-with-line method.

In a multicenter randomized controlled trial, the clip slip-off rate was reported as 16.4%[6]. If clip slip-off occurs, there is a possibility that histopathological evaluation for the margin is made difficult due to damage to the specimen. Moreover, clip slip-off requires reattaching the clip-with-line, which is time-consuming. Interference between the endoscope and the line due to friction causes slip-off. Therefore, once the clip-with-line is attached to the lesion, unnecessary movement and withdrawal of the endoscope should be avoided.

A propensity score matching analysis[58] showed that ESTD had a shorter median ESD procedure time (38.0 min *vs* 48.0 min; $P = 0.006$) and lower muscle injury rate (28.9% *vs* 52.6%; $P = 0.036$) compared with conventional ESD. Furthermore, a meta-analysis including 17 studies[59] showed that ESTD had significantly higher en bloc resection rate, shorter ESD procedure time, and lower muscle injury rate. In ESTD, the endoscope tip is held by the submucosal tunnel, which allows stabilization of the endoscope and a parallel approach to the muscle layer. The endoscope tip inside the submucosal tunnel pushes up the lesion, providing sufficient tension at the dissection plane. These advantages of the ESTD may provide a shorter ESD procedure time and a lower muscle injury rate.

In conclusion, many promising results have been reported for the clip-with-line method and ESTD. At present, it may be better to select either of these two methods. Most studies on traction method for esophageal ESD have been reported from Asia, mainly targeting squamous cell carcinoma. There are not many reports of traction-assisted ESD for Barrett's esophageal adenocarcinoma, located around the esophagogastric junction; future studies should focus on this issue.

Gastric ESD

As the stomach lumen is large, both the forward and retroflexed endoscopic positions are common, unlike in esophageal, duodenal, and colonic ESD. Therefore, it is desirable that a traction method for gastric ESD is easy to utilize in both forward and retroflexed endoscopic positions. The popular traction methods for gastric ESD include clip-with-line, internal traction, sheath traction, and ESTD.

The clip-with-line method may be the first traction method for gastric ESD, and was reported in 2002[3,4]. However, a multicenter randomized controlled trial[5] comparing the conventional ESD ($n = 316$) and the clip-with-line method ($n = 319$) failed to show a reduction in the mean procedure time for gastric ESD in the total population (conventional ESD, 60.7 min *vs* clip-with-line method, 58.1 min; $P = 0.45$). Since traction by the clip-with-line method in gastric ESD is limited to the cardia, the direction of traction varies depending on the lesion location. In the retroflexed endoscopic position, the traction is likely to be distal, especially for lesions located at the lesser couverture side of the gastric body (Figure 15A). Distal traction may cause the submucosal dissection plane to fall distally, making the procedure difficult and prolonging the procedure time in some cases. In the forward endoscopic position, the traction may be proximal or diagonally proximal (Figure 15B). If the endoscope tip cannot be parallel to the gastric wall, proximal traction may cause the mucosal flap to fall proximally, making it difficult to approach the submucosal layer. In contrast, a subgroup analysis based on lesion location demonstrated that the mean ESD procedure time for lesions located at the greater curvature of the upper and middle third of the stomach was significantly shorter in the clip-with-line method (104.1 min *vs* 57.2 min; $P = 0.01$). From an anatomical point of view, these results seem reasonable, because it is difficult for the clip-with-line method to provide vertical traction unless the lesion is located at the greater curvature (Figure 15C). In a subgroup analysis based on operator experience, the mean ESD procedure time was not significantly different between the conventional and clip-with-line methods in an expert group (58.0 min *vs* 58.0 min; $P = 1.00$). Conversely, the mean ESD procedure time in a trainee group tended to be better in the clip-with-line method (68.9 min *vs* 58.3 min; $P = 0.13$). However, the lesions managed by trainees were primarily easy cases; therefore, a simple comparison may be inaccurate. Nonetheless, the analysis result suggests that the benefit from clip-with-line method differs depending on the operator experience.

S–O clip-assisted ESD is classified as internal traction, and can provide traction in any direction. Since the use of the S–O clip in gastric ESD has a potential for the endoscope in the retroflexed position to stretch the spring, we have developed a modified attachment method for the S–O clip, to avoid interference between the endoscope and spring part of the clip[9,10]. We reported a single-center randomized

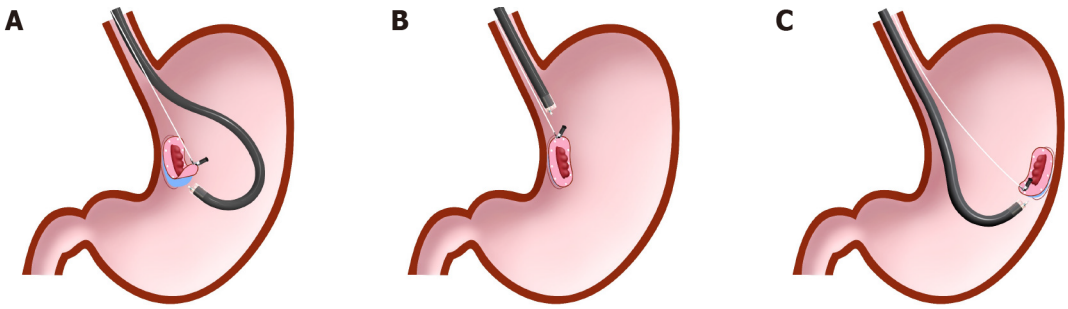

Figure 15 Difference in traction direction depending on the lesion location in the clip-with-line method. A: Distal traction; B: Proximal traction; C: Vertical traction.

controlled trial comparing conventional (n = 40) and S–O clip-assisted ESD (n = 40), which showed that the median ESD procedure time was significantly shorter in S–O clip-assisted ESD than in conventional ESD (29.1 min *vs* 52.6 min; P = 0.005)[11]. According to the subgroup analysis comparing the ESD procedure time by lesion location, the median ESD procedure time of S–O clip-assisted ESD was significantly shorter than that of conventional ESD in the upper and middle third of the stomach (39.4 min *vs* 58.3 min; P = 0.005). In the lower third of the stomach, the two methods were not significantly different (22.8 min *vs* 36.2 min; P = 0.146). Essentially, the ESD procedure performed in the lower third of the stomach is easier than that performed in the upper and middle third of the stomach[60,61]. The difference in the difficulty of the ESD procedure may explain the difference in the subgroup analysis outcomes. Therefore, the S–O clip-assisted ESD is especially recommended for lesions in the upper and middle third of the stomach. Meanwhile, en bloc resection, R0 resection, perforation, and post-ESD bleeding showed no significant difference between the two groups. The S–O clip slip-off rate was only 2.5%, probably because the modified attachment method prevented interference between the endoscope and spring part of the clip. In this trial, vertical traction was selected for the S–O clip-assisted ESD using its multidirectional traction function. Considering this result and the result of subgroup analysis of the above-mentioned multicenter randomized controlled trial of the clip-with-line method, vertical traction may be the optimal traction direction for gastric ESD. Although other internal traction methods, including the pulley, double scope, and magnetic anchor methods, may be able to provide vertical traction, their feasibility in gastric ESD is unclear and needed to be assessed.

The usefulness of the sheath traction method in gastric ESD has been reported. Unlike the clip-with-line method, the sheath traction method allows traction not only in the pulling direction but also in the pushing direction, so distal traction can be avoided in both forward and retroflexed endoscopic positions. A retrospective study comparing conventional ESD (n = 20) and the clip-and-snare method (n = 20) demonstrated that the clip-and-snare method significantly reduced the median ESD procedure time (38.5 min *vs* 59.5 min; P = 0.023)[29]. En bloc resection was achieved without perforation in all the patients in both groups. A case series of 21 challenging gastric ESD cases treated using the Endo Trac reported that the ability to change the traction direction in both proximal and distal sides was 100%[31]. Although these results are promising, the stress on the operator due to possible interference between the sheath and the endoscope is concerning. Moreover, these studies have the limitations of being retrospective studies with small numbers of cases. Therefore, evaluation in large-scale studies is warranted.

ESTD was reported to be useful not only for esophageal ESD but also for gastric ESD. A retrospective study evaluating 799 consecutive cases of gastric ESD in single institution showed that resection speed using ESTD was greater than with conventional ESD (19.3 mm^2/min *vs* 17.7 mm^2/min; P = 0.009)[62]. Perforation was significantly less frequent in ESTD (0.9% *vs* 6.0%; P = 0.035). However, the creation of a submucosal tunnel in the stomach may be more difficult compared to the esophagus because the stomach lumen is not straight. It has been reported difficult to form a submucosal tunnel for a lesion located at the pylorus ring or the greater curvature side of the fornix[62]. On the other hand, lesions located at the cardia, the lesser curvature of the gastric corpus, and the greater curvature of the antrum are reported to be suitable for ESTD[63].

In summary, the internal traction method using the S–O clip with modified attachment method has the potential to be the most appropriate traction method for

gastric ESD. The clip-with-line method and ESTD may be effective methods in gastric ESD if the lesion location is appropriate for these methods.

Colon and rectal ESD

Colonic ESD is more challenging than esophageal and gastric ESD because the maneuverability of the endoscope is limited, the colorectal lumen is angulated, and the muscle layer is thin and easy to perforate. Withdrawal and reinsertion of the endoscope is time-consuming in colonic ESD, unlike ESD in the upper gastrointestinal tract and rectum. Therefore, for colonic ESD, a traction method that does not require withdrawal and reinsertion of the endoscope is suitable. By contrast, it is easy to utilize most traction methods in rectal ESD.

Internal traction methods are suitable not only for rectal ESD but also colonic ESD because they do not require withdrawal and reinsertion of the endoscope. Recently, several novel devices for internal traction in colorectal ESD have been reported, such as S–O clip[7,8], ring thread[47], multiloop[48], double clip and rubber band[49], and clip band[50]. These devices have the common advantage of controlling traction direction at anchor site. Among them, the S–O clip is made of a highly elastic spring that can be used flexibly, regardless of the lesion location. A prospective randomized controlled trial comparing conventional (n = 27) and S–O clip-assisted ESD (n = 23) demonstrated that the mean ESD procedure time for S–O clip-assisted ESD was significantly shorter than that for conventional ESD (37.4 min *vs* 67.1 min; P = 0.03)[64]. No significant differences were observed in en bloc resection, perforation, and post-ESD bleeding. Although the conventional ESD was converted into the S–O clip-assisted ESD in eight cases, these cases remained in the conventional ESD group. In most of these conversion cases, the lesions were located in flexural areas where endoscope maneuverability is poor; these areas were the sigmoid colon, hepatic flexure, and splenic flexure. In these areas, reaching under the mucosal flap by the endoscope tip is difficult when only used with hood traction. The S–O clip helps the endoscope tip reach under the mucosal flap, providing proper visualization of the submucosa, despite poor endoscope maneuverability. Traction-assisted ESD using ring thread[47], multiloop[65], or double clip and rubber band[49] also showed promising treatment results in clinical trial, compared with conventional ESD. Further studies should focus on which traction direction is appropriate for colorectal ESD by using multidirectional traction function of internal traction methods.

PCM is another traction method that does not require withdrawal and reinsertion of the endoscope. In this method, the submucosal pocket holds the endoscope, allowing stable endoscope maneuverability. Moreover, the endoscope inside the submucosal pocket pushes up the lesion and provides sufficient tension at the dissection plane. A prospective randomized controlled trial comparing PCM (n = 59) and the conventional method (n = 55), conducted at three Japanese institutions, reported that the rate of ESD completion (defined as completion of colorectal ESD in three hours with en bloc resection using the assigned ESD method without changing to other methods or other devices and without perforation during the procedure) was significantly higher in PCM compared with conventional ESD (93% *vs* 73%; P = 0.01)[66]. By contrast, the median dissection speed was not significantly different between the two methods (15.9 mm^2/min *vs* 17.4 mm^2/min; P = 0.81). This was unforeseen, as several retrospective studies had reported dissection speed significantly greater in PCM than in the conventional method[67,68]. A novel method that combines the PCM and internal traction has been developed[52,53], and it can possibly accelerate the dissection speed. A meta-analysis including five studies (two randomized controlled trials and three retrospective studies) evaluated the efficacy and safety of PCM in comparison with the conventional method for superficial colorectal neoplasms[69]. PCM achieved a higher R0 resection rate (93.5% *vs* 78.1%; OR, 3.4; 95%: 1.3–8.9; I^2 = 58%), a higher en bloc resection rate (99.8% *vs* 92.8%; OR, 9.9; 95%CI: 2.7–36.2; I^2 = 0), a shorter procedure time (min) [mean difference (MD), -11.5; 95%CI: -19.9 to -3.1; I^2 = 72%], a faster dissection speed (mm^2/min) (MD, 3.6; 95%CI: 2.8–4.5; I^2 = 0), and a lower overall adverse event rate (4.4% *vs* 6.6%; OR, 0.6; 95%CI: 0.3–1.0; I^2 = 0) than the conventional method. However, all the included studies were conducted in Japan, with only two randomized controlled trials. Hence, further study is needed, especially regarding dissection speed.

The conventional clip-with-line method requires withdrawal and reinsertion of the endoscope, which may be troublesome during colonic ESD for lesions located where it is difficult to insert the endoscope. Modified preparation techniques for the clip-with-line method have been developed that eliminate withdrawal and reinsertion of the endoscope[70]. A single-center prospective randomized controlled trial comparing the clip-with-line method with modified preparation technique (n = 42) against the

conventional method (n = 42) demonstrated that the median colorectal ESD procedure time was significantly shorter in the modified clip-with-line method than in the conventional method (40 min *vs* 70 min; P < 0.0001)[71]. No significant differences were noted in en bloc resection, R0 resection, perforation, and post-ESD bleeding. In this study, two experts and two intermediates performed the colorectal ESD procedures. When the intermediates encountered difficult situations, the experts took over the procedure. The intermediates' self-completion rate was significantly higher in the modified clip-with-line method than in the conventional method (100% *vs* 90%; P = 0.04). Although this modified preparation technique is a little tricky, and the clip-with-line method is not able to control the traction direction, it has the advantage that does not require any special device. Since colorectal ESD is generally performed in the forward endoscopic position, the clip-with-line method may provide diagonally proximal or proximal traction for colorectal ESD. The clip-with-line method may be effective, as long as the endoscope has a parallel approach to colorectal wall.

The sheath traction, clip-and-snare, and Endo Trac methods also requires withdrawal and reinsertion of the endoscope. However, the sheath traction method was reported to be utilized even for colonic lesions[27,31]. A retrospective study reported that the clip-and-snare method (n = 17) significantly reduced mean colorectal ESD procedure time compared with conventional method (n = 123) (45.6 min *vs* 70.1 min; P = 0.047)[28]. There were no significant differences in en bloc resection, curative resection and adverse events (perforation and post-ESD bleeding). The sheath traction method has the great advantage that it can control traction direction to some extent by pushing or pulling the sheath, which may facilitate the ESD procedure. Moreover, the clip-and-snare method may be useful in any country, because it does not require any special device. Although reinsertion of the endoscope during the sheath traction method is occasionally troublesome, a balloon overtube may help address the issue, simplifying insertion of the endoscope.

The usefulness of underwater techniques during colorectal ESD has been reported [20,72-75]. Underwater conditions provide buoyancy (classified as natural traction) which can help turn over the mucosal flap of a lesion located lower gravitationally (Figure 7C and D). Although colorectal ESD is generally performed with the patient's posture such that the target lesion is on the upper side of gravity, to open the mucosal flap by gravity, it is difficult to select this posture in some cases due to poor endoscope maneuverability, a vertical approach to the lesion, and difficulty opening the lumen. Water pressure from the endoscope using its water supply function can be used as a traction method at any time. Water pressure can be used even in the conventional method. However, splashing can sometimes obstruct the visual field. In underwater conditions, splashing can be avoidable, which makes it easier to get under the mucosal flap. The underwater condition provides a good field of vision through a zoom effect and the disappearance of halation; this facilitates colorectal ESD in a poor field of vision due to severe submucosal fibrosis or fat tissue (Figure 16). We reported a case series study that demonstrated the feasibility and safety of underwater techniques for colorectal ESD[20]. However, additional studies are needed to evaluate whether underwater techniques improve colorectal ESD procedures compared with conventional methods.

In conclusion, PCM and internal traction methods (*e.g.*, S–O clip, ring thread, multiloop, double clip and rubber band, clip band) are especially recommended for colorectal ESD, based on the results of recent studies.

Duodenal ESD

Duodenal ESD is extremely challenging due to the fragile muscle layer, thin submucosal layer, and poor maneuverability of the endoscope, resulting in a high perforation rate which has been reported as 8.8% to 27.0%[76-78]. However, there are few reports of effective methods for reducing the procedural difficulty of duodenal ESD, because superficial duodenal epithelial tumors indicated for ESD are rare.

PCM has been reported to be useful for the treatment of superficial duodenal epithelial tumors. A retrospective study showed that perforation was significantly less frequent in PCM [7% (2/28)] than in the conventional method [29% (5/17; P = 0.046)] [79]. PCM demonstrated a faster dissection speed (9.4 mm^2/min *vs* 6.5 mm^2/min; P = 0.09) and a higher en bloc resection rate (100% *vs* 88%; P = 0.07) than the conventional method, although the differences were not statistically significant. PCM may prevent leakage of the injected solution from the submucosa due to minimal mucosal incision until completion of submucosal dissection. Moreover, the submucosal pocket holds the endoscope, providing stable endoscope maneuverability. In the conventional method for duodenal ESD, the mucosa around the lesion is incised widely before the completion of submucosal dissection, while the injected solution in the submucosa may

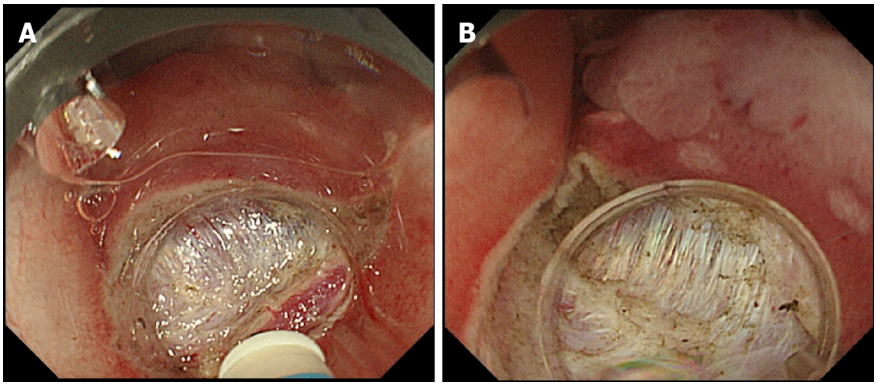

Figure 16 Comparison of aerial and underwater endoscopic view. A: Severe fibrosis and halation makes the boundary between the submucosal layer and muscle layer unclear; B: Submergence enables a detailed observation through a natural zoom effect and causes halation disappearance, thereby clarifying the boundary between the submucosal layer and muscle layer. Citation: Nagata M. Usefulness of underwater endoscopic submucosal dissection in saline solution with a monopolar knife for colorectal tumors (with videos). *Gastrointest Endosc* 2018; 87(5): 1345-1353. Copyright © 2018 American Society for Gastrointestinal Endoscopy. Published by Elsevier Inc[20].

easily flow out and the endoscope operability deteriorates.

Underwater techniques are another option for the treatment of superficial duodenal epithelial tumors[21,80]. Severe submucosal fibrosis in duodenal submucosa occasionally exists and causes insufficient submucosal elevation, even with a large quantity of injection. Although submucosal fibrosis generally makes it difficult to get under the mucosal flap during the first half of duodenal ESD, water pressure from the endoscope using its water supply function helps turn over the mucosal flap and enable the endoscope to get under the mucosal flap. Since the underwater condition eliminates splashing, unlike under gas supply conditions, the endoscope can get under the mucosal flap seamlessly after generating water pressure. This technique is classified as natural traction, and can be used repeatedly at any time.

The underwater condition has several useful effects based on nature, such as a zoom effect, the disappearance of halation, buoyancy, and a heat sink effect. The zoom effect and the disappearance of halation allow recognition of the proper dissection plane despite severe submucosal fibrosis. Buoyancy can be classified as natural traction and aids the opening of the mucosal flap when the lesion is gravitationally lower (Figure 7C and D). The heat sink effect minimizes thermal damage to the muscle layer from the ESD procedure. Thermal damage may increase the risk of delayed perforation after duodenal ESD, which leads to serious complications[81]; therefore, the underwater techniques may be suitable for duodenal ESD.

The underwater techniques may be able to be combined with other traction methods. In fact, a case report showed the usefulness of the underwater techniques with PCM and internal traction during duodenal ESD[82]. Although underwater techniques have major disadvantages, such as visual field loss due to active bleeding, gel immersion endoscopy, which secures the visual field during bleeding, may help address this issue[83]. Underwater techniques have the potential to reduce difficulty in ESD procedures through unique effects not found in conventional methods under gas supply condition. Further study should focus on the efficacy of ESD with underwater techniques, especially in the duodenum.

Several traction methods other than PCM and underwater techniques for duodenal ESD have been reported, such as internal traction using the S–O clip[84] and the sheath traction method[31]. However, there are few reports about these methods, and the efficacy of these methods is still unclear.

In summary, PCM and underwater techniques have the potential to facilitate the duodenal ESD procedure, decreasing the risk of perforation.

CONCLUSION

The purpose of traction during ESD is to create a visual field by turning over the mucosal flap and facilitate dissection by providing tension for the dissection plane. In order to achieve these effects, it is important to understand the advantages and disadvantages of each traction method and to use a traction method that is most appropriate as per the situation. The results of previous studies suggest that traction

direction affects the effectiveness of the traction method. Therefore, traction direction should be considered when choosing a traction method. Although there are increasing reports of methods that can control the traction direction, further studies should focus on investigating the optimal traction direction and its influence on the effectiveness of the traction method.

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FRONTIER

Cytoprotective gastric pentadecapeptide BPC 157 resolves major vessel occlusion disturbances, ischemia-reperfusion injury following Pringle maneuver, and Budd-Chiari syndrome

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Abstract

The stable gastric pentadecapeptide BPC 157 counteracts various venous occlusion-induced syndromes. Summarized are all these arguments, in the Robert's cytoprotection concept, to substantiate the resolution of different major vessel occlusion disturbances, in particular ischemia-reperfusion injury following the Pringle maneuver and Budd-Chiari syndrome, which was obtained by BPC 157 therapy. Conceptually, there is a new point, namely, endothelium maintenance to epithelium maintenance (the recruitment of collateral blood vessels to compensate for vessel occlusion and reestablish blood flow or bypass the occluded or ruptured vessel). In this paper, we summarize the evidence of the native cytoprotective gastric pentadecapeptide BPC 157, which is stable in the human gastric juice, is a membrane stabilizer and counteracts gut-leaky syndrome. As a particular target, it is distinctive from the standard peptide growth factors, involving particular molecular pathways and controlling VEGF and NO pathways. In the early 1990s, BPC 157 appeared as a late outbreak of the Robert's and Szabo's cytoprotection-organoprotection concept, like the previous theoretical/practical breakthrough in the 1980s and the brain-gut axis and gut-brain axis. As the time went on, with its reported effects, it is likely most useful theory practical implementation and justification. Meantime, several reviews suggest that BPC 157, which does not have a lethal dose, has profound cytoprotective activity, used to be demonstrated in ulcerative colitis and multiple sclerosis trials. Likely, it may bring the theory to practical application, starting with the

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initial argument, no degradation in human gastric juice for more than 24 h, and thereby, the therapeutic effectiveness (including *via* a therapeutic per-oral regimen) and pleiotropic beneficial effects.

Key Words: Gastric pentadecapeptide BPC 157; Cytoprotection; Major vessel occlusion disturbances; Pringle maneuver; Budd-Chiari syndrome; Therapy

Core Tip: Summarizing the essential epithelium and endothelium protection interplay described in Robert's and Szabo's cytoprotection concept, and the role of the stable pentadecapeptide BPC 157 as a likely mediator, we suggest that BPC 157 may be a useful cytoprotective therapy. The hope is that it could finally bring into practice the huge theoretical importance of all aspects of the cytoprotection concept. Conceptually, there is a new point to discuss, namely, endothelium maintenance to epithelium maintenance (recruitment of collateral blood vessels to compensate for vessel occlusion and reestablish blood flow or bypass the occluded or ruptured vessel). BPC 157 counteracts various venous occlusion-induced syndromes, as well as inferior caval vein syndrome, ischemia-reperfusion injury following Pringle maneuver, and Budd-Chiari syndrome in rats. Activation of the alternative collateral pathways to bypass occlusion and reestablish alternative blood flow, results in the counteraction of the consequent syndromes. The severe venous occlusion-induced disturbances, the high portal and caval hypertension, aortal hypotension, arterial and venous thrombosis, both peripherally and centrally, and various organ lesions (*i.e.*, gastrointestinal, liver, kidney, heart, and brain) were all attenuated and/or eliminated. Furthermore, this particular beneficial effect may be competing with the Virchow's triad that can be a common presentation [*i.e.*, duodenal venous congestion lesions, perforated cecum, ischemic/reperfusion colitis, bile duct ligation-induced liver cirrhosis and portal hypertension, temporary portal triad occlusion (ischemia-reperfusion injury following the Pringle maneuver), and suprahepatic occlusion of the inferior caval vein (Budd-Chiari syndrome)]. The resolution of these various venous occlusion-induced syndromes emphasizes the evidence that as the native cytoprotective gastric peptide and a stable gastric pentadecapeptide membrane stabilizer, BPC 157, which is stable in the human gastric juice and counteracts gut-leaky syndrome, is a particular target and easily distinguished from standard peptide growth factors, involving particular molecular pathways, and specifically controlling the VEGF and NO pathways, in particular the prostaglandin pathway.

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INTRODUCTION

The current review aims to evaluate whether the stable gastric pentadecapeptide BPC 157, which has consistent efficacy in the co-, pre-, and post-treatment regimens, with a rapid onset of the therapeutic effect, as well as the parenteral and per-oral effectiveness, may bring the Robert's cytoprotection theory into practical application[1].

As previously stated[1], all of the studies to date that have tested the stable gastric pentadecapeptide BPC 157 as a treatment have demonstrated extremely positive healing effects for various injury types in numerous organ systems. Its practical significance as a prototypic cytoprotective agent and an important mediator of Robert's cytoprotection[1], and its contribution to resolving Selye's stress response[1], and brain-gut and gut-brain axis activity have been reported[2]. Additional particular points are its wide interactions with the nitric oxide (NO) system[1] and prostaglandin

system and counteraction of the toxicity of non-steroidal-anti-inflammatory drugs[1]. Its therapeutic effects on fistula healing[1] and damaged skin, muscles, tendons, ligaments, and bone comparable to those in the gastrointestinal tract[3,4] and wound healing, in particular[3], are also reviewed. The counteraction of tumor-induced muscle cachexia and the signaling process implicated in cancer cachexia[5] and leaky gut, and its membrane stabilizer and free radical scavenger activity[6] are highlighted. The final focus is on the particular effect of BPC 157 on blood vessels and vessel recruitment[1,7]. In addition, BPC 157, due to its profound cytoprotective activity, which has been demonstrated in ulcerative colitis and applied to multiple sclerosis trials, may be used, since it does not have a lethal dose (LD1)[1,6]. In one of the most recent studies[3], BPC 157 was found to be distributed in the gastrointestinal mucosa, lung, bronchial epithelium, epidermal layer of the skin, and kidney glomeruli by *in situ* hybridization and immunostaining. These data suggest that BPC 157 may have additional regulatory roles in the function of the lungs, kidneys, and skin in humans, in addition to being isolated from gastric juice and primarily acting in the gastrointestinal system[3]. BPC 157 has also been reviewed in several other articles[8].

In the present review, we discuss the cytoprotective activity of the gastric pentadecapeptide BPC 157[1] to resolve major vessel occlusion disturbances, ischemia-reperfusion injury following the Pringle maneuver, and Budd-Chiari syndrome[9-11], providing evidence that it may bring the cytoprotection theory to practical application. On the other hand, as mentioned above, the stable gastric pentadecapeptide BPC 157 perfectly matched with the original Robert's cytoprotective requirements for the stomach, or even extended it[1]. These requirements are the protection of the epithelium ("epithelial pathway") and endothelium ("endothelial pathway"), and the maintenance of gastrointestinal mucosal integrity to obtain a large beneficial effect inside and outside the gastrointestinal tract. Typically, human gastric juice rapidly destroyed standard growth factors within 15 min[12,13]. In contrast, BPC 157, with its essential gastric juice origin and stability in human gastric juice for more than 24 h [12], was matched in the local level (stomach, the permanent maintenance of the mucosal integrity, and thereby the entire gastrointestinal tract)[1]. Therefore, BPC 157 has particular therapeutic effectiveness, including *via* a therapeutic per-oral regimen, and pleiotropic beneficial effects. This local stomach and gastrointestinal tract protection was further extended to the general level (protection of other organs) (cytoprotection to organoprotection)[1]. As previously mentioned, BPC 157 could follow both the "epithelial" and "endothelial" pathways in Robert's cytoprotection[1,7, 12,14].

According to Andre Robert[14] in 1975, the first indication for cytoprotection was the evidence that certain prostaglandins (PGF2 and PGFB, which could not affect gastric acid secretion) protected the gastric mucosa against indomethacin, in a gastric acid-independent manner, *via* a mechanism other than the inhibition of gastric acid secretion[14-16]. Therefore, the term *cytoprotection* pioneered by Robert[14-16] was introduced in 1979 against the noxious effect of both intragastric alcohol application and the use of nonsteroidal anti-inflammatory drugs (NSAIDs). According to Robert's concept, the rapid onset of gastric cytoprotection would be the most remarkable aspect [14-16]. Prostaglandins reduce the development of gastric necrotic lesions when given orally at an appropriate dose as late as 1 min before the administration of absolute ethanol. Knowing this "before but not after effectiveness" as a limitation, Robert appreciated the curing of the already existing lesions as the further possibility[14-16]. The full explanation was provided in a few subsequent reviews[14-16] and the full argument was later substantiated[14-16]. In our view of the new cytoprotection principle, the essential evidence of Robert (epithelial protection) is the remarkable ability of endogenous and exogenous cytoprotective agents (*i.e.*, prostaglandins) to prevent rapidly acute gastric hemorrhagic lesions induced by diverse noxious stimuli such as ethanol, bile acids, hyperosmolar solutions, and NSAIDs such as aspirin or indomethacin. According to the claims of Robert [*i.e.*, cytoprotection preventing mucosal necrosis caused by noxious agents due to the direct damage of cells or a local deficiency of cytoprotective mediators (*i.e.*, prostaglandins)], the cytoprotection concept also goes beyond peptic ulcer therapy[14-16]. Moreover, in Robert's view[14], the demonstration of adaptive cytoprotection suggests that cytoprotection by prostaglandins may be a physiological phenomenon[14]. One milliliter of 20% ethanol (as small irritant) given orally (note, in Robert's publication, "orally" implies administration *via* a tube into the stomach, or rather intragastric application, see Chapter *2.1.3. Epithelial pathway to adaptive cytoprotection*) to fasted rats 15 min prior to giving absolute ethanol (regarded as a strong irritant) prevented the gastric mucosal necrosis caused by the latter[17]. For the effectiveness of cytoprotective agents, the concept holds the protection of stomach cells (cytoprotection) and other epithelia (organoprotection)

against direct injury to cells induced by various noxious agents[14-16]. In addition, see the notation for the cytoprotection-organoprotection for other agents[18,19].

In the early 1980s, the concept obtained an additional key, the concomitant protection of the stomach endothelium[20,21] or Szabo's vascular injury, as an early pathogenic factor in the development of ethanol-induced gastric hemorrhagic erosions. Demonstration of the vascular injury was seen within 1 min, as was the estimated effect of the agents[20,21]. This vascular point in ethanol-induced gastric lesions was fully elaborated in a series of subsequent reports[22]. Since then, the rapid recovery of damaged endothelium may be considered a shared effect of the cytoprotective agents [23].

Thus, the cytoprotection theory holds that cytoprotective agents should exert direct epithelial and endothelial cell protection inside and outside of the gastrointestinal tract [14-16], *via* the "epithelial pathway" and "endothelial pathway". There is an essential evolvement in the stomach protection: Endothelium protection to epithelium protection[14-16]. Each of these pathways originates as a result of the increase in cytoprotective activity, together manifested as an increased therapeutic effect in both the prophylactic process (important for maintaining undisturbed organ function) and the therapeutic process (important for the possible reversal of the damaged tissue to a normal structure, and the interruption of damaging events). Unfortunately, such practical realization of the highly conceptualized theory is lacking. The anticipated huge range of organ lesions that should be counteracted and protection against non-specific lesions[1], as well as the rapid onset of action implemented in the agents' efficacy, as a resolving outcome, remains obscure.

On the other hand, in addition to the proposed role of BPC 157[1,7], within Robert's concept of cytoprotection, different points of view and different highlights can be clearly seen. Certainly, such a multitude illustrates the essential value of the potential application of the concept. The emphasis was on NO, carbon monoxide and hydrogen sulfide[24-26], sulfhydryls (SH)[20,27,28] in parallel with prostaglandins, as well as histamine[23], prostaglandins[17], EP1 and IP receptors[29,30], the healing action of antacid[31], sucralfate[31,32], heat shock protein 70 (HSP70)[33], and the renin-angiotensin system and active angiotensin metabolites[34]. Further illustrative emphases include opioids[35], alpha-2 adrenoreceptors[36], glucocorticoids[20,37-39], thyrotropin-releasing hormone (TRH)[40-43], capsaicin[44-46], dopamine[19,47-51], somatostatin[18,52,53], epidermal growth factor (EGF)[53-55], bombesin[56], ghrelin [17,34,57], cholecystokinin (CCK) and leptin[58,59], melatonin[60], neurotensin[61], fibroblast growth factor (FGF)[62,63], agmatine[64], amino acids[65], second-generation histamine H(2)-receptor antagonists[66], hemeoxygenase-1[67,68], and the molecular basis of alcohol-related gastric and colon cancer (acetaldehyde)[69].

Finally, a historical cytoprotection review, along with many original details, is given by Mozsik[70].

The term cytoprotection was commonly coined in other organ studies, *i.e.*, the heart and brain[71,72], kidney[73], liver[74], eye[75], skin and wounds[76], bone[77], and skeletal muscle[78].

Unfortunately, the multitude of agents supposed to be involved did not resolve the conceptual problems that were initially shown with the prime agents, providing the limited therapeutic potential of prostaglandins in stomach lesions (*i.e.*, prostaglandins might only prevent rather than cure any already established stomach lesions)[14-16]. Likewise, there was an even more limited therapeutic potential in the healing of other organs (prostaglandins were only effective in a few organ lesions)[79-81]. The switching to other cytoprotective agents (*i.e.*, sulfhydryls[19,21], somatostatin[18], EGF [53], TRH[41,67,82], opioids[83], dopamine[50,51,61], and CCK) led to similar incomplete results in both stomach and other organ lesions (for review see[1,7]). Consequently, considering the application and efficacy of standard agents, a considerable gap remains between the theoretical potential and practical realization[1,7]. Considering the supplemental endothelium protection, after initial demonstration in the stomach, no endothelial protection outside the stomach was investigated at the time[22]. Of note, BPC 157 appears to resolve both of these issues, *i.e.*, the "epithelium pathway" and "endothelium pathway" in cytoprotection[1,7], and may both prevent lesion development and cure any established lesions.

Likewise, to illustrate the failed realization of the concept with standard antiulcer agents, in addition to only prophylactic effectiveness in stomach lesions and a few other organs in which effectiveness was shown, the theoretical/practical problem is that standard cytoprotective agents also demonstrated the opposite outcome[42,84]. The intriguing point is the sulfhydryl prototype, cysteamine, and sulfhydryl conceptual involvement[19,21]. Cysteamine is highly protective in alcohol-induced stomach lesions[19,21], but, in contrast, cysteamine application provided the most

valuable standard model for the induction of duodenal[85,86] and colon[87] lesions. Also, we emphasized[1] that Robert's concept[14-17] largely applied the antecedent Selye's stress concept[88,89] which essentially contributed[90] to the introduction of corticosteroid therapy[91]. Of note, both concepts act against various noxious non-specific agents that would induce non-specific lesions[1]. Both concepts also hold organoprotection (Selye's concept of homeostasis that should be reestablished by the stress response[88] *vs* Robert's direct stomach cell protection that should be generalized by the application of cytoprotective agents[14-16]), and adaptation [Selye's small stress that protects against severe stress[89] *vs* Robert's small irritants that protect against strong irritants (adaptive cytoprotection[17])]. However, the essential first mediator of Selye's stress concept[88,89], which would integrate the adaptive bodily stress response and reestablish organoprotective bodily homeostasis, remained undiscovered, and appeared to be a major weakness of the concept that would preclude its practical realization[92,93].

For the classic concepts of Robert and Selye[14-16,88], the adverse effects of the prototype agents (*i.e.*, mediators) appeared to be an additional pitfall. Obviously, protection against direct injury to the cell in Robert's cytoprotection concept[14-16] certainly precludes any adverse effects, which are quite common with the application of prostaglandin analogues[65]. Likewise, the reestablishing of homeostasis (Selye's stress response defined "as such")[88] does not include the adverse effects that have been commonly known for the application of corticosteroids since early times[94]. As BPC 157 appears to be very safe and LD1 was not achieved, with no side effects reported in clinical trials, the possible switching of beneficial effects to negative ones (over-shutting phenomenon) appears to be highly unlikely[1].

However, whatever the pitfalls may be, these two concepts[14-16,88] provided a firm theoretical frame for the development of novel agents and therapies. It should be practically realized and demonstrated, in addition to the local (stomach) beneficial effect, by the agents' pleiotropic beneficial effects[1,7]. If properly followed, it may fulfill the first conceptual beneficial point (starting with Robert's cytoprotection[14-16], local protection and therapy of the stomach and gastrointestinal tract achieved) by the next extended beneficial point (the protection of other organs [epithelia] and the achievement of therapy), and bring them together to a reality that can no longer be disputed.

Thus, in the early 1990s, pentadecapeptide BPC 157[1,7] appeared as a late outbreak of the cytoprotection-organoprotection concept of Robert and Szabo[14-16,18,19], for epithelial and endothelial protection, like the previous theoretical/practical breakthrough in the 1980s[14-16,18,19] and the brain-gut axis and gut-brain axis[3]. As time went on, with its reported effects, BPC 157 could be most useful in the practical implementation and justification of the theory[1]. All arguments were given to bring the long-standing theory into practice, starting with the initial argument of the lack of degradation in human gastric juice for more than 24 h[12], and thereby the therapeutic effectiveness (including *via* a therapeutic per-oral regimen) and pleiotropic beneficial effect[1,7].

BPC 157 IN CYTOPROTECTION

Overall, and in particular for the role and cytoprotective effectiveness of BPC 157, it is safe to speculate that the efficacy and activity limitation of this agent, and thereby its practical application, would be determined by the foundation of the standing concepts, and *vice versa*. Briefly, the agent "runs" within the concept frame, and *vice versa*. Ideally, agent and concept can match completely (as may be seen with the achieved extent of the obtained beneficial effects; cytoprotection can be manifested as a huge range of beneficial effects, both inside and outside the gastrointestinal tract). General pitfalls may be the number of mentioned cytoprotective agents that have previously failed to match the required cytoprotection concept. In general, this means more problems with the use of new agents, and more problems for the concept to maintain its validity and less possibility (enthusiasm and belief) to be once applied. Alternatively, if there were no known agents which fulfilled the requirements of the standing concepts, the agent's efficacy and activity would determine the opposition to the "law" of the standing concepts and form a new relevant concept.

Illustratively, regarding the sympathetic system function, Alhquist's receptor concept[95] (*i.e.*, six catecholamines, and their different order of potency depending on the tissue involved, to anticipate the presentation of the particular alpha and beta receptors) discharged the long-standing "law" of physiology, Cannon's concept of two

mediator substances (sympathin E and sympathin I)[96]. Although there was an overlap of several years, Alhquist's receptor concept accuracy[95] envisaged the development of specific blocking agents in the subsequent years[97], and the consequent regular use of beta blockers in a large range of indications[98]. However, similar general acceptance and applicability did not arrive for the cytoprotection concept, nor was there any proof[99,100], when years later, as alternative gastric acid-nondependent, Robert's cytoprotection theory challenged the peptic ulcer therapy[14-16]. The lack of a practical solution and the absence of any commonly applicable cytoprotective therapy[99,100] mean that the "law" "no acid-no ulcer" and the superiority of H2-blockers were not discharged until the present time[101].

In the early 1990s, BPC 157 was introduced as a pentadecapeptide with cytoprotective effects[1,18,19], many years after the breakthrough of the original concepts of Robert[14-16] and Selye[88]. The surveillance of these two major concepts[14-16,88] and their development and achievements lacking full realization and adequate practical application[92,93,99,100] considered the introduction of BPC 157 to be too late a challenge. Seeing from the achieved perspective of all agents tested as standard cytoprotective agents, it was safe to speculate that a novel agent would hardly achieve a wider range of pleiotropic beneficial effects and drug characteristics that remained elusive for years.

However, conceptually, there is a new point, namely, endothelium maintenance to epithelium maintenance is upgraded to endothelium maintenance to epithelium maintenance (collateral blood vessels to compensate for vessel occlusion and reestablish blood flow or bypass the occluded or ruptured vessel)[1,7]. The recruitment of collateral blood vessels would compensate for vessel occlusion and reestablish blood flow[1,7,9-11]. BPC 157 counteracted various venous occlusion-induced syndromes[9-11], inferior caval vein syndrome[9], ischemia-reperfusion injury following Pringle maneuver[10], and Budd-Chiari syndrome[11] in rats. This beneficial effect was also shown for other syndromes, *i.e.*, duodenal venous congestion lesions, perforated cecum, ischemic/reperfusion colitis, and bile duct ligation induced liver cirrhosis and portal hypertension[1,7]. The resolution of these various venous occlusion-induced syndromes[1,7,9-11] emphasized the practical evidence. The stable gastric pentadecapeptide BPC 157, as a membrane stabilizer[5], likely acts as the native cytoprotective gastric peptide[1,3,7], which is resistant and stable in human gastric juice[12], and counteracts gut-leaky syndrome[6]. As a particular target, it is distinct from the standard peptide growth factors[3], involving particular molecular pathways [102-105], particularly controlling VEGF and NO pathways[1,106,107], and the prostaglandin pathway[1].

Epithelial pathway in stomach and gastrointestinal tract healing for cytoprotection against direct cell injury produced by direct contact with noxious agents

BPC 157 consistently counteracted the gastric lesions induced by 96% alcohol[1]. Of note, epithelial protection, as direct cytoprotection against direct cell injury produced by direct contact with noxious agents (*i.e.*, alcohol)[14-16], appears to be essential to resolve the follow-up of Robert's stomach cytoprotection ("epithelial pathway")[14-16]. As with Robert's alcohol intragastric application, this was a more advantageous therapeutic effect, overriding previous common limitations shared by standard cytoprotective agents (*i.e.*, prophylactic effect that may only counteract lesion development, but is unable to cure already existing lesions upgraded to the equal therapeutic ability[1]). BPC 157 demonstrated very consistent efficacy in alcohol-induced gastric lesions for co-, pre-, and post-treatment regimens, with a rapid onset of therapeutic effect, thereby providing consistent evidence for undistributed pertinent and specific effects, such as protection and healing, and the likely positive effects of an unusually high range[1]. This essential stomach point is confirmed and appreciated by others[108]. The BPC 157 equipotent (co-, pre-, and post-treatment regimens, per-oral and parenteral) beneficial effect is particular. There are constant interactions with the NO system and capsaicin-sensitive somatosensory neurons, since it consistently appears in naive rats as well as in those challenged with NOS blockade (NOS blocker L-NAME), NOS substrate L-arginine (NOS over-activity), NO system immobilization (concomitant application of L-NAME and L-arginine), capsaicin (as newborn or adult), or small exogenous or endogenous irritants[1,7]. A comparable beneficial effect was also achieved *in vitro* (denervated (isolated) gastric mucosal cells)[109,110].

Further supporting evidence included a strong reduction of the Monastral blue staining in ethanol-treated rats and, thereby, endothelium maintenance[1,7] and comparable beneficial effect in the stress gastric ulcer model[1] and cysteamine-duodenal ulcer model[1]. The same high efficacy was observed for both intragastric

and intraperitoneal regimens[1,7]. The evidence that BPC 157 fully counteracted NSAID-induced gastric and intestinal lesions is consistent with the prostaglandin requirement of Robert's model, and the beneficial effect of BPC 157 in the entire gastrointestinal tract[1]. Also, in addition to cysteamine- or ischemia/reperfusion-induced colitis[1,7], BPC 157 counteracted trinitrobenzene sulfonic acid (TNBS)[111] or iodoacetamide[112,113]-induced ulcerative colitis. Of note, the beneficial effect of BPC 157 is long-lasting, and may also counteract ulcer recidivation (*i.e.*, cysteamine ulcerative colitis)[1,7]. Also important for the issue of cytoprotection is the evidence that BPC 157 may counteract stomach ulcer and induce ulcer regression (*i.e.*, clopidogrel-induced)[114], as recently demonstrated in another prototype model of direct injury, Okabe's direct acetic acid application into stomach-induced gastric lesions[115,116], which is also commonly used in cytoprotection studies[1].

Provided that the essential point for lesions in Robert's cytoprotection model would be the injury made by direct contact (damage) to the cells[14-16], the perforation lesion instantly made by surgery is thereby a prototype[1]. The healing of perforated injury by the application of BPC 157 is an important conceptual point[1]. Further consequent evidence includes the healing of skin wounds and other wounds[1,3]. Importantly, proper wound healing includes the achievement of all four major events (vascular constriction, loose platelet plug, fibrin mesh to ensure stability of platelet plug, and dissolution of the clot) that occur in a set order following the loss of vascular integrity [3]. As a result, an agent applied in wound healing, such as stable gastric pentadecapeptide BPC 157, which is shown to be effective in wound healing, should also be effective in bleeding disorders[3].

Together, these consistent beneficial effects clearly indicated a full potential, in addition to the achievement of local protection and therapy of the stomach and gastrointestinal tract[1], toward Robert's point (other organ (epithelia) protection and therapy achieved)[14-16]. Of note, as pointed out, these studies indicated the use of the stress gastric ulcer models as a "cytoprotective" model (*i.e.*, not related to gastric acid secretion)[39]. The significance of the stress gastric ulcer models is fairly described in several reviews[117-121]. Likewise, the connection with the prostaglandin system (and thereby, Robert's cytoprotection) is fully substantiated[31]. For BPC 157, the use of the prolonged restraint stress procedure[1] was important, provided that the use of the restraint stress methodology by gradually modulating/increasing the level of the stress[39,120] (*e.g.*, usual cold + 3 h[39,120] *vs* 48 h restraint stress[1]) fully highlighted its efficacy[1]. Thereby, we could consistently suggest the effectiveness of BPC 157 over the application of standard H2-blockers or dopamine agonists[1].

Likewise, providing protection against the possible negative influence of gastric acid (hyper)secretion, in addition to the counteraction of Shay stomach ulcers induced by pylorus ligation[122] (but no influence on gastric acid secretion[123]) by BPC 157, there is some antagonism of the cysteamine-induced duodenal ulcer[1]. Since the Szabo's study[86], cysteamine-duodenal ulcers are commonly related to gastric acid hypersecretion[124-127]. However, we should consider stress lesions[85] as cytoprotection before Robert's cytoprotection[14-16], and thereby, Selye's "stress view"[85]. The introduction of cysteamine duodenal ulcers in rats will overcome the problems arising from multiple gastric erosions as the most characteristic rat gastrointestinal manifestations of exposure to stress, and would closely mimic human "stress ulcers", which are frequently localized in the duodenum[85]. Selye and Szabo considered the duodenal ulcer potency of various agents, and also emphasized "some relation to nonspecific stress" since cysteamine was the most potent agent of the other agents assessed (acetanilide, allylchloride, acetaminophen, 4,4-diaminodiphenylmethane, proprionitrile, and 3,4-toluendiamine) which were capable of inducing such lesions [85]. Yet, at that time, no mention was made on any influence of dopamine or gastric acid secretion[85]. With such particular "stress" notation to the duodenal lesions[85], initiation goes along with the emergence of the histamine, and the H2 receptor blocker-mediated resolution of peptic ulcers[128]. The subsequent cysteamine report by Szabo in the Lancet revealed the dopamine and gastric acid hypersecretion background, meaning that it became a seminal dopamine paper[86].

Also, this beneficial effect in cysteamine-induced duodenal ulcers[1] combined BPC 157 application with the dopamine system. Szabo provided cysteamine as a dopamine antagonist and its close similarity with the parkinsongenic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (that also induced duodenal ulcers)[129, 130] in support of the theory holding schizophrenia, Parkinson's disease, and ulcer disease as dopamine system failures, and dopamine antagonists (ulcerogenic potential)/dopamine agonists (therapy) in peptic ulcer therapy[131]. Lately, the interaction of BPC 157 with the dopamine system was reviewed[2]; BPC 157 counteracted the effect of neuroleptics (haloperidol), MPTP, and reserpine (*i.e.*,

akinesia, catalepsy, hypothermia, and gastric lesions). Also, BPC 157 counteracted the models resembling positive-like symptoms of schizophrenia[55], and haloperidol-induced catalepsy and gastric ulcers[2]. This effect, as a close interaction with dopamine system functioning, was able to determine an active gut-brain axis or brain-gut axis functioning[2]. It should be noted that BPC 157 also counteracted various encephalopathies and behavioral disturbances, and may therefore represent essential brain-gut and gut-brain axis activities[2]. As an extension of the therapeutic effect, BPC 157 also counteracted the typical and atypical neuroleptic-induced arrhythmias, QTc-interval prolongation[1]. The counteraction of the prolonged QT interval appeared as part of the large therapeutic effect of BPC 157 on the heart disturbances noted in the prevention and reversal of doxorubicin-induced chronic heart failure[1], and the counteraction of various arrhythmias[1], including those induced by venous occlusion [9-11].

Subsequently, again with the 96% alcohol-induced gastric lesion[1], the cytoprotective effect of BPC 157 was closely related to the NO system that should have an essential role in the maintenance of gastrointestinal mucosa integrity, and, more importantly, in endothelial functioning[1,8]. BPC 157 induced NO release from homogenate supernatants of the gastric mucosa from the rat stomach, which is particularly resistant to the NOS blocker N(G)-nitro-L-arginine methylester (L-NAME), and may counteract the NOS substrate L-arginine-induced NO over-release[1]. This particular interaction may be seen in various models and species with the ability of BPC 157 to counteract the adverse effects of L-NAME and L-arginine application[1].

Also, an essential point to remember in the cytoprotective effect of agents is capsaicin-sensitive afferent neurons[45,46], which regulate vascular function in many somatic and visceral tissues, including the regulation of local blood flow in the gastrointestinal tract. Thereby, the important point is that the beneficial effect of BPC 157 in gastric lesions induced by ethanol, restraint stress, or indomethacin was combined with the maintained as well as restored capsaicin-sensitive afferent neurons [1]. Quite recently, this cytoprotective notation was confirmed with the evidence that BPC 157 acts *via* inhibition of the release of enteric serotonin, an increase in the rat and human survival rate of cultured enteric neurons, and the proliferation of cultured enteric glial cells (EGCs)[132]. It was suggested that the inhibition of the release of enteric serotonin may be related to the release of serotonin noted in several brain areas (*i.e.*, nigrostriatum) after the administration of BPC 157[2,132].

Together, these findings clearly indicate a complex involvement of BPC 157 in the practical realization of cytoprotection as a non-gastric acid dependent phenomenon and "direct cell injury to cell – direct cell protection" principle. Furthermore, unlike its ulcerogenic effect ascribed to gastric acid hypersecretion[86], we showed that the application of cysteamine after gastrectomy induced duodenal ulcers in gastrectomized rats, and BPC 157, as well as all standard anti-ulcer agents, may clearly antagonize these cysteamine-induced ulcers in gastrectomized rats[1]. Interestingly, sialoadenectomy abolished the beneficial effect of standard antiulcer agents on cysteamine-induced duodenal ulcers, while BPC 157 was also effective in sialectomized rats[1]. As mentioned above, further evidence showed cysteamine enema-induced ulcerative colitis[1]. Thus, these findings may be used as a full argument that cysteamine-induced ulcer appears as originally suggested (stress ulcer, non-gastric acid-dependent)[5,8] while cytoprotection, as the non-gastric acid-dependent phenomenon and "direct cell injury to cell – direct cell protection" principle, is continuously operating[1].

Epithelial pathway for innate cytoprotection both inside and outside the gastrointestinal tract

The wider range of BPC 157 therapy[1,7] follows the definition of the innate cytoprotective potential in additional circumstances (*i.e.*, other epithelial healing), which has to combine the healing of different tissues, and is thus a pleiotropic beneficial effect[14-16]. Provided that cytoprotection represents a huge range of beneficial effects as the prototype model[1], there was a consistent demonstration of the strong therapeutic effect of BPC 157[1]. As emphasized, it not only occurred in the entire gastrointestinal tract[1,2], but also in various liver lesions, acute pancreatitis, and heart, lung, and kidney disturbances[1]. The consistent beneficial effects that include a considerable number of models may clearly verify the large range of therapeutic effects[1]. For instance, there are therapeutic effects in the liver lesion network against prolonged restraint stress, bile duct and hepatic artery ligation, CCl4 application, chronic alcohol drinking, NSAID over-dose application, insulin over-dose, and bile duct ligation-induced cirrhosis[1]. In particular, the beneficial effects occur against ischemia-reperfusion injury following Pringle maneuver[10], and Budd-Chiari syndrome[11] in

rats. Acute pancreatitis models were represented by bile duct ligation or lower esophageal and pyloric sphincter dysfunction[1]. As already mentioned, heart disturbance counteraction[1] was based on doxorubicin-induced chronic heart failure [57], and the counteraction of various, quite distinctive arrhythmias. This may be clearly seen providing the wide range of noxious events tested (*i.e.*, digitalis, hyperkalemia, bupivacaine, and lidocaine)[1] and venous occlusion procedures applied[9-11]. Likewise, the lung lesion counteraction is based on edema of the interstitium, and substantial dilatation and congestion of the capillaries in the alveolar septum in the lung of rats with venous occlusion syndromes[10,11]. If not corrected, the lung congestion appears as a common outcome (*i.e.*, time-dependent and time-independent features that can be acute respiratory distress syndrome exudative phase features); acute lung injury is a primary component of multiple organ dysfunction syndromes triggered by intestinal ischemia-reperfusion, which results in high mortality and acute lung injury[133,134].

Likewise, as a general follow-up of Robert's cytoprotection, the BPC 157 wound healing studies appear to be well founded[1,3]. As well as gastrointestinal ulcers, consistent evidence includes various skin wounds. In addition to the incisional wound and deep burns and fistula wounds[1,3], there were also diabetic ulcers[102,135] and alkali wounds[105]. These beneficial effects also include the healing of muscle (*i.e.*, the healing of the transected, crushed and denervated muscle), tendons (transected Achilles' tendon and Achilles' tendon detached from the calcaneus), ligaments (transected medial collateral ligament), and bone (alveolar bone loss and radial pseudoarthrosis)[3]. The delivery of BPC 157 was through local (*i.e.*, cream application) and systemic (*i.e.*, intraperitoneally, or intragastrically, or per-orally in drinking water) methods[1,3]. The therapeutic effects of BPC 157 on tendon and muscle healing was also investigated[3,103,104,107]. Moreover, there is a strong practical distinction from the standard angiogenic factors[3]. As pointed out, bFGF, EGF, and VEGF gastrointestinal tract studies demonstrated improved healing[3]. However, most of their corresponding studies on tendon, muscle, and bone injuries provide evidence of their increased presentation along with various procedures used to produce beneficial effects, compared to fewer studies *in vitro*[3]. *In vivo* healing evidence of these standard angiogenic growth factors was limited, commonly to local application. Evidently, providing the use of different carriers with corresponding peptides, there is an obvious attribution problem due to different combinations of peptide + carrier complex. Thereby, for the standard growth factors and use of different carriers, there is inadequate evidence due to diverse healing evidence with diverse carriers and delivery systems[3]. Contrary to this, BPC 157, using the same regimens as gastrointestinal healing studies (always given alone, without carrier), improves tendon, ligament and bone healing, accurately implementing its own angiogenic effect in healing[3]. Important for the particular effect on angiogenesis (particular in consideration of the corneal avascularity as "angiogenic privilege", no formation of corneal neovascularization which is essential for corneal wound healing)[136], later studies also included corneal wound healing and maintained corneal transparency (rescued total debridement of the corneal epithelium and perforating corneal incisions)[3]. The evidence that BPC 157 eye drops successfully close perforating corneal incisions in live rats is consistent with the cytoprotection/endothelial/mucosal protection model[3]. Regardless of its complex function in the corneal endothelium, endothelial maintenance by BPC 157 is also implicated in the healing of corneal ulcers in live rats [3]. Since this model is sensible, we suggest that BPC 157 should have tissue-specific healing effects[3]. Thus, we can envisage a particular healing potential in cytoprotection terms. From the method viewpoint[1,3], all of these lesions are within the scope of Robert's direct cell injury produced by direct contact[14-16].

In addition, there is quite indicative evidence about the simultaneous healing of different tissues. There is healing of various anastomoses (vessel, nerve, and gastrointestinal tract) and of various fistulas (surgically induced by defects and anastomosis creation), both external and internal[1]. Together, these findings showed that this additional extent (*i.e.*, the healing of other epithelia) may be combined in the simultaneous healing of different tissues, such as the simultaneous healing of fistula defects and the closing of fistulas[1]. A particular point is that these rat fistulas are severe, considering the significant size of the defect relative to the small size of the corresponding rat tissue[1]. Illustratively, rectovaginal fistulas in rats, with a 5 mm defect *vs* a 2.4 cm vaginal length, result in long-lasting defects and spontaneous patency of the fistula, leading to fecal matter leaking through the vagina; this actually mimics severe fistulas that may not spontaneously heal, thereby clearly emphasizing the beneficial effect of BPC 157[1].

Further, for BPC 157, in an additional cytoprotective extent (*i.e.*, other epithelia healing), epithelium protection is based on the extended relevance of the intragastric alcohol or NSAIDs on the stomach lesions commonly used in Robert's cytoprotection studies[1,14-16]. Namely, Robert's first epithelium protection, or the direct cell protection against cell injury produced by direct contact with the noxious agents, used intragastric alcohol or NSAIDs to induce stomach lesions[14-16]. Consequently, further evidence toward an additional extent (*i.e.*, other epithelia healing) follows other adverse effects of alcohol and NSAIDs and their consistent counteraction[1,7].

In addition to the 96% alcohol intragastric application-induced gastric lesions, BPC 157 largely counteracted chronic alcohol drinking-induced stomach lesions, liver failure, and portal hypertension, providing evidence that it may act as an alcohol antagonist[1]. Likewise, BPC 157 promptly counteracted acute alcohol (4 g/kg intraperitoneally) intoxication (*i.e.*, quickly produced and sustained anesthesia, hypothermia, increased ethanol blood values, 25% fatality, 90-min assessment period) given before or after ethanol[1]. In addition, BPC 157 counteracted chronic (withdrawal) alcohol intoxication, and was suggested as an alcohol antagonist[1], peripherally and centrally (of note, BPC 157 may attenuate the effect of thiopental anesthesia)[2].

Confronted with the over-dose application of various NSAIDs[1], similar beneficial effects occurred against various gastrointestinal lesions, and liver and encephalopathies; the worst damaged areas showed the most evident therapeutic effect[1]. Prolonged bleeding, consequent thrombocytopenia, and thrombocyte malfunctioning were also attenuated and/or counteracted[1,3,7]. Therefore, it seems that BPC 157 may particularly affect the functioning of the prostaglandins system[1,3,7] (interestingly, unlike NSAIDs and corticosteroids, BPC 157 strongly prevented adjuvant arthritis development and reversed the already formed adjuvant arthritis in rats[1]). The final clue may be that BPC 157 counteracted indomethacin-induced leaky gut syndrome[6]. It acts *via* increasing tight junction protein ZO-1 expression, and transepithelial resistance, inhibiting the mRNA of inflammatory mediators (*iNOS, IL-6, IFNγ*, and *TNF-α*), and increasing the expression of HSP 70 and 90, and antioxidant proteins, such as HO-1,NQO-1, glutathione reductase, glutathione peroxidase 2, and GST-pi[6]. Considering the importance of the leaky gut as an essential mechanism responsible for various severe systemic diseases, this may fully substantiate the significance of BPC 157 in the realization of that additional cytoprotective extent (*i.e.*, other epithelia healing)[6]. Also, BPC 157 counteracted other encephalopathies induced by various noxious events (insulin over-dose, cuprizone, multiple sclerosis mimicking neurotoxin, magnesium over-dose, brain trauma, spinal cord compression, and stroke)[1,2].

Epithelial pathway for adaptive cytoprotection

We also demonstrated that BPC 157 may regulate cytoprotection adaptation processes (adaptive cytoprotection)[1], functioning of the endogenous adaptive processes essential for permanent mucosal maintenance, and afford defensive reactions that start after any injurious event[1]. This follows Robert's connotation about the cytoprotection as a physiologic process[14-16] based on the adaptive cytoprotection evidence of Robert's small irritant to the stomach that precedes and protects against any subsequent major injurious event (*i.e.*, Robert's strong irritant to the stomach)[17]. Evidently, cytoprotective agents should have a more extensive action, participate in Robert's first epithelium protection, exhibit direct cell protection against cell injury produced by direct contact with noxious agents, and also participate in adaptive cytoprotection, in the next defensive reaction, and afford its final beneficial effects (*i.e.*, permanently attenuated lesion consequences)[1]. Thus, whatever the small irritant may be, whether exogenous (mild alcohol) or endogenous (*i.e.*, accumulated gastric juice, gastric acid, *i.e.*, made by gastrojejunal anastomosis), BPC 157 administration strongly contributed to the final attenuation of stomach lesions[1]. Thus, BPC 157 strongly contributes to and improves the presentation of adaptive cytoprotection processes[1]. Specifically, BPC 157 would improve adaptation processes in the damaged intestine, through a prostaglandin-related process, as it may be strongly aggravated by the application of NSAIDs[1]. In rats with short bowel surgery, the BPC 157 therapy, per-oral (in drinking water) and parenteral, causes constant weight gain (even more than preoperative values), with all three wall layers accordingly increased (*i.e.*, villus height, crypt depth, and muscle thickness [inner (circular) muscular layer] also increased), but no difference in jejunal and ileal diameters, and increased anastomosis strength. These beneficial effects of BPC 157 (*i.e.*, the weight gain in the BPC 157 rats with short bowel, all three wall layers accordingly increased) appear to be particular[1]. Namely, standard growth factors [even using a special application route (*e.g.*, subcutaneous pump)][137,138] at best may induce a decrease in weight loss[139-

142], with an increase in one layer, but not in the other. There is also some caution about the use of peptidergic agents, and adaptation processes, particularly on a long-term basis[140]. There is some growth of several tumor cell lines (EGF)[143,144], and hyperplastic lesions in the colon (subjects treated with GLP-2[145]). In contrast to adequately controlled adaptive processes, supportive evidence for BPC 157 (*i.e.*, BPC 157 administration showed no toxic effect and was limit test negative, with LD1 not achieved, and no side-effects in trials[1,3]) shows that it inhibits the growth of several tumor cell lines and counteracts the tumor-promoting effect of vascular endothelial growth factor (VEGF)[1,4]. In mice with C26 colon adenocarcinoma, BPC 157 counteracted tumor-cachexia and markedly prolonged survival[5]. BPC 157 afforded significant mitigating action against cancer cachexia-induced muscle degeneration, inflammation, and catabolism. BPC 157 significantly corrected deranged muscle proliferation as well as myogenesis, counteracted an increase in proinflammatory cytokines such as IL-6 and TNF-α looking at muscle metabolism relevant to cancer cachexia, as well as any changes in the expression of FoxO3a, p-AKT, p-mTOR, and P-GSK-3β[5]. Also important for its likely control of the adaptation processes, and prostaglandins-system function, in the short bowel rats, BPC 157 may counteract gastrointestinal lesions and the concomitant liver and brain lesions, and the additional aggravation that would otherwise appear with the application of diclofenac[1].

Also, it is possible that BPC 157 would afford an adaptive cytoprotective reaction regardless of the site of its initiation in the gastrointestinal tract[1]. Supporting evidence was also provided showing that adaptation cytoprotection accordingly occurs in the complete gastrointestinal tract, lasting for a considerable time, depending on the part that is initially targeted by the small irritant, stomach, duodenum, or colon, enabling the other parts to be more resistant to any subsequent strong irritant challenge[1]. Considering the eating and drinking habits, the adaptive cytoprotection in the gastrointestinal tract starts in the upper parts, in the stomach and duodenum, and may beneficially affect other parts (and thereby, adaptive cytoprotection occurs between stomach → stomach; stomach → duodenum, stomach → colon; duodenum → duodenum; duodenum → stomach, duodenum → colon)[1]. The colon seems to be distinctive and passive, as it could not initiate an adaptive cytoprotection response[1]. We used combinations of specific agents for initial small lesion and final more severe lesion [1 mL/rat of 25% or 96% ethanol intragastrically (stomach); cysteamine 40 mg/kg or 400 mg/kg subcutaneously (duodenum); cysteamine 40 mg/kg or 400 mg/kg intrarectally (colon)][1]. All of these ulcerogens were known to be inhibited by BPC 157[1].

Finally, with normal eating and drinking, Robert's adaptive cytoprotection (*i.e.*, Robert's small irritant to the stomach and Robert's strong irritant to the stomach) showed another essential point. We used the tongue as the initial target[1]. Within the very short time needed to swallow, the stomach is immediately affected, and the lesions are considerably less than those obtained with the direct instillation of alcohol into the stomach[1]. The application of BPC 157 considerably afforded this spontaneous healing effect, and additionally mitigated tongue, esophageal, gastric, and duodenal lesions, and reversed lower esophageal and pyloric sphincter impairment, through an action which seems to be NO system dependent[1]. Actually, it means that Robert's cytoprotection and adaptive cytoprotection following the direct application of noxious agents into the stomach completely avoid the regular defensive response that would occur with the tongue (and not the stomach) as the initial target.

On the other hand, this emphasizes the original significance of Robert's application of alcohol directly into the stomach, and thereby cytoprotection, as the direct cell protection against direct cell injury produced by direct contact with the noxious agents. Robert's regimen (alcohol applied in the stomach directly, by tube) regularly skips the existing defensive system (*i.e.*, starting with the tongue). Consequently, spontaneous rapid healing mechanisms remain skipped and not activated. Thereby, the essential ability of the cytoprotective agents would depend more on their own healing capacity, and their ability to act rapidly to induce healing.

Thus, such a huge range of healing effects, as noted with the applications of BPC 157, should be a prerequisite for realizing the endothelium pathway (blood vessel recruitment and activation towards defect or bypassing vessel occlusion)[1,7].

Endothelium pathway (endothelium maintenance to epithelium maintenance)

We already emphasized[1,7] the original cytoprotection studies[14-16,20,21] from the 1980s, which demonstrated significant stomach endothelium lesions, and verified the consequent change in stomach injuries *via* the endothelium pathway (endothelium maintenance → epithelium maintenance)[14-16,20,21]. Since that time, the cytoprotective endothelium pathway remained to be fully elaborated for therapeutic

purposes, both as a stomach therapy and as a more general therapy. With this high therapy requirement, we summarize the additional evidence. These highlights include the Virchow triad situation, endothelium injury, thrombus, and stasis, preceding the current demonstration that the administration of BPC 157 may finally induce the rapid recruitment of existing blood vessels and activate particular collateral pathways when confronted with vessel occlusion[1,7,9-11]. That pathway activation would accordingly compensate for the occlusion of vessels, and mitigate the consequent noxious chain of events[1,7,9-11]. We would analyze the possible cause in the indicated cytoprotection terms leading to an extension of the endothelium pathway to blood vessel recruitment and activation towards defect or bypassing vessel occlusion[1,7,9-11].

After presenting the initial cytoprotection concept (*i.e.*, epithelial pathway)[14-16], the endothelial pathway appeared as a further clarification of the development of stomach lesions in the concept of cytoprotection[14-16,20,21]. As a common point[47] appeared the evidence that with alcohol intragastric instillation, these vascular changes are early events, even before the appearance of gross hemorrhagic lesions, occurring within seconds or minutes during the development of moderate or severe gastric mucosal injury with interstitial hemorrhage and the necrosis of glandular epithelial cells[14-16,20,21]. Additionally, there is early stasis of mucosal blood flow and thrombi formation (within 30 s), often in regions without deep necrotic lesions. Even more, there was the rapid and complete cessation of blood flow to areas of mucosal damage consequent to ethanol administration[146]. Thus, although this was not initially claimed for the beneficial effect of prostaglandin and cysteamine application[14-16], we can envisage the particular Virchow triad presentation[1,7]. Unlike the initial claim for generalization of the epithelial stomach protection to other epithelial protection (cytoprotection → organoprotection)[14-16,19], at that time, these studies[14-16,20,21] made no attempt to generalize the findings seen for endothelium recovery in the stomach.

A strong reduction of Monastral blue staining and maintenance of the endothelium integrity after alcohol intragastric application was considered to be essential for the healing effect of BPC 157[1]. An interesting insight appeared after absolute alcohol instillation in the fully distended rat stomach, and gastric, esophageal, and duodenal lesions. Throughout the next 3 min, left gastric artery blood vessels clearly disappeared at the serosal site, indicative of the loss of vessel integrity and function. In contrast, constant vessel presentation could predict the beneficial effect of the applied agent. After pentadecapeptide BPC 157 instillation into the stomach, the vessel presentation remained constant, and lesions of the stomach, esophagus, and duodenum were inhibited[1]. Standards (atropine, ranitidine, and omeprazole) could only slightly improve the vessel presentation compared to control values, and only had a partial effect on the lesions[1]. Furthermore, for BPC 157, this maintenance of the endothelium integrity initially revealed a strong inhering angiogenic effect, which was more potent than those noted for standard antiulcer agents[1,7]. This appeared as a follow-up of "direct" cellular pharmacological treatment for ulcer with growth factors, notably bFGF and PDGF, that should result in the superior quality of ulcer healing by optimal angiogenesis, and thereby dense granulation tissue, as well as the complete re-epithelization and restoration with minimal inflammation[32]. Moreover, with BPC 157, it appears that angiogenesis was closely related to its wound healing and promotion, as well as healing in other tissues (*i.e.*, muscle, tendon, and ligament, known to be hypovascular tissues)[3]. In particular, in both muscle (transected or crushed) and transected tendon healing, we noted an increase in early angiogenesis (and the increased expression of VEGF, Factor VIII, and CD34), while late angiogenesis decreased (and the expression of VEGF, Factor VIII, and CD34 was decreased)[3]. The therapeutic potential (*i.e.*, acceleration of the blood flow recovery and vessel number in rats with hind limb ischemia) of pro-angiogenic BPC 157 is associated with VEGFR2 activation and up-regulation[107]. It also immediately triggered the internalization of VEGFR2 and subsequently activated the phosphorylation of VEGFR2 and Akt, and the eNOS signaling pathway without the need for other known ligands or shear stress [107].

On the other hand, as the reduction of Monastral blue staining and maintenance of the endothelium integrity after alcohol intragastric application is an immediate effect of BPC 157, we should consider the pleiotropic beneficial effects of BPC 157 in the entire gastrointestinal tract[1,7]. This should provide evidence that it effectively combines its particular mediator role (as an original anti-ulcer peptide which is stable in human gastric juice for longer than 24 h[12]) and thereby, in Robert's stomach cytoprotection, protection against direct cell injury made by direct contact of various noxious agents and required endothelium protection and maintenance of the endothelium function[1,7]. This has to be an immediate and rapid effect[1,7]. Thereby,

BPC 157, as a cytoprotective agent in the entire gastrointestinal tract, may both prevent and reverse the Virchow triad situation, and have an additional modulatory role[1,7].

As the first evidence of the implementation of the endothelium maintenance originally noted in stomach cytoprotection studies[1], BPC 157 prevents and reverses thrombosis formation after abdominal aorta anastomosis, or major vein occlusion[1,7,9-11]. Furthermore, BPC 157 may attenuate the prolonged bleeding that appeared after different injuries (*i.e.*, tail or leg amputation, organ perforation, and prolonged occlusion of the inferior caval vein) or anticoagulants, such as heparin or warfarin, and aspirin and the NOS substrate L-arginine application[1,7,9]. Also, it was shown that BPC 157 maintains thrombocyte function, without interfering with coagulation pathways[1,7]. Furthermore, there is evidence that BPC 157 counteracted stroke, given in reperfusion, after clamping of the common carotid arteries [*i.e.*, both early and delayed neural hippocampal damage, achieving full functional recovery (Morris water maze test, inclined beam-walking test, and lateral push test)][147]. Together, this may be a particular modulatory effect or NO-system-related effect[1]. BPC 157 may counteract both the NOS blocker L-NAME's pro-thrombotic effect and the NOS substrate L-arginine's anti-thrombotic effect in the same way that it counteracted both L-NAME-induced hypertension and L-arginine-induced hypotension, and could induce the NO release on its own, which is quite resistant to L-NAME application[1]. Finally, in addition to the VEGFR2-Akt-eNOS signaling pathway being activated without the need for other known ligands or shear stress[107], there is a direct effect on vasomotor tone (*i.e.*, specific activation of Src-Caveolin-1-endothelial nitric oxide synthase (eNOS) pathway)[106]. Also, it should be recalled that four major events (vascular constriction, loose platelet plug, fibrin mesh to insure stability of platelet plug, and dissolution of the clot) are implicated in the wound healing process and occur in a set order following the loss of vascular integrity[3]. Consequently, it may be not surprising that an agent implemented in wound healing, such as stable gastric pentadecapeptide BPC 157, should be effective in this particular way also in bleeding disorders[1,3], due to its innate cytoprotective effect, and the fact that it has been shown to be an effective therapy in wound healing[3].

Finally, in consideration of the previous original findings in cytoprotection endothelium studies (complete cessation of blood flow to areas of mucosal damage and rapid cloth formation consequent to ethanol administration[146]), and resolving of the presented Virchow triad circumstances, we suggested that the beneficial effect of cytoprotective agents should be related to the resolution of this noxious chain of events[1,7]. Thus, conclusive evidence involves confrontation with permanent major vessel occlusion, and therapeutic evidence that BPC 157 administration quickly recruits vessels to rapidly activate the collateral pathway which would adequately compensate for vessel occlusion and reestablish blood flow[1,7,9-11]. There, the alleviated peripheral vascular occlusion disturbances rapidly activated alternative bypassing pathways[1,7,9-11], appears to be an additional follow-up of its essential endothelium protection[1], which was long ago implemented as an essential class activity of cytoprotective agents[13]; however, in this way, this has so far only been implemented by the application and beneficial effects of the stable gastric pentadecapeptide BPC 157[1,7,9-11].

Rapid activation of a bypassing loop from the existing vessels

With BPC 157 therapy, when confronted with the occluded vessel in rats with distinctive vascular occlusion disturbances, we first reported the rapid activation of a bypassing loop recruited from the existing vessels (*i.e.*, intestinal arcade vessel network, or the left ovarian vein)[1,7,9-11]. The evidence [1,7,9-11] included the infrarenal occlusion of the inferior caval vein, left colic artery and vein occlusion ischemic/reperfusion ulcerative colitis, superior anterior pancreaticoduodenal vein-induced duodenal venous congestion lesions, bile duct ligation-induced liver cirrhosis and portal hypertension, temporary occlusion of the portal triad (Pringle maneuver)-induced ischemia-reperfusion injury[10], and suprahepatic occlusion of the inferior caval vein-induced Budd-Chiari syndrome[11]. This occurred in rats with a ligated part of the left colic artery and vein, ischemic/reperfusion colitis, or an infrarenal ligation of the inferior vena cava[1,7,9-11]. Evidently, the BPC 157 application-induced activation of the collateral pathways (the left ovarian vein and other veins in rats with infrarenal occlusion of the inferior caval vein) may rapidly resolve any systemic disturbances (*i.e.*, caval hypertension, aortal hypotension, heart dysfunction, thrombosis, and consequent thrombocytopenia, and induced bleeding prolongation in rats with infrarenal occlusion of the inferior caval vein)[1,7,9-11]. Likewise, there is also the local injury counteraction (attenuated/counteracted ischemia/reperfusion injury) in a rat study of the ischemic/reperfusion colitis[1,7]. As emphasized[1], with

part of the left colic vein and artery excluded by two ligations, along with BPC 157 application, blood vessels propagated toward the injury obstruction, bypassing it, interconnecting collaterals between arcades, and reestablishing the inside-outside point. In reperfusion, the application of BPC 157 after the initiation of full reperfusion with both ligations removed resulted in increased vessel presentation and arcade interconnections. With application of BPC 157 in ischemia as well as in reperfusion, the mucosal folds were recovered, and the pale areas were small and markedly reduced in size[1]. In the ischemia and even more so in the reperfusion, oxidative stress was counteracted, and the otherwise increased MDA (as a result of the lysis of endothelial cells[148,149]) and NO levels in colon tissue were found to be normal in rats that received BPC 157 bath treatment[1]. This occurs as before in both ischemic and reperfusion conditions in the various tissues (*i.e.*, the colon, duodenum, cecum, liver, and veins) and plasma[1,7,9-11]. Thus, the action of BPC 157 as a free radical scavenger (noted also in the other tissues, *i.e.*, gastrointestinal sphincters, stomach, duodenum, bowel adhesions, bladder, and brain[1,7,9-11]) may considerably contribute to its pleiotropic beneficial effects and maintain endothelial function. Notably, BPC 157 contains four carboxylic groups that could be active in scavenger process, and if they are reactivated (by, *e.g.*, glutathione or enzymes), the overall antioxidant activity could be very high [1].

Thus, relieving Virchow's triad situation is the particular activation of collateral pathways corresponding to the damaging occlusion [*i.e.*, mentioned passing through arcade vessels (occlusion of the left colic artery and vein) or the left ovarian vein (infrarenal occlusion of the inferior caval vein)][1,7,9-11]. As pointed out[1,7,9-11], the superior anterior pancreaticoduodenal vein-inferior anterior pancreaticoduodenal vein-superior mesenteric vein appears to counteract duodenal congestion lesions[1,7,9-11]. A porto-caval shunt appears with the portal vein-superior mesenteric vein-inferior mesenteric vein-rectal vein-left iliac vein-inferior caval vein pathway to counteract portal hypertension in rats with bile duct occlusion or ischemia-reperfusion injury following Pringle maneuver[1,7,9-11]. The inferior caval vein - azygos vein - left superior caval vein pathway appears to counteract Budd-Chiari syndrome in rats[1,7,9-11].

Of note, an adequate compensation regularly occurred. As pointed out in our venous occlusion studies[10,11], there is consistent evidence in rats with bile duct ligation. Preventing the development of portal hypertension, and the rapid reversal of the already established portal hypertension, are both among its additional beneficial effects[150]. We noted that BPC 157 therapy markedly abated jaundice, ascites, and nodular, steatotic livers with large dilatation of the main bile duct, increased liver and/or cyst weight, and decreased body weight[150]. Furthermore, the piecemeal necrosis, focal lytic necrosis, apoptosis, and focal inflammation, disturbed cell proliferation (Ki-67-staining), cytoskeletal structure in the hepatic stellate cell (α-SMA staining), and collagen presentation (Mallory staining) were all counteracted, providing evidence that BPC 157 may affect both liver fibrosis and portal hypertension [150]. Thus, this may be the principle seen in venous occlusion studies[10,11].

As previously reviewed[1], in rats with a perforated cecum, BPC 157 application rapidly reversed the regular noxious course, with the rapid disappearance of blood vessels at the cecum serosa (emptied/disappeared), thereby producing a large immediate defect, with bleeding, the leakage of fluid, increased oxidative stress, and disturbed NO-levels in cecal tissue. With BPC 157, there is immediate blood vessel recruitment and activation ("running") towards the site of injury[150], as was described in the "bypassing" of vessel occlusion *via* alternative pathways[9-11], which can likely cure rats and reestablish blood flow. Also, a small-vessel network appeared around the perforated defect with BPC 157 bath administration; cecal defect enlargement reversed to defect contraction (*i.e.*, each defect breaks blood flow) may be a result of the reestablishment of blood flow as well as the shortened bleeding time from the perforated cecum[1]. Less bleeding corresponds to the beneficial effects in rats with amputation, anticoagulant or aspirin application, or vein obstruction; direct defect closing corresponds to the closing of various fistula defects, which were also surgically created in corresponding tissues[1,7,9] (*i.e.*, all by Robert's direct injury to the cell by direct contact).

Along with these findings[1,7,9-11] is the beneficial effect of BPC 157 in rats with a damaged peritoneum. Endothelium maintenance → epithelium maintenance = blood vessel recruitment and activation ("running") towards the site of injury, also described as "bypassing" the occlusion *via* alternative ways[1,7,9-11], was seen with BPC 157 administration after parietal peritoneum excision with an underlying superficial layer of muscle tissue in rats to counteract failed vasculature, and finally to counteract the increased formation of adhesions. Rapid abundant vascular vessels in and close to the

defect mean that BPC 157 could interfere with the motion of the coagulation cascade once the peritoneum is damaged[1,7,9-11]. When two damaged peritoneal surfaces come into contact with each other, BPC 157 is likely to interfere the temporary role of fibrin in healing without adhesions that must be degraded by the fibrinolytic system for the restoration of normal tissue structure and function, as it reversed the healing that would result in fusion to form a connection, *e.g.*, an adhesion[151,152].

Finally, with the BPC 157 therapy in the Pringle maneuver in rats[10], severe preportal hypertension, temporary portal triad obstruction, ischemia, and short and prolonged reperfusion, we resolved the regular lack of adequate portocaval shunting as the most detrimental feature that should be counteracted[10]. With the stable gastric pentadecapeptide BPC 157, we noted the resolution of damage, either following occlusion or following the re-opening of the hepatic artery, portal vein, and bile duct. Therefore, in the portal triad obstruction syndrome in rats, in the rapidly activated manner, portal vein-superior mesenteric vein-inferior mesenteric vein-rectal vein-left iliac vein-inferior caval vein pathway would appear as specific activation of the collateral circulation, as the bypassing loop that can rapidly circumvent occlusions and decompress portal triad obstruction in rats upon BPC 157 administration[10]. That solution in rats with ischemia and reperfusion following the Pringle maneuver goes along with the resolution of oxidative stress, hemodynamic disturbances, severe portal and caval hypertension, aortic hypotension, rapid cloth formation in the portal vein, superior mesenteric vein, lienal vein, inferior caval vein, and hepatic artery, ascites, peaked P waves, tachycardia, increased serum values, and gross intestine, liver, lung, spleen, and heart lesions[19]. In particular, it goes along with the application of agents during reperfusion. Furthermore, the pentadecapeptide BPC 157 resolved the suprahepatic occlusion of the inferior caval vein in a Budd-Chiari syndrome model in rats[11]. Budd-Chiari syndrome was perceived as originally suggested[153,154], a hepatic venous outflow obstruction and its manifestation, regardless of cause, but this was mostly attributed to thrombosis, which can be located anywhere from the small hepatic veins to the entrance of the inferior vena cava into the right atrium[153,154]. Thereby, bypassing the occlusion in the rat Budd-Chiari syndrome along with pharmacotherapy treatment should be essential. BPC 157 therapy results in the rapidly activated azygos/hemiazygos vein bypassing pathway, upgrading an inadequate rescuing inferior-superior vena cava shunt to an adequate one, as well as a portocaval shunt[11]. Consequently, the caval and portal hypertension and aortal hypotension presented by Budd-Chiari syndrome rats were largely eliminated by BPC 157 therapy [11]. Largely attenuated consequent disturbances (rapid clot formation in the portal vein, superior mesenteric vein, splenic vein, inferior vena cava, hepatic artery, and coronary artery, as well as peaked P waves, significant ST elevation, tachycardia, gross organ lesions, and liver and spleen weight increases) together support this contention [11].

Thus, BPC 157 application may counteract a life-threatening syndrome[9-11]. Characterized by the multiple mutual cause-consequence relationships in vascular occlusion-induced syndrome presentation in rats, the generalized thrombosis and stasis, vascular failure, and heart dysfunction, lung congestion appears to be a common outcome (*i.e.*, time-dependent and time-independent features that the exudative phase features of acute respiratory distress syndrome)[9-11]. Acute lung injury is a primary component of multiple organ dysfunction syndrome triggered by intestinal ischemia-reperfusion. The results may be high mortality and acute lung injury[155,156], followed by liver failure (substantial congestion of central vein as well as branches of portal veins in portal triads), kidney congestion, prominent portal and caval hypertension, aortal hypotension, and consequential gastrointestinal hemorrhagic lesions[9-11]. Therefore, the previously mentioned beneficial effects, in elaborating the cytoprotective "epithelial pathway" (*i.e.*, counteracted various heart or liver lesions), including the combined and simultaneous healing of different tissues[1], may also be essential. In particular, the compensatory efficacy of new functional equilibrium ("endothelium pathway") with the activated specific functioning collateral pathways[13-15] is also ascertained with an important notification for the general pathology of the portal hypertension[1,7]. Namely, BPC 157 counteracted all portal hypertension presentations whatever the cause, post-hepatic, hepatic, and pre-hepatic [1,9-11].

In addition, as in venous-occlusion syndromes[9-11], BPC 157 also counteracted various lung lesions[1,7].

Finally, with holistic concepts, any criticisms about the cytoprotection concept, such as "cytoprotection", "as everything and nothing", and "cytoprotection which is not mechanism", and thereby, criticisms about peptides and cytoprotection, could not be avoided. The general point that animal studies *per se* may be cautious regarding their

results and the relative paucity of BPC 157 clinical data was also reported[1]. On the other hand, it should be noted that BPC 157 was proven to be efficacious in ulcerative colitis, both in clinical settings[157,158] and in experimental ischemic/reperfusion ulcerative colitis studies in rats and other ulcerative colitis models[1]. A particular point is the very safe profile (LD1 could be not achieved)[1,3,7], a point that was recently confirmed in a large study by Xu *et al*[159]. In this context, the role of the animal model is indispensable, and the practical evidence is even more important. Besides the majority of studies with BPC 157 conducted on rodents that were given an injection of the supplement, there have also been a considerable number of studies, particularly in gastrointestinal research, with intragastric application or peroral application in drinking water (regularly used in fistulas studies[1,3]), that are correspondingly effective. There are also studies in other species, *i.e.*, birds and insects (given in the food), which favor a more general effect of BPC 157 application[1,7]. Lastly, the suitability of the models used for the topic of cytoprotection, in particular, since Robert's original description of the cytoprotection in rats[14-16], evidently resolves the practical/theoretical consideration of the cause-consequence issue. Thus, the suited models and lesion counteraction clearly indicate the beneficial effects. The deciding result exemplified the resolved endothelium pathway (blood vessel recruitment and activation towards defect or bypassing vessel occlusion)[1], but the particular background still needs to be further elaborated. Note, the consistently used range of BPC 157 application (μg-ng) may also suggest a physiological role, in accordance with *in situ* hybridization and immunostaining for BPC 157 in the gastrointestinal mucosa, lung bronchial epithelium, epidermal layer of the skin, and kidney glomeruli[3]. Thereby, illustrative examples for further research may be the evidence that BPC 157 exhibited a specific effect on the Egr, Nos, Srf, Vegfr, Akt1, Plcγ, and Kras pathways in infrarenal occlusion-induced inferior caval vein syndrome in rats. This appears in a timely manner, to be increased, decreased, or unchanged, depending on whether the vessel was blinded (the right ovarian vein and inferior caval vein) or open and served as an alternative operating pathway (the left ovarian vein)[9]. Also, to support the beneficial effect of BPC 157 on brain lesions, given in reperfusion in stroke rats[147], BPC 157 therapy counteracted both early and delayed neural hippocampal damage, showing that achieving full functional recovery can restore recognition memory deficits along with a therapeutic effect[147]. mRNA expression studies at 1 h and 24 h, provided strongly elevated (*Egr1, Akt1, Kras, Src, Foxo, Srf, Vegfr2, Nos3,* and *Nos1*) and decreased (*Nos2* and *Nfkb*) gene expression (*Mapk1* not changed). This may be how BPC 157 acts[147].

In conclusion, Robert's cytoprotection concept[14-16] was initially of intense interest, but lately received the claim that the concept's foundation ("gastric cytoprotection") is still relevant[23]. Anyway, the essential rebuilding was lacking. Now, the concept has been reexamined for many major reasons (Figure 1): (1) The gastric pentadecapeptide BPC 157, thought to be an essential cytoprotective mediator that is native to and stable in human gastric juice, was noted to have a pleiotropic beneficial effect[1,3]; (2) With the administration of BPC 157, in prophylactic as well as in therapeutic regimens, there is evidence of the innate Robert's cell protection in the stomach epithelium against direct injury (which may be induced by various noxious agents) using either method of application, which provides the ability to realize the protection of other epithelia as well[1-3]; (3) BPC 157 effectively combines its particular mediator role (as an original anti-ulcer peptide that is stable in human gastric juice for longer than 24 h); therefore, in Robert's stomach cytoprotection, it has a protective effect against direct cell injury made by the direct contact of various noxious agents, requiring endothelial protection and the maintenance of endothelial function. This has to be an immediate and rapid effect[1]; (4) As first evidence of the implementation of the endothelial maintenance originally noted in stomach cytoprotection studies, BPC 157 prevents and reverses thrombosis after abdominal aorta anastomosis, or major vein occlusion[1,7,9-11]. Furthermore, BPC 157 may attenuate the prolonged bleeding that appeared after different injuries or anticoagulant, heparin or warfarin, and aspirin application[1,7,9-11]. Also, BPC 157 maintains thrombocyte function, without interfering with coagulation pathways[1,7,9-11]; and (5) The vessel recruitment activated collateral pathways to bypass vessel occlusion as a new conceptual point in the cytoprotection concept, and cytoprotective agent activity[1,7,9-11]. BPC 157 counteracted various venous occlusion-induced syndromes, inferior caval vein syndrome[9], ischemia/reperfusion injury following Pringle maneuver[10], and Budd-Chiari syndrome[11] in rats. Activation of the alternative collateral pathways to bypass occlusion, and reestablishing alternative blood flow result in counteraction of the consequent syndromes[1,7,9-11]. Due to the severe venous occlusion-induced disturbances, the high portal and caval hypertension and aortal hypotension, arterial

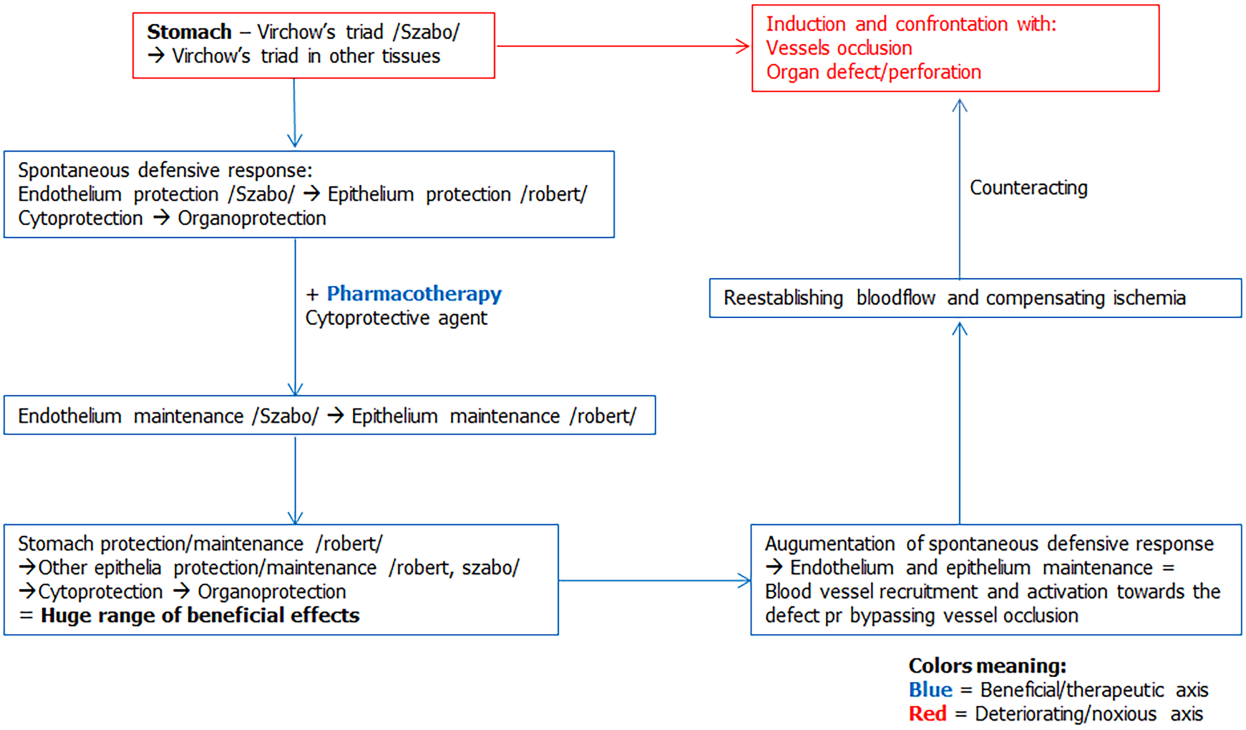

Figure 1 Summarizing the essential epithelium and endothelium protection interplay known in Robert's and Szabo's cytoprotection concept, and the role of the stable pentadecapeptide BPC 157 as a likely mediator, we suggest that BPC 157 may be a useful cytoprotective therapy. Hopefully, it may finally realize in the practice the huge theoretical importance of all aspects of the cytoprotection concept. Conceptually, there is a new point, namely, endothelium maintenance to epithelium maintenance (the recruitment of collateral blood vessels to compensate for vessel occlusion and reestablish blood flow or bypass the occluded or ruptured vessel). BPC 157 counteracts various venous occlusion-induced syndromes, inferior caval vein syndrome, ischemia-reperfusion injury following the Pringle maneuver, and Budd-Chiari syndrome in rats. Activation of the alternative collateral pathways to bypass occlusion, and reestablishing alternative blood flow, result in the counteraction of the full consequent perilous syndromes.

and venous thrombosis, both peripherally and centrally, and various organs lesions (*i.e.*, gastrointestinal, liver, kidney, heart, and brain) were all attenuated and/or eliminated[1,7,9-11]. Furthermore, this particular beneficial effect may be competing with the Virchow's triad situation that is commonly presented [*i.e.*, duodenal venous congestion lesions, perforated cecum, ischemic/reperfusion colitis, bile duct ligation-induced liver cirrhosis and portal hypertension, portal triad temporary occlusion (ischemia-reperfusion injury following the Pringle maneuver), and suprahepatic occlusion of the inferior caval vein (Budd-Chiari-syndrome)][1,7,9-11,150].

CONCLUSION

BPC 157 may be a useful cytoprotective therapy, which may finally result in the huge theoretical to practical importance of all aspects of the cytoprotection concept[1,7,9-11].

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REVIEW

Transfusion-transmitted hepatitis E: What we know so far?

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Abstract

Hepatitis E virus (HEV) is a major cause of viral hepatitis globally. There is growing concern about transfusion-transmitted HEV (TT-HEV) as an emerging global health problem. HEV can potentially result in chronic infection in immunocompromised patients, leading to a higher risk of liver cirrhosis and even death. Between 0.0013% and 0.281% of asymptomatic blood donors around the world have HEV viremia, and 0.27% to 60.5% have anti-HEV immunoglobulin G. HEV is infectious even at very low blood concentrations of the virus. Immunosuppressed patients who develop persistent hepatitis E infection should have their immunosuppressant regimen reduced; ribavirin may be considered as treatment. Pegylated interferon can be considered in those who are refractory or intolerant to ribavirin. Sofosbuvir, a nucleotide analog, showed modest antiviral activity in some clinical studies but sustained viral response was not achieved. Therefore, rescue treatment remains an unmet need. The need for HEV screening of all blood donations remains controversial. Universal screening has been adopted in some countries after consideration of risk and resource availability. Various pathogen reduction methods have also been proposed to reduce the risk of TT-HEV. Future studies are needed to define the incidence of transmission through transfusion, their clinical features, outcomes and prognosis.

Key Words: Hepatitis E virus; Acute and chronic hepatitis; Immunosuppression; Blood transfusion; Transplantation

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Core Tip: Transfusion-transmitted hepatitis E virus (HEV) is an emerging global health concern. In immunocompromised patients, chronic HEV infection increases the risk of liver cirrhosis. The prevalence of viremia and anti-HEV immunoglobulin G in asymptomatic blood donors varies widely between countries but even low concentrations of HEV in blood components are infectious, and in most countries blood donations are not routinely screened for HEV. Treatment of persistent infection includes modification of the immunosuppressant regimen followed by ribavirin. The need for screening of HEV in all blood donations remains controversial. Strategies to reduce de novo HEV infection should also be emphasized.

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INTRODUCTION

Hepatitis E virus (HEV) was first discovered as an epidemic of non-A, non-B hepatitis in the 1980s[1], and has since become one of the major global causes of viral hepatitis. The World Health Organization estimated that HEV caused approximately 44000 deaths in 2015, and accounted for 3.3% of global deaths related to viral hepatitis[2]. A recent meta-analysis concluded that approximately 939 million of the global population have ever experienced HEV infection, and 15 to 110 million individuals have recent or ongoing infection[3]. The infection is generally self-limiting; however, it poses a threat to some vulnerable patients resulting in a significant burden of inpatient admissions, chronic infection, organ failure, and death[4]. The mortality rate can be greater than 20% in patients with chronic liver disease, cirrhosis, or pregnancy [4,5]. With a high HEV serological prevalence among the global population, the safety of blood products has become a public health concern. Herein, we review existing evidence on transfusion-transmitted HEV (TT-HEV), and the implications for screening of blood donations.

VIROLOGY

HEV is a positive-sense, single-stranded RNA icosahedral virus belonging to the genus *Orthohepevirus* within the Hepeviridae family[6]. *Orthohepevirus A* has eight distinct genotypes, of which HEV-1, -2, -3 and -4 infect humans[7]. HEV genotype C1, belonging to the species *Orthohepevirus C*, circulates in rats and can cause cross-species infection and sporadic zoonotic transmission to humans[8].

HEV exists in urine or feces as non-enveloped virions encased by a capsid. It circulates in blood in a membrane-associated, quasi-enveloped form (eHEV) which is considered to be less contagious[9]. The entry mechanisms for HEV are not well characterized, but once the genomic RNA is uncoated and delivered to the cytosol, the replication cycle is initiated[10]. The viral release that initiates subsequent infection requires multivesicular bodies through endosomal sorting complexes required for transport[11].

EPIDEMIOLOGY

The prevalence rates of HEV antibody are higher in developing countries than in developed countries[12]. The highest anti-HEV immunoglobulin G (IgG) seropositivity rate has been reported in Africa with a mean of 21.76%, followed by Asia (15.80%), Europe (9.31%), North America (8.05%), South America (7.28%), and Oceania (5.99%). In addition, the reported anti-HEV immunoglobulin M (IgM) seroprevalence rate was 3.09%, 1.86%, 0.79%, 0.22% and 2.43% in Africa, Asia, Europe, North America, and South America, respectively[3].

Among the four major genotypes that can infect humans, HEV-1 and -2 are mostly found in developing countries including Asia, Africa, Latin America, and Mexico. Infection is mainly transmitted *via* fecally contaminated water, but occasionally also by person-to-person and vertical transmission[13]. Hepatitis E occurs as outbreaks as well as sporadic cases of acute hepatitis, with the preponderance of cases among adolescents and young adults. When stratified by age, the estimated incidence of HEV-1 and -2 infection is roughly between 0.5% and 1.0% for ages 0 to 15 years, with rates increasing to between 1.0% and 1.4% for ages 15 years to 20 years, then falling rapidly to a lower rate of 0.2% and below in individuals older than 30 years[14].

HEV-3 accounts for most of the autochthonous infection in developed countries while HEV-4 is mainly found in Asia and sporadically in Europe[15,16]. The reported seroprevalence of HEV-3 ranged from 0.6% to 52.5% in Europe, 6% in United States, 3 to 16% in United Kingdom and up to 52% in some regions of France[17]. HEV-3 and HEV-4 are zoonotic viruses which are frequently transmitted *via* food, close contact with animals, or transfusion of viremic blood units[18].

CLINICAL FEATURES AND EXTRAHEPATIC MANIFESTATIONS

The incubation period following exposure to HEV ranges from 2 to 6 wks. HEV infection commonly takes a clinically silent, asymptomatic course with around 5% to 30% of infected individuals developing acute hepatitis[19]. Symptoms of acute hepatitis include fever, malaise, anorexia, vomiting, followed by jaundice, tea-colored urine, and hepatomegaly[20]. It is then followed by a convalescent phase with gradual recovery within a few weeks in immunocompetent patients[21]. Acute liver failure is rare and occurs more frequently in middle-aged/elderly patients[22]. Fulminant hepatitis with fatal outcome is uncommon, but has been observed in pregnant women or in patients with pre-existing liver disease. The development of fulminant hepatitis appears to be related to host-specific factors rather than virus genotype, variants, or specific substitutions[23]. HEV superinfection may trigger liver decompensation in patients with chronic liver disease or cirrhosis, resulting in acute-on-chronic liver failure, which is associated with significant short-term mortality[24,25]. Further research is needed to clarify the clinical features, course of illness, and prognosis of patients with decompensated cirrhosis who develop HEV infection.

HEV-3 and HEV-4 can persist in immunocompromised patients resulting in chronic infection, defined as viral replication lasting for more than 3 to 6 mo[26]. It has been well described in patients after solid organ or stem cell transplant, hematology patients receiving chemotherapy, or HIV-infected patients[27-32]. The prevalence of anti-HEV IgG was about 11.6% and viral RNA was 2% in solid organ transplant recipients[33]. In solid organ transplant recipients who were positive for HEV RNA, more than 60% developed chronic hepatitis[33].

The natural history of chronic hepatitis E infection is not well understood[34]. In liver transplant recipients infected by HEV, histological analyses of liver biopsy revealed atypical morphology that is distinct from those in immunocompetent patients during early phases of infection[35]. Proliferation of, and cytokine production by, CD4+ and CD8+ T-cells were impaired in patients with persistent HEV viremia[36]. Chronic hepatitis E leads to liver fibrosis and cirrhosis. Cases of HEV-related hepatocellular carcinoma have been reported[37].

Although HEV predominantly infects hepatocytes, it may also affect other organs and present as extrahepatic manifestations. The mechanisms by which HEV can induce extrahepatic manifestations are not fully understood, but hypotheses include direct cytopathic tissue damage by extrahepatic replication, or immunological processes induced by an overwhelming host immune response[38]. Details of extrahepatic manifestations are shown in Table 1[39-44].

PREVALENCE IN BLOOD DONORS

Viremia

The prevalence of HEV RNA in blood donors varies around the world. (Table 2)[45-78]. Most countries have a low prevalence of HEV viremia, ranging from 0.0013% to 0.086%. A relatively higher rate of viremia was reported in Germany (0.12%) and China (0.281%)[49,70]. A meta-analysis of 10 studies from China showed a pooled prevalence of HEV RNA of 0.1%[79]. The actual prevalence might have been underes-

Table 1 Extrahepatic manifestations associated with hepatitis E virus infection

System	Extrahepatic manifestations
Neurological	Guillain-Barré syndrome (GBS)
	Neuralgic amyotrophy
	Neuropathy
	Bell's palsy
	Encephalitis
	Transverse myelitis
	Myositis
	Myasthenia gravis
	Pseudotumor cerebri
	Seizure
Renal	Decrease glomerular filtration rate
	Glomerulonephritis
	Nephrotic syndrome
	Mixed cryoglobulinemia
Hematological	Thrombocytopenia
	Hemolytic anemia
	Aplastic anemia
	Hemophagocytic syndrome
	Monoclonal gammopathy of uncertain significance (MGUS)
Others	Thyroiditis
	Pancreatitis
	Myocarditis
	Polyarthritis

timated as some studies included in the meta-analysis conducted RNA detection only in those donors who were positive for anti-HEV IgM or antigen[79].

The prevalence of HEV-3 and -4 is affected by dietary habits[80]. Consumption of raw pork tartare and undercooked pork liver may represent a relevant risk factor for HEV infection in Germany[49]. Regular consumption of pork meat and shellfish were also reported in the viremic donors in China[70].

Since 70% of infections with HEV-3 and -4 are asymptomatic[81], it can be difficult to identify infected blood donors, as viremia occurs primarily during the pre-icteric phase[82]. Katiyar *et al*[72] described anti-HEV IgG positivity in 60.5% of the tested donors in India and yet none of them were positive for HEV RNA. In India, human HEV is caused exclusively by the HEV-1 genotype, which causes brief hepatitis and seldom results in chronic infection[83,84]. The difference in endemicity between HEV genotypes may affect the propensity to cause symptomatic disease and viral persistence, which in turn influences the likelihood of viremia among blood donors.

Other factors influencing the reported prevalence of HEV viremia are the sensitivity and plasma pool size of the various nucleic acid test screening platforms used[85]. For example, 33 of 90 donations with a viral load of 20-750 IU/mL were positive when tested individually but missed in the pooled screening in a study by Hogema *et al*[57]. Delage *et al*[66] revealed a low prevalence (n = 11/50765) and viral loads of HEV-RNA in Canadian blood donors based on individual nucleic acid amplification techniques (NAT). They postulated that if pooled NAT was used, only two positive donations with viral loads > 1000 IU/mL would have been detected. The true frequency of viremia in blood donors in studies using pooled NAT could be underestimated due to a dilution effect. Vollmer *et al*[86] found that screening using individual NAT yielded an approximately 50% higher detection frequency compared with NAT of a mini-pool of 96 samples; nevertheless, samples exclusively positive for individual NAT had a

Table 2 Hepatitis E virus ribonucleic acid prevalence in donor, only studies include more than 1000 study subjects are included

Ref.	Country	Initial screening method	Number of donations screened	Number positive donations	Prevalence (95%CI)	HEV genotype: *n*/*N*	Median (range) viral load, IU/mL	Outcome of recipient
Europe								
Fischer *et al*[45], 2015	Austria	RT-PCR (plasma pool of 96 samples) with 95% LOD 11.6 IU/mL	58915	7	0.012%	3: 7/7	(2200 to 290000)	N/A
Vercouter *et al* [46], 2019	Belgium	RT-PCR (plasma pool of 6 samples) with 95% LOD 18.6 IU/mL	38137	7	0.018%	N/A	(153 to 8710)	N/A
Harritshøj *et al* [47], 2016	Denmark	TMA assay on individual plasma with 95% LOD 7.9 IU/mL	25637	11	0.043% (0.02% - 0.07%)	3 (in 2 samples)	13 (unquantifiable to 920)	(1) Look-back testing was performed in 7 recipients; all were tested negative for HEV RNA and anti-HEV IgM; (2) No recipient developed transaminitis; and (3) One patient had strongly positive anti-HEV IgG assay which may indicate recent HEV infection or secondary immune response by HEV re-exposure.
Gallian *et al*[48], 2014	France	RT-PCR (plasma pool of 96 samples) with 95% LOD 23 IU/mL	53234	22	0.045% (0.043%-0.047%).	3c: 5/14; 3f: 8/14; 3 1/14	(468 to 5155800)	N/A
Westhölter *et al* [49], 2018	Germany	RT-PCR (plasma pool of 24 samples) with 95% LOD 18.6 IU/mL	18737	23	0.123%	3: 6/7	(120 to 11200000)	(1) Retrospective analysis of 4 viremic donors showed that they were HEV-positive in previous donations; (2) In 3 donors, testing of the previously donated blood in pools of 24 samples failed to identify viremic donations but were tpositive in unpooled samples; (3) Fourteen recipients had received HEV RNA positive blood products; (4) One immunosuppressed recipient tested positive for HEV RNA, developed acute on chronic liver failure, and died; and (5) One immunocompetent recipient developed acute self-limited episode of hepatitis E
Dreier *et al*[50], 2018	Germany	RT-PCR with 95% LOD 4.7 IU/ml for FFP, platelet concentrates, and RBC supernatant; 95% LOD 8.9 IU/mL for RBCs.	235524	182	0.077%	3: 4/4	(< 25 to 69.4)	(1) Nine viremic donations were transfused to 6 different recipients; (2) Two recipients were immunocompromised (heart transplantation and leukemia); (3) Two recipients died shortly after transfusion for reasons other than HEV infection; and (4) None of the other 4 recipients developed acute HEV infection or had detectable HEV RNA / anti-HEV IgG
Corman *et al*[51], 2013	Germany	RT-PCR (plasma pool of 96 samples mixed in metapools of 20)	93955	14	0.015%	3: 14/14	(3.1 to 4.8 Log_{10} IU/mL)	N/A
Vollmer *et al*[52], 2012	Germany	RT-PCR (plasma pool of 48 samples) with 95% LOD 4.7 IU/ml	16125	13	0.081%	3: 13/13	(13 to 68100)	N/A
Baylis *et al*[53], 2012	Germany	RT-PCR (plasma pool of 96 samples) with 95% LOD 250 IU/mL	18,100	4	0.022%	3	(3.26 to 5.35 log_{10} copies/mL)	Donations screened positive for HEV were excluded from pharmaceutical production
O'Riordan *et al*	Ireland	TMA assay with 95% LOD	24985	5	0.020%	3: 3/3	(10 to 44550)	N/A

[54], 2016		5.5 IU/mL			(0.0065%-0.0467%)			
Spreafico *et al* [55], 2020	Italy	TMA assay on individual plasma with 95% LOD 7.9 IU/mL	9726	1	0.010% (0.00%-0.06%)	N/A	N/A	N/A
Spada *et al*[56], 2018	Italy	RT-PCR, plasma pool and sensitivity varies according to anti-HEV IgG and IgM status	10011	0	N/A	N/A	N/A	N/A
Hogema *et al*[57], 2015	Netherlands	RT-PCR (plasma pool of 96 samples) with 95% LOD 38.4 IU/mL with the EasyMag extraction method and 10.3 IU/mL using Qiagen extracts	59474	41	0.069%	3c: 15/17; 3f: 2/17	N/A	N/A
Slot *et al*[58], 2013	Netherlands	RT-PCR (plasma pool of 48 or 480 samples) with 95% LOD 25 IU/mL	40176	13	0.032%	3: 16/17	(< 25 to 470000)	N/A
Grabarczyk *et al* [59], 2018	Poland	TMA assay on individual plasma with 95% LOD 7.9 IU/mL	12664	10	0.079% (0.043%-0.145%)	3i: 2/3; 3c: 1/3	(16 to 6586 in 4 patients with positive results in qPCR)	N/A
Rivero-Juarez *et al* [60], 2019	Spain	RT-PCR (plasma pool of 8 samples) with sensitivity 670 IU/mL	11313	4	0.035% (0.01%-0.09%)	3: 4/4	(10788 to 2000000)	(1) Five patients received transfusions from HEV-infected donors; and (2) None of them showed an increase in alanine aminotransferase levels after transfusion
Sauleda *et al*[61], 2015	Spain	TMA assay on individual plasma with 95% LOD 7.9 IU/mL	9998	3	0.030% (0.01%-0.09%)	3f (in 1 sample)	(250 to 2755)	N/A
Baylis *et al*[53], 2012	Sweden	RT-PCR (plasma pool of 96 samples) with 95% LOD 250 IU/mL	95835	12	0.013%	3	(3.20 to 5.68 $\log_{10}$ copies/mL)	Donations screened positive for HEV were excluded from pharmaceutical production
Harvala *et al*[62], 2019	United Kingdom	RT-PCR (plasma pool of 24 samples) with 95% LOD 18.6 IU/mL	1838747	480	0.026%	3c: 112/149; 3e: 21/1493f: 12/149; 3a: 1/149; 2 distantly related to 3h, and 1 clustered distantly with 3a	883 (1 to 3230000)	N/A
Thom *et al*[63], 2018	United Kingdom	RT-PCR (plasma pool of 24 samples)	94302	38	0.040%	3: 10/10	N/A	N/A
Hewitt *et al*[64], 2014	United Kingdom	RT-PCR (plasma pool of 24 samples)	225000	79	0.035%	3: 79/79	3900 (50 to 2.37 × 10^6)	(1) Forty-three patients who had received blood components from HEV-infected donors were followed up; (2) The overall transmission rate was 42% (18 of 43 exposed patients); (3) One recipient developed clinical hepatitis and 4 recipients developed asymptomatic transaminitis; and (4) Four heavily

								immunosuppressed patients had delayed (37-38 wk) seroconversion or no antibodies detected
Cleland *et al*[65], 2013	United Kingdom	Nested PCR (plasma pool of 24 samples) with 95% LOD 201 IU/mL	43560	3	0.0069%	3: 3/3	N/A	N/A
North America								
Delage *et al*[66], 2019	United States	RT-PCR on individual samples with 95% LOD 18.6 IU/mL	50724	3	0.0059%	3: 2/3; genotyping was unsuccessful in 1 patient	(23 to 1420)	N/A
	Canada		50765	11	0.022%	3 (in 1 sample)	(< 10 to 3080)	
Roth *et al*[67], 2017	United States	RT-PCR (plasma pool of 96 samples) with 95% LOD 18.6 IU/mL	128021	4	0.003%	3a: 3/3	(3.0 to 3.8 log IU/mL)	N/A
Stramer *et al*[68], 2016	United States	TMA assay on individual plasma with 95% LOD 7.9 IU/mL	18829	2	0.011% (0.0018%-0.351%)	N/A	14 IU/mL in one sample	N/A
Xu *et al*[69], 2013	United States	RT-PCR (plasma pool of 7 to 8 samples) with 95% LOD 400 IU/mL and nested PCR with 95% LOD 200 IU/mL	1939	0	N/A	N/A	N/A	N/A
Baylis *et al*[53], 2012	United States	RT-PCR (plasma pool of 96 samples) with 95% LOD 250 IU/mL	51075	0	N/A	N/A	N/A	N/A
Asia								
Wen *et al*[70], 2018	China	RT-PCR on individual plasma	5345	15	0.281%	One 4h, another one clustered between genotype 2 and 4i	N/A	N/A
Tsoi *et al*[71], 2019	Hong Kong	RT-PCR with 95% LOD 7.89 IU/mL	10000	2	0.02%	4 (in 1 sample)	N/A	N/A
Katiyar H *et al* [72], 2018	India	RT-PCR (plasma pool of 3 samples) with LOD 100 IU/mL	1799	0	N/A	N/A	N/A	N/A
Minagi T *et al* [73], 2016	Japan	RT-PCR (plasma pool of 50 or 500 samples) with 95% LOD 152 IU/mL	620140	36	0.0058%	3: 36/36	(< 1.69 to 7.22 $\log_{10}$ copies/mL)	N/A
Intharasongkroh *et al*[74], 2019	Thailand	RT-PCR (plasma pool of 6 samples) with 95% LOD 53.5 IU/mL	30115	26	0.086%	3: 6/6	N/A	N/A

Others								
Hoad *et al*[75], 2017	Australia	TMA (plasma pool of 6 samples)	74131	1	0.0013%	N/A	180	N/A
Shrestha *et al*[76], 2016	Australia	TMA assay on individual plasma with 95% LOD 7.9 IU/mL	14799	1	0.0068% (0.0002%-0.0376%	3	15000	N/A
Hewitt *et al*[77], 2018	New Zealand	RT-PCR (plasma pool of 8 to 12 samples)	5000	0	N/A	N/A	N/A	N/A
Maponga *et al* [78], 2020	South Africa	TMA assay on individual plasma with 95% LOD 7.9IU/mL	10000	1	0.01%	3	79000	All donations from donors with active HEV infection were discarded

CI: Confidence interval; FFP: Fresh frozen plasma; HEV: Hepatitis E virus; Ig: Immunoglobulin; LOD: Limit of detection; RBC: Red blood cells; RNA: Ribonucleic acid; RT-PCR: Real time polymerase chain reaction; TMA: Transcription mediated amplification.

corresponding viral load of < 25 IU/mL. High-sensitivity individual NAT can yield false-positive results[55]. Whether the identification of low-level HEV-positive donors translates into clinical significance and whether a single individual NAT is adequate remain undefined.

Antibodies

In addition to direct detection of HEV RNA, another important indirect assessment of HEV burden is the prevalence of anti-HEV IgM and IgG in blood donors (Table 3)[45,46,54-56,58,59,61,63,65,68,69,71,72,77,87-124]. HEV IgG prevalence increases with age which likely represents the cumulative effect of HEV exposure over a lifetime, especially as IgG antibodies can persist for decades[81]. The absence of detectable antibodies in donors was related to an increased risk of transfusion transmission of HEV[64]. However, the presence of anti-HEV IgG may not always be protective as multiple HEV reinfections could occur despite pre-existing antibodies[125]. Various HEV strains in serum are capable of replication in cell culture and generate infectious particles in the culture supernatant despite the coexistence of antibodies[126]. Anti-HEV IgM could be used to detect recent infection yet it failed to identify infected donors during the window period. For example, a meta-analysis of data from 28 countries found that only 26.6% of viremic blood units had positive anti-HEV antibodies[127]. In another study by Tedder *et*[128] *al*, a significant portion of viremic individuals (n = 57/79) were seronegative at the time of donation. Anti-HEV IgM sometimes exhibits unexpectedly long persistence for up to 3 years after a self-limiting acute hepatitis E episode[129]. Only a minority of anti-HEV IgM-positive donors have detectable RNA[58,93,103,109]. All these findings suggest that detection of anti-HEV IgG or IgM alone may not provide effective screening of HEV in blood donors.

Table 3 Seroprevalence of hepatitis E in blood donors

Ref.	Country	Number of donations screened	Assay used	Number of samples positive for HEV IgG antibodies	Anti-HEV IgG prevalence (95%CI)	Number of samples positive for HEV IgM antibodies	Anti-HEV IgM prevalence (95%CI)	Number of samples positive for HEV RNA in anti-HEV IgM positive	Viral load, IU/mL	Genotype
Europe										
Fischer *et al*[45], 2015	Austria	1203 (from HEV RNA negative donors)	Wantai	163	13.55% (11.6%-15.5%)	N/A	N/A	0	N/A	N/A
Vercouter *et al* [46], 2019	Belgium	356 (from HEV RNA negative donors)	Wantai	31	8.71% (6.20%-12.10%)	0	N/A	0	N/A	N/A
Miletić *et al*[87], 2019	Croatia	1036	3 commercial ELISA assays were used, only findings with highest prevalence are shown	209	20.17%	46	4.44%	0	N/A	N/A
Holm *et al*[88], 2015	Denmark	504	In-house NIH assay	54	10.7% (8.2%-13.7%)	N/A	N/A	N/A	N/A	N/A
			Wantai	100	19.8% (16.4%-23.6%)					
Dimeglio *et al* [89], 2018	France	300	Wantai	23	7.7% (4.9%-11.3%)	2	0.6% (0.1%-2.4%)	0	N/A	N/A
Juhl *et al*[90], 2013	Germany	1019	RecomWell assay and Western blot	69	6.8% (5.3%-8.3%)	N/A	N/A	N/A	N/A	N/A
Dalekos *et al* [91], 1998	Greece	3016	Abbott assay and Western blot	8	0.27%	0	N/A	N/A	N/A	N/A
O'Riordan *et al* [54], 2016	Ireland	1076	Wantai	57	5.3% (4.0%-6.8%)	2	0.19%	0	N/A	N/A
Spreafico *et al* [55], 2020	Italy	767	DiaPro	52	6.8% (5.1%-8.8%)	0	N/A	0	N/A	N/A
Spada *et al*[56], 2018	Italy	10011	Wantai	869	8.7% (8.14%-9.25%)	46	0.4% (0.34% - 0.61%)	0	N/A	N/A
De Sabato *et al* [92], 2017	Italy	170	Bio-Chain Institute and Western blot	15	8.82%	3	1.76%	0	N/A	N/A
Lucarelli *et al* [93], 2016	Italy	313	Wantai	153	48.9% (43%-54%)	2	0.6% (0.08%-2.3%)	1	100	3
Puttini *et al*[94], 2015	Italy	132	EIAgen HEV IgG kit	12	9.1%	N/A	N/A	N/A	N/A	N/A

Hogema *et al* [95], 2014	Netherlands	513	Wantai	58	11.31%	N/A	N/A	N/A	N/A	N/A
Slot *et al*[58], 2013	Netherlands	5239	Wantai	1401	26.7% (25.6%-28.0%)	49	0.94%	4	Range: < 25 to 3700	3
Grabarczyk *et al* [59], 2018	Poland	3079	Wantai	1340	43.52% (41.78%-45.28%)	39	1.27% (0.93%-1.73%)	N/A	N/A	N/A
Sauleda *et al* [61], 2015	Spain	1082	Wantai	216	19.96% (17.60%-22.32%)	13	1.20%	0	N/A	N/A
			Mikrogen	116	10.72% (8.90%-12.60%)					
Mateos *et al*[96], 1999	Spain	863	Abbott assay and Western blot	34	3.9%	0	N/A	N/A	N/A	N/A
Niederhauser *et al*[97], 2018	Switzerland	3609	Wantai	737	20.4% (19.1%-21.8%)	N/A	N/A	N/A	N/A	N/A
Kaufmann *et al* [98], 2011	Switzerland	550	MP Biomedicals	27	4.9%	N/A	N/A	N/A	N/A	N/A
Thom *et al*[63], 2018	United Kingdom	1714	Wantai	104	6.1% (5.0%-7.3%)	N/A	N/A	N/A	N/A	N/A
Cleland *et al*[65], 2013	United Kingdom	1559	Wantai	73	4.7% (3.6%-5.8%)	0	N/A	N/A	N/A	N/A
Beale *et al*[99], 2011	United Kingdom	262	Wantai	31	11.8%	4	1.5%	0	N/A	N/A
North America										
Zafrullah *et al* [100], 2018	United States	5040 (from HEV RNA negative donor)	DSI	569	11.29%	146	2.90%	0	N/A	N/A
			MP Biomedicals	537	10.65%	93	1.85%			
			Wantai	619	12.28%	34	0.67%			
Stramer *et al* [68], 2016	United States	4499	MP Biomedicals	329	7.3% (6.6%-8.1%)	26	0.58% (0.39%-0.85%)	N/A	N/A	N/A
Xu *et al*[69], 2013	United States	1939	Wantai	364	18.8% (17.0%-20.5%)	8	0.4% (0.1%-0.7%)	0	N/A	N/A
South America										
Di Lello *et al* [101], 2020	Argentina	391	DiaPro	44	11.3%	8	2.0%	0	N/A	N/A
Bangueses *et al* [102], 2020	Uruguay	400	DiaPro	40	10%	19	4.75%	3	N/A	3

Asia										
Nouhin *et al* [103], 2016	Cambodia	301	Wantai	85	28.2% (23.4%-33.5%)	3	1.0% (0.01%-1.8%)	1	956	3
Chen *et al*[104], 2019	China	4044	Wantai	799	19.8% (18.6%-21.0%)	43	1.1% (0.8%-1.4%)	2	N/A	4
Wen *et al*[70], 2018	China	5345	Wantai	1227	22.96%	38	0.71%	15	N/A	N/A
Wang *et al*[105], 2017	China	9069	Wantai	2682	29.57%	131	1.44%	5	N/A	N/A
Ma *et al*[106], 2015	China	816	Wantai	172	21.1%	4	0.5%	0	N/A	N/A
Ren *et al*[107], 2014	China	10741	Wantai	2945	27.42%	109	1.01%	0	N/A	N/A
Zhuang *et al* [108], 2014	China	486	ELISA based on antigen protein pB166 and MPII	113	23.3%	N/A	N/A	N/A	N/A	N/A
Tsoi *et al*[71], 2019	Hong Kong	2000	Wantai	315	15.8% (14.2%-17.4%)	16	0.8%	0	N/A	N/A
Tripathy *et al* [109], 2019	India	2447	Wantai	433	17.70% (16.23%-19.26%)	5	0.20%	2	Ranged from 3.5×10^4 to 4.6×10^5 copies/mL	1
Katiyar *et al*[72], 2018	India	633	Wantai	383	60.5%	N/A	N/A	0	N/A	N/A
Gajjar *et al*[110], 2014	India	460	DiaPro	N/A	N/A	22	4.78%	N/A	N/A	N/A
Parsa *et al*[111], 2016	Iran	700	DiaPro	42	6.0%	5	0.71%	5 (only 50 seropositive blood donors were tested)	N/A	1
Hesamizadeh *et al*[112], 2016	Iran	559	DiaPro	45	8.05%	N/A	N/A	N/A	N/A	N/A
Naeimi *et al* [113], 2015	Iran	628	HEV IgG, Pasto, Iran	105	16.72%	N/A	N/A	N/A	N/A	N/A
Ehteram *et al* [114], 2013	Iran	530	DiaPro	76	14.3%	N/A	N/A	N/A	N/A	N/A
Taremi *et al* [115], 2007	Iran	399	DiaPro	31	7.8%	N/A	N/A	N/A	N/A	N/A
Takeda *et al* [116], 2010	Japan	12600	in-house ELISA	431	3.42%	N/A	N/A	N/A	N/A	N/A
Shrestha *et al*	Nepal	1845	Wantai	773	41.9% (39.7%-	55	3.0% (2.2%-3.8%)	N/A	N/A	N/A

[117], 2016					44.2%)					
Nasrallah *et al* [118], 2017	Qatar	5854	Wantai	1198	20.46%	34	0.58%	4	N/A	N/A
Jupattanasin *et al* [119], 2019	Thailand	630	EUROIMMUN test kit	187	29.7% (26.2%-33.4%)	N/A	N/A	N/A	N/A	N/A
Africa										
Traoré *et al*[120], 2016	Burkina Faso	1497	DiaPro and Wantai	584	39%	13	0.87%	N/A	N/A	N/A
Ibrahim *et al* [121], 2011	Egypt	760	N/A	N/A	N/A	3	0.39%	2	N/A	N/A
Meldal *et al* [122], 2013	Ghana	239	4 commercial assays were used, findings reactive in; at least two serological assays are shown	11	4.6%	14	5.9%	0	N/A	N/A
Lopes *et al*[123], 2017	South Africa	300	Fortress Diagnostics	76	25.3%	0	N/A	N/A	N/A	N/A
Ben-Ayed *et al* [124], 2015	Tunisia	426	Globe; Diagnostics Srl ELISA	19	4.46%	N/A	N/A	N/A	N/A	N/A
Others										
Hewitt *et al*[77], 2018	New Zealand	1013	Wantai	98	9.7% (7.9%-11.7%)	N/A	N/A	N/A	N/A	N/A
			MP Biomedicals	82	8.1% (6.5%-10.0%)					

ALT: Alanine aminotransferase; CI: Confidence interval; DSI: Diagnostic Systems Incorporated; ELISA: Enzyme-linked immunosorbent assay; HEV: Hepatitis E virus; NIH: National Institutes of Health.

Geographical variation, racial differences, and diverse study methodology and laboratory techniques all contribute to differences in HEV seroprevalence. More than one-third of donors had evidence of past HEV infection in Poland, India, Nepal and Burkina Faso[59,72,117,120]. Lucarelli *et al*[93] reported an unexpectedly high prevalence (48.9%) of anti-HEV IgG among 313 donors in central Italy. Eating raw dried pig liver sausage was the only independent risk factor for HEV IgG in their study, but the authors speculated that the uncontrolled expansion of the wild boar population had resulted in contamination of the soil and watercourses for people living in rural areas, and this may also have also contributed to the high prevalence of HEV[93].

Caution is needed when interpreting the HEV serology results because commercial kits for serological detection show marked variation in sensitivity and specificity. Despite the relatively high sensitivity of the IgM assay, the sensitivity of IgG detection kits is highly dependent on a patient's immune status, being 80% to 90% in immunocompetent individuals, but falling dramatically to 15% to 45% in immunocom-

promised patients[130]. In a meta-analysis conducted in Europe, the pooled anti-HEV IgG seroprevalence rates determined by different commercial assays showed large variability with reported seroprevalence rates ranging from 2% to 17%[131]. Poor concordance of test results between the Wantai, Dia.Pro and MP Diagnostics HEV enzyme-linked immunosorbent assays (ELISA) were observed[132,133]. This may partly explain the broad ranges of anti-HEV IgG prevalence (5.3% to 48.9%) reported in Italy[55,56,92-94]. In contrast, most studies conducted in China used the Wantai assay and revealed a similar seroprevalence of around 20% to 30%. This assay is believed to be more sensitive than other commercial assays in detecting anti-HEV IgG [134,135].

TRANSFUSION-TRANSMITTED HEPATITIS E

HEV transmission *via* transfusion has been reported since 2004[136] and there has been increasing recognition of the risk of transmitting HEV by transfusion in recent years. Cases of TT-HEV are shown in Table 4[137-150]. Identical genomic sequences were identified in most infected patients and blood donors. Table 4 Likely only represents the tip of the iceberg as other probable or possible cases have been reported in the literature[151,152]. At the same time, patients with mild symptoms of hepatitis E may have gone undiagnosed. Physicians should stay vigilant for HEV infection in patients who have received a blood transfusion.

Although blood components that contain larger plasma volumes, principally fresh frozen plasma and platelet components, are believed to transmit HEV more readily [64], a number of TT-HEV cases associated with red blood cell transfusion have also been described[138,140,141,143,144,148-150]. Red blood cell transfusion was a significant risk factor for HEV seropositivity in patients on hemodialysis in Croatia[153]. Twenty percent (n = 8/40) of multiply transfused thalassemia patients were anti-HEV IgG positive compared with 11.0% (n = 10/91) in blood donors[154]. In contrast, a study in Iran found anti-HEV antibodies in only 1.67% of patients with thalassemia, suggesting a low rate of TT-HEV in that country[155]. Results from these two studies in thalassemia patients were limited by the small sample size. Ankcorn *et al*[156] analyzed 1591 patients with hematologic malignancy and found that the more transfusions of non-HEV screened blood products the patients had received, the higher their likelihood of being IgG seroreactive was, suggesting HEV acquisition *via* transfusion in these patients.

A study by Hewitt *et al*[64] indicated that a viral concentration of between 407 and 257039 IU/mL in blood products was associated with TT-HEV, and that a high viral load in donors rendered infection more likely (P < 0.0001). However, this may not be true in immunocompromised patients. In a systematic review, Dreier *et al*[50] calculated the median transfused viral load in HEV-infected and non-infected immunocompromised patients. Although the transfused viral load was higher in the infected than the non-infected individuals (4.80×10^5 IU *vs* 1.55×10^4 IU), the between-group difference was not statistically significant (P = 0.1006)[50]. A potential reason for this finding is that a low viral concentration (150 IU/mL) of the blood component could already be infectious[140].

Most cases of TT-HEV occur in immunocompromised recipients, such as patients with hematologic malignancies, or recipients of solid organ or hematopoietic stem cell transplants. However, patients on simple immunosuppressants like corticosteroids and cyclosporine or even immunocompetent individuals are also at risk[157]. Massive transfusion increased the risk of HEV transmission in an immunocompetent trauma patient[158]. Spontaneous resolution, viral eradication by immunosuppressant reduction and/or ribavirin are possible[159] but occasionally there are cases which have progressed into chronic hepatitis, liver cirrhosis or multi-organ failure. Transfusion recipients are more vulnerable to chronic liver injury than the general population as a result of foodborne infection[140]. More than 60% (n = 56/85) of solid organ transplant recipients infected with HEV developed chronic hepatitis, with tacrolimus use as an independent predictive factor[160]. Pas *et al*[161] screened 1200 solid-organ transplant recipients in the Netherlands for HEV RNA and identified 12 patients with HEV infection. Nine of these 12 patients had been treated with a tacrolimus-based regimen postoperatively. In liver transplant recipients, graft hepatitis with rapid histological disease progression and requirement of re-transplantation due to liver cirrhosis has been reported[162,163]. The rapid progression of HEV infection to advanced fibrosis and cirrhosis has also been observed in individuals receiving kidney or heart transplants[33]. In 50 patients with hematologic malignancy and clinically

Table 4 Reported cases of transfusion transmitted hepatitis E

Study	Number of patients	Comorbidity	Blood component received (*n*)	Viral load of transfused blood product IU/mL	Genotype	Treatment	Outcome
Okano *et al* [137], 2020	1	AML on chemotherapy	Plt	N/A	3b	Nil	Spontaneous resolution and developed HEV antibodies after cessation of chemotherapy for AML
Gallian *et al* [138], 2019	23	Solid organ transplant, *n* = 9; allogeneic hematopoietic stem cells transplant, *n* = 4; hematologic malignancies, *n* = 5; immunosuppressant, *n* = 2; immunocompetent, *n* = 3	RBC *n* = 7; apheresis Plt *n* = 3; whole blood-derived pooled Plt *n* = 6; FFP *n* = 7	Ranged from 1.14 × 10^3 to 31 × 62.10 ^{6}	3a, *n* = 1; 3c, *n* = 4; 3f, *n* = 16; 3, *n* = 1; 4d, *n* = 1	Ribavirin, *n* = 15	Acute HEV infection, *n* = 8; spontaneous resolution, *n* = 4; ribavirin treatment, *n* = 3; immunosuppressant reduction, *n* = 1; chronic HEV infection, *n* = 14, all immunosuppressed; resolution with ribavirin, *n* = 10; resolution with immunosuppressant reduction, *n* = 4; One solid organ transplant recipient did not clear HEV infection despite ribavirin and died of multiorgan failure
Ledesma *et al* [139], 2019	2	Allogeneic BMT, *n* = 1; liver transplant, *n* = 1	Plt	3 × 10^4	3e	Ribavirin, *n* = 1	The patient received BMT remained HEV-infected and IgM/IgG-negative until death; the patient with liver transplant was treated successfully with a course of ribavirin
Satake *et al* [140][a], 2017	19	Hematologic malignancies, *n* = 6; organ transplant, *n* = 2; systemic disease, *n* = 8; no major comorbidity, *n* = 3	RBC *n* = 10; Plt *n* = 6; FFP *n* = 3	Ranged from 1.5 × 10^2 to 5.3 × 10^6	4, *n* = 2	N/A	Two patients with malignant lymphoma and two who had received liver transplant developed chronic hepatitis E; the two liver transplant recipients were successfully cleared of HEV by ribavirin
Lhomme *et al* [141], 2017	3	Solid organ transplant	One patient received RBC; one patient received RBC and Plt; one patient received Plt and FFP	Ranged from 3.6 to 8.2 log IU	3, *n* = 1; 3f, *n* = 2	N/A	N/A
Yamazaki *et al*[142], 2017	2	Hematologic malignancies treated with chemotherapy	N/A	N/A	3b	N/A	Did not become chronic hepatitis E
Belliere *et al* [143], 2017	1	Heart transplant	RBC	1430 copies/mL	3	Ribavirin	Died from multi-organ failure despite treatment
Riveiro-Barciela *et al* [144], 2017	1	Immunocompetent, admitted for disseminated infection	RBC	75000	3	Nil	Spontaneous resolution
Hoad *et al* [145], 2017	1	Liver transplant	FFP	947	3	Ribavirin	Resolved with treatment
Matsui *et al* [146], 2015	1	AMI post CABG with hemorrhagic cardiac tamponade	Plt	$10^{6.8}$ copies	3	Nil	Spontaneous resolution
Huzly *et al* [147], 2013	1	Immunocompromised	Apheresis Plt	30888-37273	3f	N/A	N/A
Coilly *et al* [148], 2013	1	Liver transplant	RBC	3.5 $\log_{10}$	3c	Ribavirin	Resolved with treatment
Boxall *et al* [149], 2006	1	Lymphoma on chemotherapy	RBC	N/A	3	Nil	Spontaneous resolution

Mitsui *et al* [150], 2004	1	Hemodialysis	RBC	N/A	3	Nil	Subclinical infection without elevated ALT

[a]Two cases were not confirmed by sequence identity and should only be considered as probable TT-HEV.

ALT: Alanine aminotransferase; AMI: Acute myocardial infarction; AML: Acute myeloid leukemia; BMT: Bone marrow transplant; CABG: Coronary artery bypass graft; FFP: Fresh-frozen plasma; HEV: Hepatitis E virus; Ig: Immunoglobulin; Plt: Platelet concentrates; RBC: Red blood cell.

overt hepatitis E, the mortality rate was 16% (n = 8), with liver-related death occurring in 4 patients[164]. HEV could actively suppress the cellular immune response and increase levels of immunosuppressive interleukin-10 that may perpetuate chronic infection and subsequent liver damage[165,166].

TREATMENT

The management strategy for HEV infection should be determined by the clinical presentation. Currently, there is limited information in the published literature that describes the clinical features of TT-HEV, or the optimal approach to management. Acute TT-HEV infections are usually subclinical or mild, with no severe or fulminant cases reported[140]. Therefore, most acute HEV infections should be treated conservatively, while waiting for spontaneous clearance, although a short course of ribavirin may also be considered. In 21 patients with acute HEV infection who were at high risk of liver failure, receiving immunosuppressive therapy for an autoimmune disease or undergoing chemotherapy, a short course of ribavirin for up to 3 mo was associated with rapid virological response and normalization of liver enzymes[167].

The current practice for management of chronic HEV infection is mainly based on observational data[18]; Figure 1 shows a proposed algorithm for management. In patients who are on immunosuppressants, the first-line intervention should be a dose reduction or discontinuation of the immunosuppressive drug[168,169]. In solid organ transplant recipients, reducing the dose of immunosuppressive therapies that principally target T-cells can achieve HEV clearance in nearly one third of patients [160]. Most immunosuppressive drugs such as cyclosporine and tacrolimus increase HEV replication in vitro; mycophenolate mofetil is the only immunosuppressant agent demonstrated to have an anti-viral effect[170].

If modification of the immunosuppressant regimen is not possible or is unsuccessful, pharmacological agents such as ribavirin and/or pegylated interferon-alpha (peg-IFN) can be used[171]. In a meta-analysis that included 395 patients with chronic hepatitis E, ribavirin monotherapy for a median of 3 mo achieved sustained virological response (SVR) in 76% of patients[172]. The reported dose of ribavirin in the literature ranged from 29 to 1200 mg/d, and the duration from 1 to 18 mo. Data on the optimal treatment regimen are needed[173]. HEV RNA should be assessed in the serum and in the stool before treatment discontinuation[169]. A second course of ribavirin for 6 mo

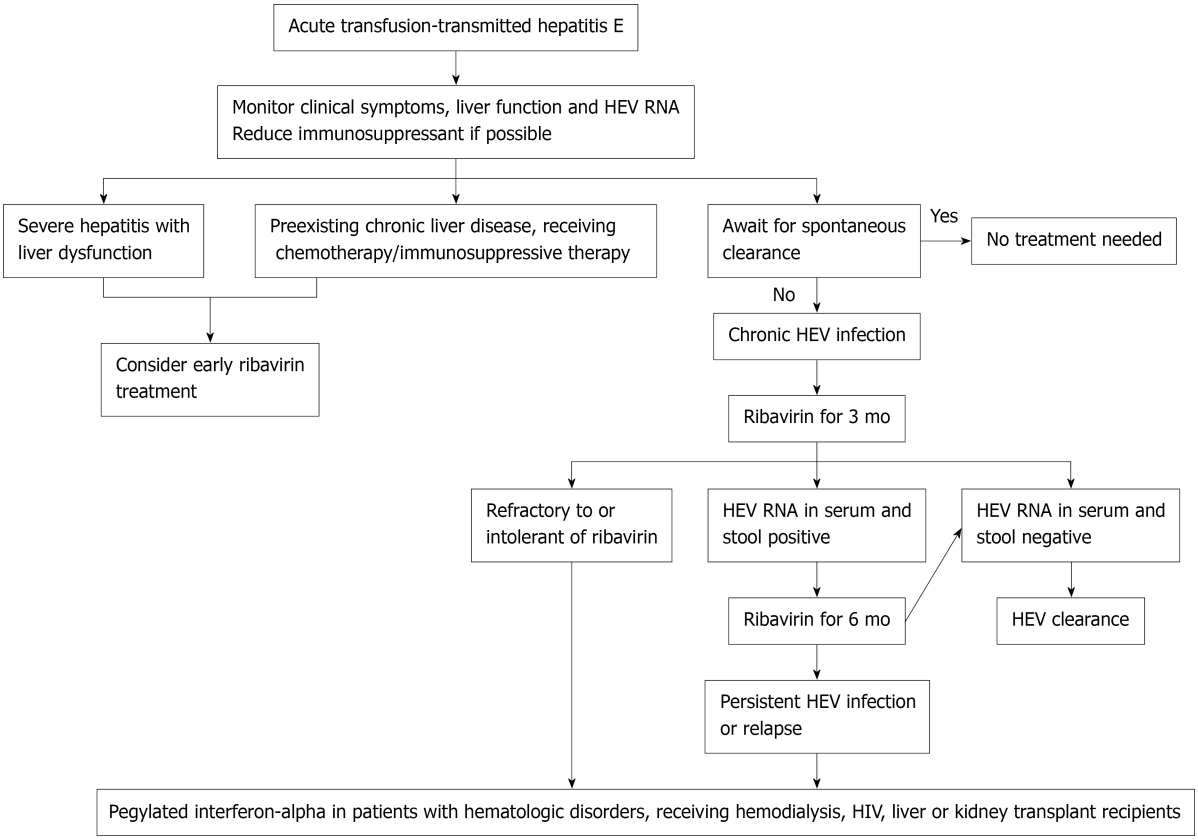

Figure 1 Recommended algorithm for management of transfusion-transmitted hepatitis E.

can be attempted in cases of treatment failure[172]. HEV RNA concentrations decrease within the first week of initiating ribavirin therapy, and a greater reduction in viral load on day 7 is an independent predictor of SVR[174]. Ribavirin failure has been linked to the presence of certain single nucleotide variants (SNVs) and in-frame insertions in the hypervariable region of open reading frame (ORF) 1 in the HEV genome[175].

For those who are refractory to, or intolerant of, ribavirin, peg-IFN can be considered. Its efficacy has been documented in patients with hematologic disorders, patients receiving hemodialysis, and in combination with ribavirin in patients with HIV[176-178]. Close monitoring is needed if it is used in transplant recipients because of an increased risk of acute humoral and cellular rejection[179,180]. Peg-IFN was thought to be safe only in liver transplant recipients until recent case reports described its successful use in a kidney transplant recipient[181-183].

Sofosbuvir is a nucleotide analog shown to decrease replication of HEV-3 *in vitro* [184]. However, in clinical studies, only modest antiviral activity was observed and SVR was not achieved[185-187]. Rescue treatment for patients who are not eligible for, or not responding to, ribavirin and/or peg-IFN remains an unmet need.

HOW TO REDUCE TRANSFUSION-TRANSMITTED HEPATITIS E

The background risk of foodborne HEV transmission to both donors and recipients of blood products is not negligible. The transfusion-related risk of infection only exceeds the annual dietary risk when more than 13 individual donor components are transfused[188]. Strategies to reduce *de novo* infection, such as modifying eating habits and eliminating HEV from pigs and other animals that are used for food production are essential[189]. The one available vaccine (HEV 239, Hecolin, Xiamen, China) is licensed only in China, and has yet to play a fundamental role in global outbreaks or pandemic control[190]. Nonetheless, the transmissibility and disease phenotype may not be the same for a person who acquires the virus orally and a person who gets infected intravenously, as there may be some protection provided by the acidic environment of the stomach and the mucosal barrier in the gut[191]. The infectivity of the non-enveloped form is different to that of enveloped HEV[9]. Data reporting

outcomes of recipients of HEV-infected blood products are sparse[47,49,50,60,64].

Policies on screening HEV in blood products differ between countries. Universal screening was adopted in the United Kingdom, Ireland, and the Netherlands. Germany and France implemented targeted screening of donated plasma intended for use in high-risk patients[192]. In Japan, the use of nucleic acid-based screening is limited to Hokkaido[193]. Blood donors are not routinely tested for HEV infection in China including Hong Kong[70,71,194]. There has been much debate on mandatory HEV screening in blood donations[195]. Key questions, such as whether or not to screen, which laboratory assay to use, which donors to screen (universal or selective screening), and which types of blood components to screen should be assessed based on risk assessment, resource availability, health economics, and political or other influences. The answers may vary considerably by geographical location[169,196]. In areas where HEV is highly endemic, most donors and/or recipients have probably been exposed to HEV previously and would have positive IgG antibodies. Therefore, the decision on serological screening should also take into consideration the prevalence of HEV infection in that particular region.

All donors should answer a questionnaire about symptoms of clinical hepatitis and potential exposure to HEV prior to blood donation. Donation should be deferred in any donors with a history of clinical hepatitis[197]. Neither alanine aminotransferase (ALT) nor anti-HEV IgM testing correlate with the presence of HEV RNA, supporting the use of NAT for screening of blood donations[60,61,105]. A simulation study by Kamp *et al*[198] reported that testing for HEV RNA by NAT with a pool size of 96, and a 95% limit of detection of 20 IU/mL will result in an 80% reduction in expected HEV transmissions as well as of consequent chronic infections with severe complications. The risk of transmission could be reduced by 90% in NAT using a mini-pool of 24 samples[198].

If opting for selective screening instead of universal screening, a clear definition of at-risk patients is warranted[199]. Targeted screening should be contemplated for blood components that will be supplied to transplant recipients, or patients with hematologic malignancies or chronic liver disease, as these individuals are at high risk of developing fulminant hepatitis, acute on chronic liver failure, or chronic hepatitis. However, it is not yet clear whether patients with rheumatologic diseases, those on low-intensity immunosuppression, or elderly individuals should only receive HEV-negative blood products. A multicenter retrospective study in Europe including 21 rheumatology and internal medicine patients found that patients with rheumatoid arthritis who were receiving methotrexate or biologics were at risk of chronic hepatitis E infection[200]. However, another study in France did not find worse hepatitis E severity or increased risk of chronicity in 23 patients with inflammatory arthritis treated with immunosuppressants[201].

Patients co-infected with HIV with CD4+ count < 200/mm^3 are at risk for persistent HEV infection[29]. In HIV patients with low CD4+ count, anti-HEV IgG seroconversion was delayed until immune reconstitution occurred[202]. A recent meta-analysis found that the HEV RNA positivity rate was significantly higher in transplant recipients than in HIV-positive patients [1.2% (95%CI: 0.9-1.6) *vs* 0.39% (95%CI: 0.2-0.7); $P = 0.0011$], possibly due to better immune status in the HIV-positive individuals using anti-retroviral therapy[203].

HEV-1 and -2 infections can take a fulminant course in pregnancy, resulting in liver failure, membrane rupture, spontaneous abortions, and stillbirths[204]. HEV-3 infection in pregnancy appears to be less virulent without significant maternal, fetal, or neonatal complications[205-207]. During pregnancy, a reduced cellular immunity and a high level of steroid hormones, in particular estrogen, progesterone, and human chorionic gonadotropin, influence viral replication/expression and possibly explain the disease severity[208]. The immune response could be influenced by HEV genotype, translating into different outcomes[209]. Ribavirin and peg-IFN are contraindicated in pregnancy due to concerns of teratogenicity[210]. Further studies are needed to clarify the risk of transmission of HEV to pregnant women *via* blood transfusion; however, in view of the potentially serious disease course and absence of a safe treatment, pregnant women are a priority group for HEV-negative blood products.

Roth *et al*[67] evaluated the safety of plasma-derived medicinal products (PDMP) and found a very low prevalence of HEV RNA (0.002%) in plasma donors. Since viral reduction methods are used in the manufacturing processes of PDMP, these data do not support routine screening of all plasma pools intended for producing PDMP. Currently there is a lack of evidence to suggest that human serum albumin or coagulation factor concentrates are a major source of HEV infection[211,212].

The cost effectiveness of HEV screening of blood donations was analyzed in the Netherlands. Screening of whole blood donations in pools of 24 would prevent 4.52 of the 4.94 TT-HEV infections annually at a cost of approximately €310000 (Euro) per prevented chronic case. The estimated cost per incurable case prevented was 10-fold higher. Costs could potentially be reduced by 85% if only the blood products intended for use by immunocompromised patients were screened. Additional costs for selective screening may arise for logistic reasons and a possible increase in the number of blood products that expire before use. They concluded that preventing HEV transmission by screening of blood donations appears not excessively expensive compared with other blood-screening measures but the impact on disease burden may be small as only a minority of all HEV cases are transmitted by blood transfusion[213]. Another economic analysis performed in North America found a very low estimated risk of TT-HEV infection risk leading to severe liver disease. When compared with no screening, the costs were $2.68 (USD) per component for a selective screening approach, and $6.68 per component for universal screening. The respective costs per quality-adjusted life-year gained were $225546 and $561810, respectively, which exceeded the threshold for what is considered as "cost-effective"[66].

In addition to screening, various pathogen reduction methods have been proposed to reduce risk of TT-HEV. Solvent/detergent treatment could not eliminate non-enveloped HEV in plasma[214]. Non-enveloped HEV is also resistant to the Intercept method, which combines a synthetic psoralen amotosalen HCl treatment with ultraviolet A light illumination to block the replication of DNA and RNA[215]. However, substantial viral reduction has been demonstrated during the manufacturing process of plasma products using immunoaffinity chromatography, nanofiltration, cold ethanol fractionation and heat treatment[216]. Anti-HEV antibodies enhanced HEV removal by nanofiltration[217]. Furthermore, ultraviolet C light provided effective inactivation of HEV in platelet concentrates[218].

CONCLUSION

To conclude, TT-HEV is gaining attention worldwide. Although the overall prevalence of viremic blood donations is low, HEV can cause sinister consequences in immunocompromised recipients. Future studies are needed to define the incidence of transmission through transfusion, clinical features, outcomes, and prognosis. The decision on a screening policy in asymptomatic blood donors should be based on local risk assessment and health economics.

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REVIEW

Viral hepatitis in 2021: The challenges remaining and how we should tackle them

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Abstract

Viral hepatitis results in 1.4 million deaths annually. The World Health Organization (WHO) set an ambitious target to eliminate viral hepatitis by 2030, but significant challenges remain. These include inequalities in access to healthcare, reaching at risk populations and providing access to screening and effective treatment. Stigma around viral hepatitis persists and must be addressed. The WHO goal of global elimination by 2030 is a worthy aim, but remains ambitious and the coronavirus 2019 pandemic undoubtedly has set back progress. This review article will focus on hepatitis A to E, highlighting problems that have been resolved in the field over the past decade, those that remain to be resolved and suggest directions for future problem solving and research.

Key Words: Hepatitis A; Hepatitis B; Hepatitis C; Hepatitis D; Hepatitis E; COVID-19

Core Tip: Viral hepatitis results in 1.4 million deaths annually. The World Health Organization set an ambitious target to eliminate viral hepatitis by 2030, but significant challenges remain. These include inequalities in access to healthcare, reaching at risk populations and providing access to screening and effective treatment. In this review article, we discuss the advances in the field of viral hepatitis over the past decade. We also discuss the remaining challenges relating to viral hepatitis A to E, and suggest strategies and pathways for their resolution.

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INTRODUCTION

Our understanding of the epidemiology of viral hepatitis and associated treatment strategies has advanced significantly over the past decade. Arguably, the most significant advances have occurred in the treatment of chronic hepatitis C, which is now curable with a short course of all oral antiviral therapy. Despite this, viral hepatitis still kills more than 1.4 million people a year[1]. As such, viral hepatitis has become a global health priority and a number of large-scale public health policies have been implemented. The World Health Organization (WHO) has set out an ambitious global elimination strategy for viral hepatitis, aiming to eliminate viral hepatitis as a public health threat by 2030[2]. Key interventions for viral elimination have been identified and include hepatitis B vaccination, facilitation of safe injection practices and safe blood transfusions, promotion of safe sex, hepatitis B treatment and hepatitis C cure. However, modelling studies suggest that up to 80% of high-income countries will not meet the WHO target[3].

This review article will focus on hepatitis A-E, highlighting problems that have been resolved in the field over the past decade, those that remain to be resolved and suggest directions for future problem solving and research. We will also discuss the impact of the coronavirus 2019 (COVID-19) pandemic on viral elimination.

METHODS

A PubMed search was performed using the following terms: "hepatitis A"; "hepatitis B"; "hepatitis C"; "hepatitis D"; "delta agent"; "hepatitis E"; "cirrhosis"; "direct acting antivirals"; "chronic kidney disease"; "chronic liver disease"; "functional cure"; "hepatocellular carcinoma"; "liver transplant"; "reinfection"; "ribavirin"; "viral elimination"; "viral resistance"; "virologic cure". Only English-language articles were included in this review. Reference lists of selected articles were reviewed for relevant studies. Published abstracts were included.

HEPATITIS A VIRUS

Worldwide, the incidence of hepatitis A virus (HAV) is decreasing[4,5], but with increasing globalization there are significant shifts in the epidemiology of HAV infection[6]. Due to a large number of cases being asymptomatic and an estimated under-reporting of up to 80% of cases, it is acknowledged that the true incidence is difficult to quantify[7]. The incidence rate of HAV infection is strongly correlated with socioeconomic indicators; the incidence decreases with increasing access to clean water and sanitation. HAV infection is commonly reported in countries where conflict leads to the displacement of people, resulting in poor sanitation and overcrowding[8].

Advances in the past decade and problems now solved

Recent studies have expanded our understanding of the molecular virology and pathobiology of HAV. It is likely that multiple immune mechanisms contribute to the development of acute liver injury due to HAV infection, including decreased frequency of regulatory T-cells due to Fas-mediated apoptosis[9] and a polymorphism in TIM1[10]. Factors now recognized to influence the clinical course of HAV infection include variations in the viral nucleotide sequence within the 5'UTR[10].

The WHO estimated that HAV infection caused approximately 7134 deaths in 2016 [11]. In the United States, case-fatality estimates range from 0.3% to 0.6% for all age groups, rising to 1.8% amongst patients aged > 50 years[12]. A safe and effective inactivated vaccine has been in use for almost 30 years[13]. It was initially developed for individual prophylaxis, but now is used to control endemics[13]. A live attenuated

vaccine has been developed and licensed in China and it is used in the Chinese national vaccination program. Use of this vaccine in children has reportedly reduced the incidence of HAV infection by 80%[14]. There are now 34 countries that use or are planning to introduce HAV vaccination into routine immunization of children in specific risk groups[11]. Within the United Kingdom, persons who are considered high-risk for HAV infection and should be offered vaccination include those in close contact with someone with HAV infection, travelers who plan to travel to parts of the world where HAV is highly endemic, persons with chronic liver disease, men who have sex with other men (MSM), people who inject drugs (PWIDs) and those who are likely to be exposed to HAV from their employment, for example workers who are exposed to raw sewage such as within the construction industry.

Another advance in the past decade has been in the area of post-exposure prophylaxis (PEP) against HAV. PEP is recommended for persons who are immunocompromised and those who have chronic liver disease[15]. Immunoglobulin was previously the only recommended PEP however due to a number of factors including declining anti-HAV IgG titres in donor pools, new strategies were sought. Recent data support post-exposure immunization with an inactivated HAV vaccine as being effective in preventing infection when given within 14 d of exposure[13].

Problems remaining to be solved

Prevention of infection in high-risk populations (including targeted vaccination): With increasing numbers of forcibly displaced persons in certain parts of the world [16], endemic HAV infection will continue to be an ongoing but preventable issue that requires a global response to provide public health infrastructure, sanitation and free HAV vaccination programmes. This approach requires significant input from public health agencies and politicians alike.

Person to person transmission is described, with infection reported amongst PWIDs and homeless populations. These populations can be difficult to engage, and vaccinating these high-risk individuals needs to be a public health priority (at least in developed countries). MSM have been linked to outbreaks of cases in developed countries, with epidemiological and laboratory investigations linking genotypes between countries[17]. It is important that high-risk groups such as MSM are identified and offered vaccination to prevent outbreaks in susceptible communities where there is lack of herd immunity[18]. Improving uptake of HAV vaccination in the MSM population is a remaining challenge. Targeting these at risk populations by methods such as social media and dating apps have been shown to improve vaccination uptake[17]. Patients with chronic liver disease should also be offered HAV vaccination due to their risk of more severe infection, however doing so has not entered widespread clinical practice[19]. In one American study of HAV vaccination in patients with chronic liver disease, 28% of patients seen in specialist centres underwent vaccination compared with 5% of patients managed in primary care[20]. In another American study of patients with hepatitis C, 7.9% of patients underwent HAV vaccination[21]. As HAV is a vaccine-preventable disease, universal vaccination of infants would be an effective method for controlling the infection going forwards.

Treatment of severe liver injury due to HAV infection: Although rare, patients with acute HAV infection can progress to acute liver failure (ALF)[7]. Whilst these patients can recover with supportive management, a small number of patients may require transplantation. Patients progressing to ALF are typically older and may not be suitable candidates for liver transplantation, and therefore other specific treatment strategies are required. Furthermore, liver transplantation is not accessible to those most at risk in displaced communities. Ribavirin has successfully been used in treatment of acute hepatitis E infection; it has been shown to have an inhibitory effect on HAV *in vitro* but has not been assessed *in vivo* for therapeutic activity[22].

HEPATITIS B + D

Hepatitis B virus

Chronic hepatitis B infection is a global problem, but the burden of disease is mostly in low to middle income countries, with 248 million of the estimated 292 million people affected residing in Asia, Africa, the Pacific and Latin America. Chronic hepatitis B virus (HBV) accounts for approximately 47% of all viral hepatitis related deaths, the vast majority of which are secondary to complications of chronic liver disease[23,24].

Advances in the past decade and problems now solved

In 2017 the nomenclature to describe the different phases of chronic HBV changed within the updated European Association for the Study of the Liver (EASL) hepatitis B guidelines[25]. This was to better reflect and highlight the two main pathological processes of chronic infection and chronic hepatitis, in particular taking into account the presence of hepatitis B e antigen (HBeAg), HBV DNA levels, alanine aminotransferase (ALT) values and the presence or absence of liver inflammation. The new definition of phases highlights the increased risk of advancing liver disease in both chronic hepatitis phases - even in HBeAg negative patients - where there is elevated HBV DNA levels and/or elevated ALT, removing the somewhat misleading term "inactive carrier". These changes in nomenclature have now been widely adopted[24].

Multiple societies now provide guidance on when to initiate treatment. Viral resistance to treatment is a problem which has now been largely overcome. The nucleos(t)ide analogues (NAs) entecavir (ETV), tenofovir disoproxil fumarate (TDF) and tenofovir alafenamide are recommended as first-line treatment in both American and European HBV guidelines[25,26]. These agents show high rates of viral suppression and high genetic barriers to resistance[27,28] and have largely replaced lamivudine (LAM) with which resistance was problematic and common. Following treatment with LAM for 1 year, 14%-32% of patients developed resistance, increasing to over 80% after 4 years[27]. Those who develop resistance to LAM and are switched to ETV are more likely to subsequently develop resistance to ETV, with resistance rates of up to 50% after 5 years of treatment compared to only 1.2% of patients developing resistance with ETV where LAM has not been previously used[27]. TDF monotherapy has been shown to be effective in patients who have previously experienced treatment failure due to LAM resistance[29] and although there have been cases reported of reduced efficacy of tenofovir, there have been very few reported cases of resistance.

Problems remaining to be solved

There remain a number of challenges in the diagnosis and management of patients with chronic hepatitis B infection - Figure 1.

Diagnosing and linking infected patients to care programmes: A significant proportion of infected persons have not been identified; current estimates suggests that only 10.5% of infected individuals have been diagnosed and only 5% of those eligible for treatment for chronic HBV infection are receiving treatment[30]. A large systemic review found that 10% of people (26 million) with HBV infection might need urgent treatment due to cirrhosis and 12%-25% of patients would also be eligible for treatment according to different international guidelines[30]. Many countries do not have the infrastructure to deliver widespread testing, vaccination or treatment; this is particularly true in low-middle income countries where resources are limited. Detailed discussion on the challenges of such health inequalities are beyond the scope of this review. The approach to up-scaling diagnostic testing needs to vary according to the target population. In the United Kingdom and other developed countries, the majority of individuals with undiagnosed hepatitis B infection are born in countries with intermediate or high prevalence rates. Identifying these individuals may increase diagnosis rates. Case finding in high-risk groups is effective; in North-East England, individuals from the British-Chinese and South Asian communities were invited to education and screening (*via* dry blood spot testing) sessions in local community centres[31]. The prevalence of hepatitis B surface antigen (HBsAg) positivity was 4.6%, which is above the 2% screening threshold recommended by the Centers for Disease Control and Prevention[31]. Another study looked at the cost-effectiveness of a one-time opt out case-finding approach in a primary care setting in the United Kingdom migrant population. This approach was deemed very likely to be cost effective amongst migrant populations with HBsAg prevalence ≥ 1%[32].

New point of care (POC) tests are also becoming available, making diagnosing infection easier and quicker. For example, the Determine HBsAg 2 test provides a HBsAg result in 15 min with high sensitivity and specificity[33]. POC tests allow testing and diagnosis to move out of established health care settings and may be of particular utility in resource poor settings and high-risk communities.

Increasing testing and subsequent diagnosis rates relies on public engagement to break down stereotypes and address stigma, improved interactions with health care services and addressing health inequalities arising from poverty and language barriers [34,35]. Collaboration and integration with other successful public health programs such as human immunodeficiency virus (HIV) services is also likely to be effective.

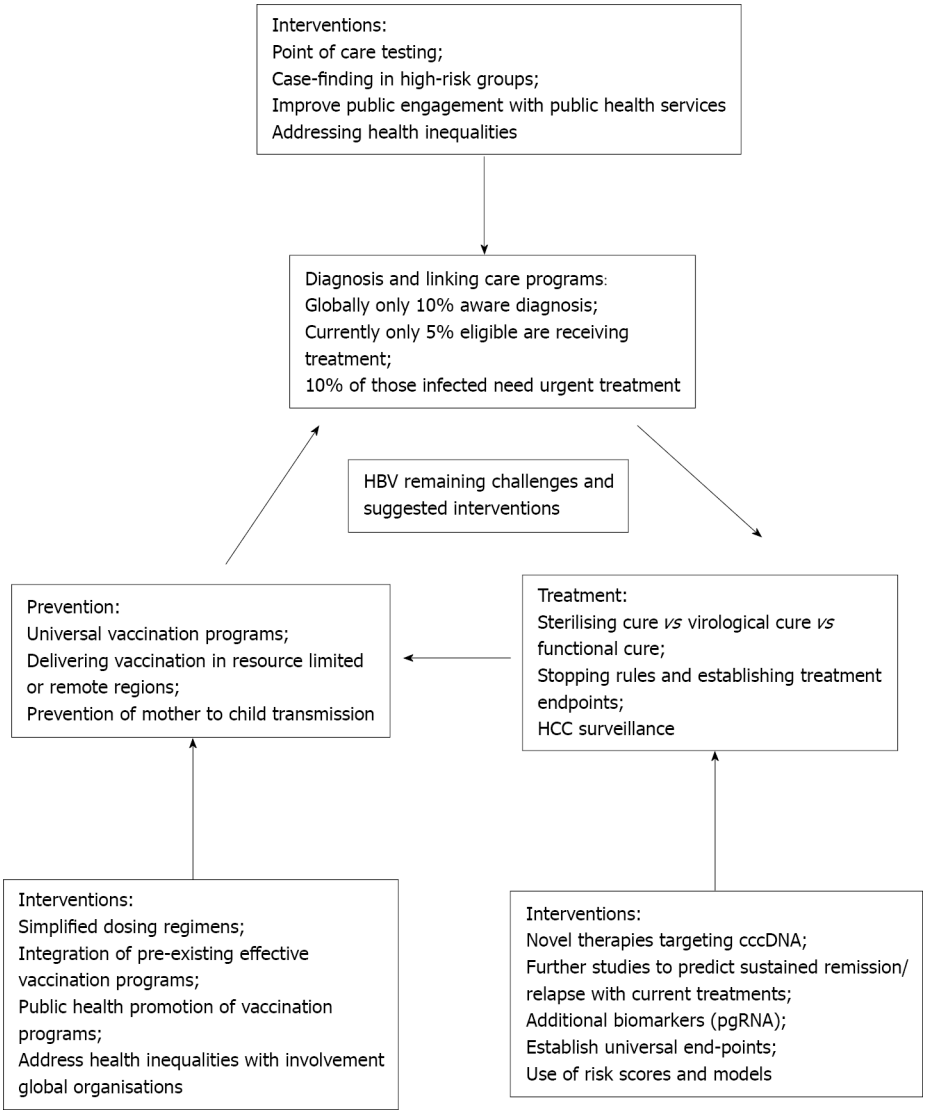

Figure 1 Remaining challenges in hepatitis B virus infection. HBV: Hepatitis B virus; cccDNA: Closed circular DNA; HCC: Hepatocellular carcinoma; pgRNA: Pregenomic RNA.

Defining cure: A 'cure' for HBV might be considered as one where the virus is completely eliminated [undetectable HBsAg, HBeAg, HBV DNA and hepatic co-valently closed circular DNA (cccDNA)] and where any (risk of) associated liver disease is also removed[36]. Consensus on definitions of cure remain contentious and as there is no current or upcoming treatment to achieve the 'holy grail' described above, there is reticence in how the word 'cure' is used. However, this is a key aspect of clinical care and research, therefore a globally accepted definition of cure needs to be obtained.

The term 'sterilising cure' (complete eradication of the virus) has been replaced with 'functional cure'. Functional cure is currently defined as sustained HBsAg loss, undetectable HBV DNA, with or without seroconversion to hepatitis B surface antibody, following a finite course of treatment[25] and it occurs in 1% of chronically infected patients annually[37]. However, HBV genomes can persist in the liver even if HBsAg is undetectable questioning the true value of achieving a functional cure. A 'partial functional cure' is considered an intermediate goal of therapy and signifies detectable HBsAg but persistent undetectable HBV DNA 6 mo post-treatment. Virologic cure is essentially 'halting' all forms of HBV replication, however difficulties with obtaining virologic cure remain due to the persistence of cccDNA in hepatocytes. To obtain virologic cure, treatments inhibiting both cccDNA and viral replication are required[38].

An agreed definition of cure remains elusive, however with clearly defining treatment endpoints and new therapies targeting different aspects of the HBV life

cycle, virologic cure may be achievable in the future.

Striving for prevention rather than cure: To prevent HBV infection, there needs to be a focus on improving vaccination strategies. Barriers to HBV vaccination, particularly in resource limited or remote regions, can be attributed to inadequate resources to acquire vaccinations, current dosing regimens, insufficient trained health staff for administration of the vaccine and lack of facilities to keep vaccinations between 2-8 °C. One study of a two-dose regime of HBsAg-1018 (containing HBsAg plus a toll-like receptor 9 agonist adjuvant) demonstrated a higher seroprotection rate at one year compared with the standard three dose regimen[39]. Simplified regimens with fewer doses over a shorter time period (HBsAg-1018 given at 0 and 4 wk) are likely to be associated with increased uptake[39]. Many countries have now instituted effective COVID-19 vaccination programmes, and similar systems could be used to roll out simplified HBV vaccination regimens.

Preventing mother to child (vertical) transmission of hepatitis B is vital if global elimination is to be achieved[40,41]. High maternal viral load is the greatest risk factor for mother to child transmission; HBeAg positivity also increases risk[41]. In resource poor settings the WHO-recommended vaccine strategy may be difficult to deliver, and diagnostic assays for HBV testing may not be readily available. A potential strategy in these settings is POC testing to establish HBeAg status, followed by empirical treatment with tenofovir in the 3rd trimester in those who are HBeAg positive to reduce viral load and the risk of perinatal transmission[42], however such diagnostic assays are not readily available and remain costly.

Defining 'stopping rules' for HBeAg negative patients treated with NAs: Where seroconversion of HBeAg occurs, 67%-85% of patients have a sustained inactive state (HBeAg negative chronic infection); this is particularly the case where seroconversion occurs below the age of 30 years and where a low or undetectable HBV DNA level has been maintained[43]. However, given significant relapse rates it remains controversial as to whether NA treatment can be stopped after HBeAg loss. A HBeAg negative state is associated with higher rates of regression of fibrosis but some patients will develop HBeAg negative hepatitis, the risk of which increases with time (22% at 10 years) and increases the risk of progression to advanced liver disease[44].

Given the low rate of clearance of HBsAg, HBeAg seroconversion is considered as a potential endpoint of treatment, where undetectable HBV DNA is achieved on three separate occasions in a 6[25] or 12-mo[26] period. If treatment is stopped at this endpoint, 50% will undergo HBeAg reversion requiring treatment with NAs to restart; close biochemical monitoring is therefore required. There is evidence to suggest that longer treatment with NAs results in a higher chance of persistent remission, with viral remission for 24-mo on NAs offering the most likely chance of sustained remission[45].

Therefore, currently there is no universal stopping rule. In real-world practice, many different factors are taken in to consideration when making the decision to stop treatment with NAs, including the stage of fibrosis and family history of hepatocellular carcinoma (HCC). Further studies are needed to more clearly define the predictors of sustained remission and/or relapse to guide stopping decisions.

Establishing treatment endpoints - aiming for viral suppression *vs* cure: Currently, long-term suppression of HBV DNA levels is the main endpoint of treatment (+/- HBeAg loss in HBeAg positive patients). It remains a subject of debate as to whether the endpoint of treatment should be viral suppression, functional cure, partial functional cure or virologic cure. The ideal goal however would be virologic cure. In 2019 the joint EASL-American Association for the Study of Liver Diseases HBV treatment endpoints conference agreed that a "functional cure" should be the primary endpoint of phase III trials; sustained HBsAg loss in more than 30% of patients was accepted as an acceptable rate of response in phase III trials[38]. The endpoint for trials may not be the same as the endpoint for real world clinical practice however.

Biomarkers continue to be developed and may prove useful in defining future treatment endpoints. These biomarkers are likely to be used in conjunction with currently utilised clinical markers. The development of hepatitis B core-related antigen (HBcrAg) as a potential serological marker for cccDNA levels may identify patients who could discontinue NA therapy, those at risk of HCC development or of recurrence following treatment[28,46]. Pregenomic RNA may be a novel marker of viral replication; evidence is emerging that this may provide an earlier predictor for HBeAg seroconversion for those patients on NAs (an important indicator for partial immune response) and may help guide future treatment in those not achieving HBeAg seroconversion[47].

Establishing a universally accepted endpoint of treatment along with biomarkers to help predict or confirm the achievement of this endpoint would be an important advance in the treatment of chronic HBV infection.

Risk of HCC and surveillance in patients on long term NAs: Chronic HBV infection is a leading cause of HCC; it is responsible for around 25% of liver cancer cases in developed countries and up to 60% of cases in developing countries[48]. NA therapy has been reported to decrease incidence of HCC[49,50]. While HBsAg loss after the development of advanced fibrosis minimizes the risk of the development of HCC, it does not negate it completely[49]. A number of factors are taken into consideration when deciding which patient to survey for HCC including disease phase, age, ethnicity and family history of HCC[49]; international guidelines do not agree on the populations for surveillance however, promoting inequalities in care.

In those on NA therapy, risk scores such as the REACH-B score[51] or PAGE-B score[52] are used to identify patients who would benefit from HCC surveillance. The REACH-B scoring system was developed in a cohort of Asian patients with chronic HBV infection who were treatment naïve; no patients with cirrhosis were included in the development of this score[51]. This score does not offer good predictability in Caucasian patients with chronic HBV infection[53]. The modified REACH-B score substituted HBV DNA levels for the liver stiffness value which increased its accuracy [54]. The PAGE-B score was developed for use in Caucasian populations receiving tenofovir or ETV. A modified PAGE-B score (addition of serum albumin) has recently been tested in Asian patients on NA therapy, with an area under the receiver operating characteristic curve of 0.82[53]. The PAGE-B score is also predictive of HCC development in untreated patients[52].

Quantitative HBsAg and HBcrAg have been proposed as new biomarkers for HCC risk which might influence patient selection for HCC surveillance[55]. Risk models incorporating these biomarkers would be an advance in the field of HBV. New models could also incorporate other novel markers such as specific HBV mutations, presence of the metabolic syndrome and HBV genotype.

Identifying new treatments with finite duration and high cure rates: Most patients with chronic HBV currently require lifelong therapy, achieving viral suppression rather than cure[25,26]. To achieve cure, combinations of therapy targeting different aspects of the HBV lifecycle are likely to be required including inhibition of cccDNA and viral replication[38].

A number of new treatments are being investigated for HBV and these are aiming to achieve clearance of HBsAg rather than just suppressing HBV DNA[36]. A detailed description of these treatments is beyond the scope of this review, but these include the development of new NAs (besifovir and metacavir), cccDNA silencers (*e.g.*, lymphotoxin beta receptor agonist) and HBV entry inhibitors (Myrcludex B)[28,38,56]. There may also be a role for immunomodulatory therapies such as toll-like receptor agonists (acting *via* activating the innate immune response), check point inhibitors (helping to restore T-cell dysfunction) or therapeutic vaccines such as TherVacB[56, 57]. Gene editing strategies and RNA interference may be other potential treatment strategies[56]. Where eligible, patients should be considered for entry into clinical trials of novel therapies.

Hepatitis D virus

The current burden of hepatitis D virus (HDV) infection is unknown; estimates from a recent meta-analysis vary considerably, ranging from 12 million to 72 million individuals infected with HDV worldwide[58]. There is geographical variation in the prevalence of HDV infection. A recent systematic review and meta-analysis estimated anti-HDV prevalence to be 4.5% amongst HBsAg positive individuals globally with rates lower in Europe (3.0%) compared with Africa (5.97%)[58-60]. However, other meta-analysis estimates differ, demonstrating higher seroprevalence amongst HBsAg positive individuals worldwide (10.58%-13.02%) and within Europe (13.81%). Such differences are likely due to variation in modelling strategies and highlight the difficulties in truly identifying the burden of HDV[58-60]. Issues and challenges remaining in the field of HDV infection include identification of infected individuals, effective treatments, treatment endpoints and prevention.

Problems remaining to be solved

Identification of infected patients: A positive HDV antibody should be accompanied by detectable serum HDV RNA to detect active infection. However, some guidelines do not explicitly make recommendations for HDV testing and therefore many patients

who are HBsAg positive are not tested for HDV. One study looking at clinic-led anti-HDV testing identified that only 40% of HBV patients were tested[61]. The same study looked at a different centre offering reflex laboratory testing and found that 99.4% of first HBsAg positive samples were tested for anti-HDV. This is a potentially reliable approach to increasing detection of patients with HDV infection, as all patients who are newly diagnosed with HBsAg positivity should be tested for serological evidence of HDV infection.

There is an epidemiological association between anti-HDV seroprevalence and PWIDs, commercial sex workers, MSM and recipients of haemodialysis[58,62]. Suggested patient groups who should be prioritised for screening for HDV include: Patients who are HBsAg positive, patients with HIV, PWIDs, MSM and migrants from highly endemic regions.

Treatment for HDV infection: Pegylated-interferon **(**PEG-IFN) is the only treatment proven to have antiviral efficacy against chronic HDV infection, however viral suppression rates with PEG-IFN are poor in HDV infection and the adverse effects of PEG-IFN therapy are well described[62,63]. Extended duration of treatment has not been associated with a consistent or significant increase in efficacy, and the addition of NAs does not improve efficacy. New treatments are urgently required; therapies currently being evaluated include HBV/HDV entry inhibitors (Myrcludex B), virion secretion inhibitors (REP 2139) and inhibitors of the prenylation of the large HDV antigen (lonafarnib)[63]. Patients with HBV/HDV co-infection should be considered for entry into clinical trials. Ultimately, global prevention of HBV infection would be the most effective means of treating HDV infection.

Establishing treatment endpoints: Unfortunately, endpoints for HDV treatment and indicators of response to treatment have not been well established[38]. Cure may not be feasible. ALT normalization, changes in HDV RNA and qHBsAg are markers of response to treatment. Barriers to establishing treatment endpoints include lack of widespread availability of HDV diagnostics and lack of standardization of HDV RNA assays. Composite endpoints are likely to be more useful than singular end-points.

HEPATITIS C VIRUS

Perhaps the greatest advances in our understanding of virology and development of treatment strategies over the past decade have occurred in relation to hepatitis C virus (HCV) infection. Despite these advances a number of challenges remain, including targeting difficult to reach populations and expanding HCV testing and treatment programmes in resource poor countries. Addressing these areas will be critical if global elimination of HCV is to be achieved by 2030.

Advances in past decade and problems now solved

Treatment and cure: HCV treatment has evolved rapidly in the last 10 years, with the emergence of direct acting antiviral (DAA) regimens. These drugs are very well tolerated and highly effective in achieving sustained virologic response (SVR), even in patients who were previously considered 'hard to treat' or in whom interferon-based treatment was contraindicated. As a result, antiviral treatment with DAAs is recommended in all patients with active HCV infection[64] and elimination of HCV is an achievable goal if these drugs can be made widely available worldwide.

In 2011 the first protease-inhibitors (telaprevir and boceprevir) were approved for use in HCV infected individuals in combination with pegylated-interferon and ribavirin, but whilst SVR rates improved so did the frequency of side effects[65]. This was quickly followed by the approval of the first interferon-free regimens for the treatment of genotype 1 HCV infection in 2014, followed by the first pangenotypic regimen, sofosbuvir-velpatasvir, in 2016[66]. Pangenotypic regimens are advantageous because they remove the need for genotype testing prior to the commencement of treatment which simplifies treatment regimens, thus reducing the frequency of patients dropping out before they start antiviral treatment.

Presently, the availability of safe and highly effective DAA regimens supports a strategy of treating all individuals with chronic HCV infection over the age of 12, irrespective of the stage of disease[67]. Current regimens offer a number of advantages over previous interferon-containing regimens including much greater efficacy, few side-effects, oral once daily dosing and shorter duration of treatment. For current DAA regimes, SVR rates (undetectable HCV RNA at 12 or 24 wk after treatment) well exceed 90% for most patient cohorts, compared with approximately 50% of patients

treated with PEG-interferon and ribavirin. Patients with chronic kidney disease (including dialysis-dependent patients) and cirrhosis were previously considered difficult to treat but now have similar SVRs when treated with DAAs to those without chronic kidney disease and cirrhosis[68,69].

Significant improvements in SVR rates with DAAs has translated into a reduction in morbidity and mortality rates in patients with HCV. A systemic review and meta-analysis concluded that there was an 87% reduction in the incidence of HCC and a 75% reduction in all-cause mortality in those who achieved SVR when compared with those who did not[70]. By 2019 in the United Kingdom, the incidence of HCV-related end stage liver disease and HCC had fallen by 24% following the introduction of DAAs and the associated increase in the number of patients completing treatment. In Scotland, new presentations of HCV-related decompensated cirrhosis decreased by 51% in the DAA area with an estimated avoidance of 330 cases of decompensated cirrhosis[73].

Problems remaining to be solved

Prevention: The ideal preventative treatment for HCV would be a vaccine. However, development of an HCV vaccine has been challenging due to the genetic diversity of the virus, the virus' ability to avoid the host immune response and a lack of *in vitro* and *in vivo* models of infection[71]. Some progress has been made, and a recent trial of a vaccine regimen to prevent chronic HCV infection was safe and induced HCV-specific T-cell responses but it did not prevent chronic HCV infection in a cohort of patients with a recent history of intravenous drug use[72]. It is therefore unlikely that an available efficacious vaccine will be available in the short-term. Work to develop a vaccine is ongoing.

In the absence of a vaccine, improving harm reduction approaches for PWIDs is vital. Existing strategies include promotion of sterile injection equipment use through needle exchange programmes and opioid substitution therapy. These services are often poorly provided and under-utilized, but they have been shown to be highly cost-effective[73]. It is been estimated that eliminating non-sterile injection techniques could prevent 43% of incident HCV infections between 2018 and 2030[74].

Difficult to reach populations: Despite advances in the medical treatment of hepatitis C, global elimination is unlikely to be achieved unless all infected patients are identified and then complete their treatment regimen. A significant proportion of people with HCV infection are unaware of their diagnosis, and our ability to find these patients is becoming increasingly challenging. Previous work has shown that HCV testing is concentrated in areas with lower risk of infection[75], commonly settings where patients are either in recovery from previous drug use or ongoing drug use is more 'controlled'. Testing needs to be expanded among 'difficult to reach' populations, especially those who may be in a more 'chaotic' phase of their drug use and are not in contact with addiction or other medical services. This group can be challenging to find and engage, but approaches such as testing and treatment in homeless hostels and food kitchens can be effective[76]. Moreover, testing delivered by peers is an approach that can increase diagnosis and subsequent treatment in patients considered hard to reach. In the United Kingdom, the hepatitis C trust run a peer-to-peer education programme, in which peer educators with personal experience of HCV deliver workshops sharing the importance of testing and treatment[77]. This has increased testing numbers and reduced attrition, whilst providing valuable education.

One important area to target to increase testing and treatment is in the prison population. Prison populations have a high prevalence of HCV infection with many studies reporting an incidence > 10 times that of the general population[78]. Drug use prior to or during imprisonment is common, yet harm reduction methods such as access to clean injecting equipment is non-existent or inadequate in the majority of prisons. Opt-out screening for blood borne viruses (BBVs) is recommended in the EASL HCV guidelines for all prison inmates[79], but even where this is practiced rates of testing are suboptimal[78]. Opt-in testing is more commonly practiced but is a far less effective approach. BBV testing can be challenging, particularly in reception prisons (prisoners awaiting sentencing) because these typically have a very large throughput of inmates and periods of incarceration can be short. However, these challenges can be effectively overcome with investment and an organized approach to testing. Effective approaches to increasing testing for HCV and scaling up of treatment with DAAs can also be used as 'treatment as prevention'. This approach was practiced in an Australian prison population and led to a significant reduction in incidence of new HCV infections[80].

Another approach that could be considered to identify undiagnosed patients with HCV is a 'track and trace' approach by mapping the social networks of individuals with a history of injecting drug use and offering HCV testing to those in a group who may not have been tested. Whilst this may sound like a practical solution, one study showed that this was ineffective in real world clinical practice with only one participant coming forward for testing[81]. Further work is needed to determine whether this approach could be refined to increase its efficacy, particularly since people are now more aware of 'track and trace' programmes as a result of the COVID-19 pandemic.

Attrition: Increasing detection rates will only help in the strive for global elimination if these are translated into increased treatment rates. An analysis of two large national laboratory databases from 2013 to 2016 found that 89.4% of patients diagnosed with chronic HCV infection did not receive a prescription for antiviral therapy[82]. In Spain, 49.8% of those with a positive anti-HCV result were not then linked into specialist care [75]. One reason for this is that care pathways have been unnecessarily complex including multiple investigations prior to treatment, which leads to patients frequently being lost to follow up and never completing treatment. Attrition appears to occur early in the treatment cascade; in one study 57.3% of patients dropped off prior to having liver enzymes checked[83]. With the advent of pangenotypic regimens and simple non-invasive fibrosis scores [*e.g.*, fibrosis-4 (FIB-4) and aminotransferase-platelet ratio index] pathways can effectively be simplified, which is likely to reduce attrition. An ideal pathway is shown in Figure 2.

Moving care delivery out of hospital settings may improve attrition rates. One study from the North-East of England found that distance from a HCV treatment service was a major predictor of patients not commencing antivirals[84]. DAAs can be effectively delivered in non-hospital settings, increasing access to treatment. A cluster-randomized trial showed that pharmacist delivered treatment in patients on opiate substitution therapy was more effective than conventionally delivered HCV therapy with more patients initiating and completing treatment, and achieving SVR[85]. Other examples of successful non-traditional HCV services have been delivered in primary care, nurse led community clinics, addiction services and homeless hostels[86]. Empowering addiction workers and those working with the homeless to become involved in the care cascade is also likely to improve attrition rates.

Reinfections: Re-infection with HCV after SVR, detected by the presence of HCV RNA rather than HCV antibodies, is largely related to an individuals' ongoing high risk behavior, inadequate harm reduction knowledge and/or lack of availability of clean injecting equipment. The true rate of reinfection is not known and is likely to vary significantly depending on the population studied. Individuals who continue to actively inject drugs after treatment have the highest rates. Very high rates of reinfection (up to 40%) have been seen in some high-risk groups, but other studies have reported lower rates[87]. Better access to harm reduction methods is vital to reduce reinfection rates. In addition, PWIDs should be tested at least annually for HCV RNA if they have ongoing high-risk behavior to identify reinfections. It is critical that they are offered re-treatment to try and reduce the risk of onward transmission of the infection[79].

Long term impact of hepatitis C infection: HCV infection is associated with multiple extrahepatic complications including increased risk of autoimmune disorders, cryoglobulinaemia and lymphoma. In addition, there is increased risk of type II diabetes, cardiovascular disease, chronic fatigue and psychological morbidity. Many of these comorbidities persist following SVR and one study found that nearly all patients have at least one co-morbidity that remains long-term[88].

Despite individuals with HCV having a significantly increased risk of cardiovascular disease, few are actively treated to reduce their cardiovascular risk[89]. Moreover, even though quality of life improves following successful antiviral treatment, this remains significantly worse than the general population[89]. This probably relates to high rates of mental health disorders, unhealthy alcohol consumption, ongoing drug use, deprivation, type II diabetes and the metabolic syndrome. Participation in physical activity in individuals with HCV is associated with improved quality of life[90]. Taking a more holistic approach to the care of individuals with HCV rather than just focusing on treating the infection may help improve long-term outcomes and improve quality of life. Use of a holistic care bundle may help achieve this[89].

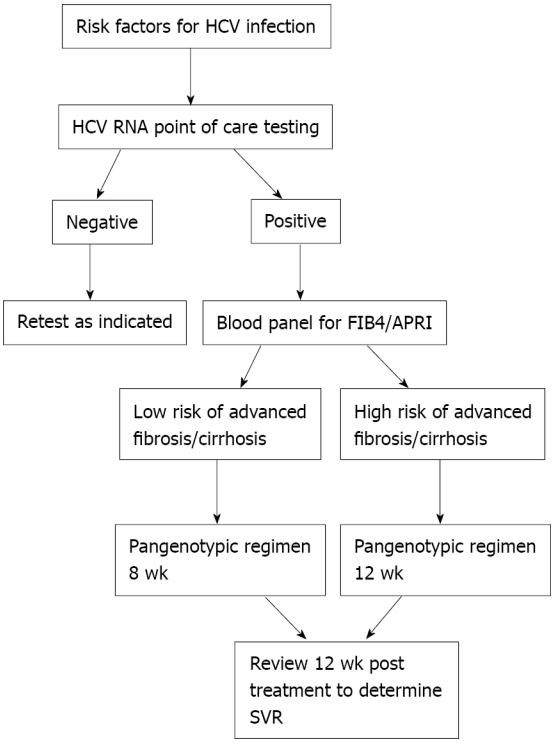

Figure 2 Proposed simplified pathway for hepatitis C virus diagnosis, staging and treatment. HCV: Hepatitis C virus; FIB-4: Fibrosis-4; APRI: Aminotransferase-platelet ratio index; SVR: Sustained virologic response.

HCC surveillance in non-cirrhotic patients: The risk of development of HCC in individuals with HCV-related cirrhosis falls following SVR, but remains approximately 2% per year and as a result, surveillance is recommended for these individuals [91]. HCC may also occur in patients with advanced fibrosis, but at a lower rate than in those with cirrhosis and it remains uncertain whether HCC surveillance is clinically effective and cost effective in this group. This is further complicated by the fact that many patients are staged using transient elastography and relevant cut-offs to identify those who are likely to benefit from HCC surveillance have not been defined. There is a clear cut need to develop better models to predict the development of HCC in individuals following SVR. International societies have different recommendations regarding HCC surveillance in those achieving SVR which reflects the overall uncertainty - Table 1.

There have been some recent studies that have attempted to more clearly define patients who would benefit from HCC surveillance post SVR[90,91]. One study developed a model to predict patients with advanced fibrosis who have a low risk of HCC and may therefore not benefit from surveillance. The model used a combination of baseline and dynamic changes in liver stiffness measurement, FIB-4 score and serum albumin after SVR and identified that nearly 20% of their cohort of patients with compensated advanced fibrosis had a very low risk of developing HCC[90]. Dynamic assessment of the FIB-4 score in isolation may also predict the risk of development of HCC after SVR[98]. In one study, no patients with a FIB-4 < 1.45 after SVR developed HCC[92]. A number of studies are underway with the aim of developing better predictive models for HCC using clinical parameters and novel biomarkers.

Increased use of hepatitis C positive donor organs: The advent of safe and highly effective DAAs for HCV infection has increased the potential to use HCV-positive organs even when the donor is viraemic, expanding the donor pool[93]. HCV positive (HCV RNA +) donor organs universally transmit HCV to the recipient[94] so prior to the widespread availability of DAAs use of these organs was restricted to those who already had HCV viraemia. However, given the efficacy of DAAs it is now possible to transplant HCV RNA + organs in to HCV negative recipients and then treat the HCV infection in the recipient.

In 2019, Kwong *et al*[95] assessed the outcomes from HCV treatment with DAAs in 10 non-viraemic patients who received HCV RNA + livers. Short-term outcomes were

Table 1 Recommendations for hepatocellular carcinoma surveillance in patients with hepatitis C virus achieving sustained virologic response

Guideline	Recommendation
EASL[114] 2020	Survey patients with advanced fibrosis (F3) or cirrhosis (F4)
AASLD[115]	Survey cirrhotic patients
Asia-Pacific[116]	Survey cirrhotic patients; Survey patients with any histologic stage of HCV with comorbidities, such as alcohol abuse and diabetes mellitus

HCV: Hepatitis C virus. AASLD: American Association for the Study of Liver Diseases; EASL: European Association for the Study of the Liver.

excellent with 100% achieving SVR at 12 wk post treatment. The practice of using HCV RNA + organs with subsequent DAA treatment is now routine in some countries around the world.

HEPATITIS E VIRUS

Hepatitis E virus (HEV) is the most common cause of acute hepatitis worldwide and carries a significant global burden of disease. HEV genotypes 1 and 2 account for approximately 20.1 million HEV infections, 3.4 million symptomatic cases, 70000 deaths, and 3000 stillbirths annually[96]. Our understanding of the impact of hepatitis E infection has advanced significantly over the past decade, with the recognition of chronic infection, risk of progression to cirrhosis, risk factors for transmission and treatment strategies. Despite these advances, there are problems that remain to be resolved.

Advances in past decades and problems now solved

There are now eight recognized genotypes of HEV. Genotypes 1-4 and 7 cause human infection. Genotypes 1 and 2 are obligate human pathogens transmitted by the faeco-oral route and cause both sporadic infection and large outbreaks. In the developed world, sporadic infections are mainly caused by genotype 3 infection.

Transmission of HEV infection

Ingestion of raw or under-cooked meat (particularly pork products), shellfish and contaminated fruits is a significant risk factor for locally-acquired infection in the Western world. Genotype 3 and 4 HEV infection can be transmitted *via* transfusion of infected blood products and solid organ transplantation, and may have a significant clinical impact upon immunosuppressed individuals. A French study looked at 23 cases of reported transfusion related HEV infections in France between 2006-2016. It reported that 14 of these cases, all of whom were immunosuppressed, went on to develop chronic HEV infection[97]. The United Kingdom introduced a universal screening policy for blood products in 2017 and also screens deceased and live organ donors for HEV RNA[98]. Other countries have a more selective strategy and only screen blood products intended for high-risk patients[99]. Universal screening has been shown to be more cost effective than selective screening if the incidence of HEV infection is above 1 in 10000 blood donations[100]. Sexual transmission in MSM has also been reported more recently[101].

Chronic infection and risk of cirrhosis

Prior to 2008, HEV was recognized to cause an acute, self-limiting illness. Genotype 3 HEV was first reported to cause chronic infection in 2008 and chronic infection has now been reported in immunocompromised individuals including solid organ transplant (SOT) recipients, patients receiving chemotherapy for haematological malignancies, HIV-1 infected patients and patients receiving immunomodulating drugs. In immunocompromised patients, the detection of HEV RNA in plasma or stool after 3 mo is defined as chronic infection[102]. Progression to cirrhosis in those with chronic hepatitis E infection occurs in 10%-15% and can occur rapidly, within 2-3 years [103]. In a study of 85 patients with chronic HEV infection in 17 transplant centres across Europe and North America, almost 66% of transplant recipients who contracted HEV developed chronic infection and 10% progressed to cirrhosis[103,104]. Chronic infection and the risk of cirrhosis is not seen with genotype 1 or 2 infection.

Treatment of chronic infection

Most published data regarding treatment of chronic HEV infection are from case series and reports in SOT recipients[105]. Reducing immunosuppression dose by around 30% has been shown to be effective in clearing HEV in around one third of patients [106]. Both PEGylated interferon and ribavirin are effective in treating chronic HEV infection. Interferon increases the risk of organ rejection in transplant recipients and therefore ribavirin monotherapy is the preferred option[107]. A systematic review has shown that 64% of patients were HEV RNA negative at 6 mo after the end of treatment with ribavirin monotherapy[108]. The optimal dose and duration of treatment is still to be determined but 3 mo courses have been used most commonly[107]. A multi-centre case series of 59 transplant recipients infected with HEV showed that ribavirin monotherapy, at a median dose of 600 mg/d for 3 mo achieved SVR in 78% of cases [107].

Problems remaining to be solved

Non-response to ribavirin: The main problem to be solved in relation to chronic HEV infection is how to manage non-response to ribavirin. Sofosbuvir has been proposed as an alternative agent to treat chronic HEV infection. It has shown promise in inhibiting HEV replication *in vitro*[109] but it had a negligible effect on improving viraemia in a case report[110]. A later study of sofosbuvir monotherapy in nine patients demonstrated a modest reduction in viral load but viral elimination was not achieved[111]. Convalescent plasma has also been trialed in a patient with persistent hepatitis E infection, and showed no effect on HEV RNA levels.

We also need to understand the relevance of HEV mutations and their effect on ribavirin resistance. Mutations have been identified in ribavirin non-responders but their impact on the treatment of these and other individuals has yet to be established. For example, the G1634R mutation does not lead to absolute ribavirin resistance and does not appear to compromise the response to a second course of treatment with ribavirin[112]. New treatments are ultimately required for those who fail treatment with ribavirin.

IMPACT OF COVID-19 ON VIRAL ELIMINATION

The COVID-19 pandemic has compromised efforts to progress towards the WHO goal of elimination of viral hepatitis. This impact of the pandemic is likely to be felt for years to come and during the initial peaks has resulted in delays in diagnosis and treatment, and reduced access to harm reduction services. In April 2020 in the United Kingdom, new diagnoses of HCV were down 85% and new treatment initiations had also fallen by 63% compared with the year prior[113]. Although there has been some recovery, pre-COVID 19 levels of testing and treatment have not yet been reached. Funding and resources have also been re-allocated to fighting the COVID-19 pandemic. In addition to the impact on global elimination, the COVID-19 pandemic has significantly impacted upon the provision of HCC surveillance programmes for patients with viral hepatitis.

However, during the pandemic many new ways of working (such as telemedicine) and care cascades have been adopted, which may in fact positively impact upon the delivery of viral hepatitis services in the years to come. For example, in some centres patients have been commenced on HCV treatment remotely using telemedicine (personal communication). The vaccination programmes and 'track and trace' systems set up during the COVID-19 pandemic could be extrapolated to viral hepatitis to improve service delivery.

SUGGESTED PUBLIC HEALTH AND RESEARCH PRIORITIES FOR THE NEXT DECADE

The global hepatology community is well placed to set public health and research priorities in viral hepatitis for the forthcoming decade, striving towards global elimination and reduced health care burden. Potential priorities for each individual virus are proposed in Table 2.

Table 2 Public health and research priorities for the next decade

Virus	Public health priorities	Research priorities
Hepatitis A	Increased vaccination of high-risk individuals; Improved sanitation and vaccination in camps for displaced persons	Medical treatments for those with acute liver failure
Hepatitis B	Increase uptake of vaccination; Identifying undiagnosed individuals; Linkage to care	Establishing treatment end-points; Identifying curative treatment
Hepatitis C	Microelimination; Reducing re-infection rates; Identifying undiagnosed individuals; Harm reduction	Vaccination; Confirming most effective HCC surveillance strategies
Hepatitis D	Identification of infected individuals; Clarifying current disease burden of HDV	Novel therapies
Hepatitis E	Increased screening of blood products/change in donor policies; Educating immunosuppressed patients of risk of food-borne transmission; Further understanding of sources of infection	RCT to confirm optimal dose and duration of ribavirin therapy; Novel treatments; Vaccination; Greater understanding of genetic mutations

HDV: Hepatitis D virus; HCC: Hepatocellular carcinoma; RCT: Randomized controlled trial.

CONCLUSION

Significant advances have occurred in the field of viral hepatitis over the past decade, particularly in relation to the treatment and cure of hepatitis C. Over the next decade – as we strive towards global elimination of viral hepatitis – the gastroenterology and hepatology community must focus on identifying the undiagnosed and engaging these individuals in to treatment programmes whilst continuing to develop novel treatments with the ultimate aim of cure.

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MINIREVIEWS

Risk of hepatocellular carcinoma after hepatitis C virus cure

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Abstract

Hepatitis C virus (HCV) is a significant cause of hepatocellular carcinoma (HCC). The direct-acting antivirals marked a new era of HCV therapy and are associated with greater than 95% cure rate. Successful treatment of chronic hepatitis C greatly reduces the risk of HCC. A proportion of patients, especially those with pre-existing cirrhosis, remain at risk for HCC despite sustained virologic response (SVR). Diabetes mellitus, hepatic steatosis, alcohol consumption and lack of fibrosis regression are associated with risks of HCC after HCV cure. Noninvasive modalities such as aspartate aminotransferase to platelet ratio index and fibrosis-4 index and transient elastography have been used to monitor hepatic fibrosis. More recently, various fibrosis scores have been combined with clinical parameters and other novel biomarkers to predict risks of HCC for patients who achieved SVR. These models still need to be validated and standardized prior to applying to routine clinical care.

Key Words: Hepatitis C virus cure; Hepatocellular carcinoma; Hepatocellular carcinoma risk models; Fibrosis markers; Transient elastography

Core Tip: Direct-acting antivirals (DAA) therapy has revolutionized the treatment for chronic hepatitis C. However, the development of hepatocellular carcinoma (HCC) after achieving DAA-induced sustained virologic response remains a significant concern, especially those with advanced fibrosis. It is critically important to monitor hepatic fibrosis and continue HCC surveillance for patients with pre-existing cirrhosis. Lack of hepatic regression and several comorbid conditions are associated with HCC

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INTRODUCTION

Hepatitis C virus (HCV) is a global health issue affecting 160-170 million people worldwide[1]. According to recent National Health and Nutrition Examination Survey data, there are approximately 2.4 million people with chronic hepatitis C (CHC) in the United States[2]. There are 6 major genotypes of HCV[3]. Globally, G1 is most common accounting for 49.1% of all infections among adults, followed by G3 (17.9%), G4 (16.8%), G2 (11.0%), G5 (2.0%) and G6 (1.4%)[3]. There are significant geographic variations in the 6 HCV genotypes (Table 1). G1 is the predominant HCV genotype, for example, in North America, Europe, Caribbean and Latin America. G4 is most common in North Africa especially Egypt and the Middle East. The high prevalence of G3 in Asia is largely contributed by South Asia in particular India and Pakistan[3].

HCV-related hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide, accounting for 85%-90% of primary liver cancers[4]. Advanced stage liver fibrosis (Metavir stage F3) carries an increased risk of HCC, and patients with cirrhosis (Metavir stage F4) have an annual HCC incidence of approximately 4%[4]. With the advent of direct-acting antivirals (DAA) therapy, over 95% of the treated patients were able to achieve sustained virologic response (SVR) or HCV cure[5]. HCV cure reduces the HCC risk but those with preexisting cirrhosis remain at risk[6,7]. This review focused on the pathogenesis and risk factors of HCC after HCV cure, and the applications of noninvasive modalities and models to predict HCC.

NATURAL HISTORY OF HCV INFECTION

The transmission of HCV occurs mainly *via* blood with the majority due to unsafe injection use (intravenous drug use, healthcare workers in underdeveloped countries) and blood transfusion recipients before 1992[8]. Moreover, sexual transmission of HCV has significantly increased in human immunodeficiency virus-infected MSM in recent years[9,10].

After the virus transmission, HCV RNA reaches a detectable level in the serum in 7 to 21 d[11,12]. HCV RNA levels rise rapidly during acute infection but it generally takes 4-12 wk for the elevation of alanine aminotransferase (ALT) (indicative of hepatic injury) with an associated increase of serum bilirubin[13]. HCV itself is not cytolytic, but it generates potent innate and adaptive immune responses with cytotoxic cytokines production and hepatic injury[14]. Acute liver failure due to HCV is rare, but its incidence increases especially in patients with pre-existing chronic liver diseases[12].

Spontaneous eradication of HCV with recovery occurs only in only 15%-25% of patients with acute hepatitis C. The presence of homozygous rs12979860-C alleles in the interferon lambda gene, however, is associated with about 80% of spontaneous recovery[15,16].

CHC is defined as the persistence of HCV RNA six months after the initial infection. CHC can lead to progressive fibrosis, cirrhosis, end-stage liver disease and complicated with HCC (Figure 1). It is estimated that 20%-30% of patients with CHC will develop cirrhosis after a period of 20 years[17]. Monitoring the development of fibrosis over time can provide a more accurate progression to cirrhosis. A study of paired liver biopsies scored by the same pathologists suggested the time to develop cirrhosis from diagnosis is about 30 to 40 years[17].

Table 1 Regional prevalence of hepatitis C virus genotypes

Regions	G1 (%)	G2 (%)	G3 (%)	G4 (%)	G5 (%)	G6 (%)	Mixed
Africa	26.3	23.7	6.3	28.1	12.2	-	3.4
North Africa/Middle East	27.3	0.8	6.3	65.3	0.3	-	-
North America	66.3	13.1	15.7	4.3	-	0.6	-
Caribbean	83	7.2	2.1	0.6	-	0.1	7.0
Central Latin America	74.6	21.6	3.3	0.1	0.1	-	0.3
Central Asia	70.4	8.6	19.6	-	-	-	1.4
South Asia	15.5	1.9	66.7	3.7	0.1	0.5	11.6
Europe	64.4	5.5	25.5	3.7	0.1	0.1	0.7
Australasia	55.0	6.5	36.0	1.2	-	1.3	-

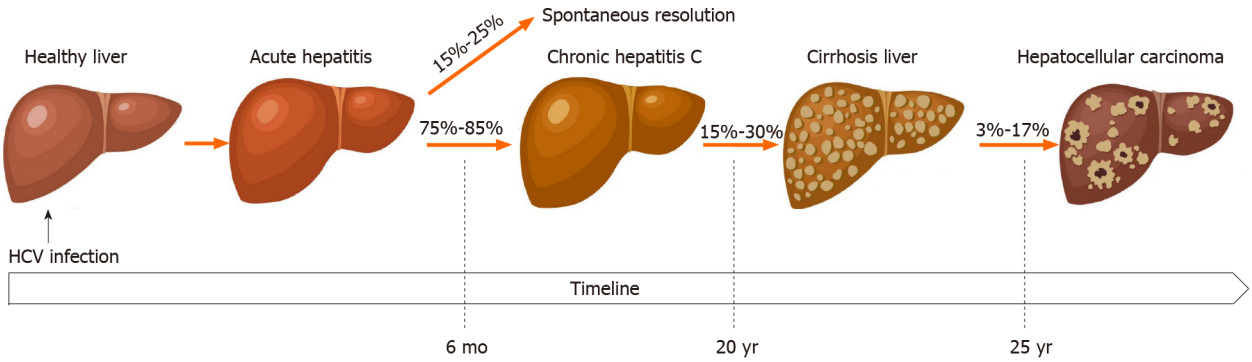

Figure 1 Natural history of chronic hepatitis. HCV: Hepatitis C virus.

After the progression to cirrhosis, patients are at increased risk of decompensated liver disease with associated complications such as ascites, spontaneous bacterial peritonitis, variceal bleeding and hepatic encephalopathy. The development of any of these complications is an indicator of increased risk of death or need for liver transplantation. Among patients with compensated cirrhosis, the 5-year and 10-year survival was 85%-91% and 60%-79% respectively[18]. The rate of clinical decompensation was 2%-5% per year and incidence of HCC was 1%-4% in these patients[18]. Generally, the risk for HCC and death increases significantly once decompensation develops[18].

HCV CURE

Treatment for HCV has revolutionized in the last decade. Before 2011, interferon was the mainstay of the therapy for HCV. Pegylated interferon combined with ribavirin had a success rate of 70% and 80% for genotype 2 and 3, respectively. However, the efficacy of interferon in HCV genotype 1 was low at 10%-20% only[19]. The advent of DAA marked the new era of HCV cure (Table 2). Boceprevir (Victrelis®) and Telaprevir (Incivek®) were the first DAA agents approved for the treatment of genotype 1 HCV infection and multiple other regimens obtained approval in the ensuing years. Since 2016, there are three pangenotypic combination therapies against genotype 1 to 6 with potent efficacy.

HCV cure or SVR is characterized by the absence of detectable HCV RNA in the serum 12 wk after the completion of DAA therapy[20]. In a meta-analysis of 43 studies, the risk of relapse or reinfection in the low-risk patients was 0.95% [95% confidence interval (CI): 0.35%-1.69%] over a 5-year period. Among the high-risk populations, such as injecting drug users or prisoners, the reinfection rate increased to 10.67% (95%CI: 6.38%-15.66%) in 5 years[21].

Table 2 Current therapies for treatment of chronic hepatitis C

Year approved	FDA approved therapy	Genotype	Trade name
2011	PegIFN/RBV + Boceprevir	Genotype-1	Victrelis®
2011	Telaprevir + PegIFNα/RBV	Genotype-1	Incivek®
2013	Sofosbuvir + RBV or Sofosbuvir + PegIFNα/RBV	Genotype-1, 2, 3 and 4	Sovaldi®
2014	Ledipasvir + Sofosbuvirwith or without RBV	Genotype-1, 4, 5 and 6	Harvoni®
2015	Daclatasvir + Sofosbuvir with or without RBV	Genotype-1, and 3	Daklinza™ + Sovaldi®
2016	Grazoprevir + Elbasvir + RBV	Genotype-1, and 4	Zepatier™
2016	Velpatasvir + Sofosbuvir	Genotype 1 to 6	Epclusa®
2017	Glecaprevir + Pibrentasvir	Genotype 1 to 6	Mavyret™
2017	Sofosbuvir + Velpatasvir + Voxilaprevir	Genotype 1 to 6	Vosevi®

FDA: Food and Drug Administration; RBV: Ribavirin; IFN: Interferon.

REGRESSION OF FIBROSIS AFTER DAA THERAPY

Liver biopsy is the gold standard to estimate liver fibrosis regression after DAA therapy. In a study by Cheng *et al*[22], the Metavir fibrosis score decreased from F3-F4 to F0-2 in more than 50% of the patients from baseline to post-therapy. Since liver biopsy is an invasive procedure that can be associated with potential adverse events, non-invasive modalities have been developed to monitor hepatic fibrosis[23,24].

Fibrosis markers: Fibrosis-4 and aminotransferase to platelet ratio index

Aminotransferase to platelet ratio index (APRI) and fibrosis-4 (FIB-4) are non-invasive serum fibrosis markers. FIB-4 and APRI values have been shown to decrease significantly during the first four weeks of DAA therapy[22]. The initial reduction in fibrosis may be related to a decrease in hepatic inflammation. They reported that aspartate aminotransferase (AST) and ALT values significantly decreased by 50.8% and 64.1% respectively after 4 wk of DAA therapy and ultimately reaching normal values[22].

Fibroscan or vibration-controlled transient elastography

Vibration-controlled transient elastography is a non-invasive and accurate measuring tool of liver fibrosis. Liver stiffness scores significantly decreased in patients who responded to DAA. Several studies have shown long-term regression of fibrosis over a follow-up period of 2 years.

Rout *et al*[25] reported that high baseline liver stiffness measurements (LSM), low platelet count, and low body mass index (BMI) were independently associated with improvement of LSM values one year after successful therapy. Furthermore, the levels of serum transaminases were not significantly associated with a reduction of LSM on multivariate analysis.

Chan *et al*[26] monitored a cohort of patients for at least a year after completion of DAA therapy to exclude the confounding effect of liver inflammation on LSM. They observed the median intra-patient LSM reduction was 0.5 kPa between the end of therapy and 12 mo after treatment.

Stasi *et al*[27] observed the greatest reduction in stiffness values at end of DAA therapy. The reduction in fibrosis was more gradual thereafter. In this group of patients, the liver stiffness values reduced progressively at 1 year, 2 years after treatment, respectively. Their findings suggested a continued reduction of fibrosis beyond the initial resolution of inflammation.

Several studies reported that patients with advanced fibrosis had significant fibrosis regression after achieving SVR. The reduction was approximately 3.1 kPa in 6-12 mo after achieving HCV cure, and the median decline in liver stiffness was 28.2% (interquartile range of 21.8% to 34.8%)[28]. Despite a reduction from baseline LSM, more than half of the patients remained cirrhotic at week 24 after treatment completion[29]. This result is consistent with previous observations that advanced fibrosis often persists after SVR[30,31].

RISKS OF HCC AFTER HCV CURE

Lack of fibrosis regression

It is crucial to explore the relationship between the lack of fibrosis regression and HCC risk especially in patients with advanced fibrosis and cirrhosis[32,33]. In a study by Ravaioli *et al*[34], 139 patients with HCV-related cirrhosis who achieved SVR after DAA treatment were included to evaluate their HCC risk by comparing LSM at baseline to end of treatment. The majority of the patients were male (65.5%) and genotype 1b (58.3%). Those who developed HCC had in average an 18% reduction in LSM compared to 28.9% among those without HCC (P = 0.005). At multivariate analysis, a less than 30% reduction in LSM was an independent HCC risk factor.

In another study, Kawagishi *et al*[35] evaluated fibrosis regression by LSM in 110 HCV patients who achieved SVR. Regression of liver fibrosis was defined as: A decrease by > 1 stage after DAA therapy in patients with liver fibrosis stage F2 to F4; and no deterioration of fibrosis in patients with liver fibrosis F0/1. They found the rate of regression was lower at 96 wk after SVR among those with higher baseline fibrosis stages.

Hepatic steatosis and non-alcoholic fatty liver disease

Hepatic steatosis is one of the histopathologic features of CHC[36]. Both *in vitro* and *in vivo* studies have shown that HCV core protein expression either in cell cultures or in transgenic mice led to the development of hepatic steatosis, contributing to carcinogenesis[37-39]. Cholet *et al*[40] in their study demonstrated a significant relationship between steatosis and hepatic fibrosis in CHC highlighting the important role played by steatosis in liver disease progression in CHC. This relationship remained significant in multivariate analysis as well[40].

Hepatic steatosis is among the factors associated with increased risk of developing HCC in HCV patients after DAA therapy[41,42]. In a large retrospective study conducted by Peleg *et al*[43] on 515 CHC patients treated with interferon-free DAA regimens, baseline liver steatosis (LS) was significantly associated with all-cause mortality and the development of HCC after treatment. Patients with LS had higher incidence rates of HCC (5.23 cases per 100 person-years, 95%CI: 4.85-5.71) compared to patients with advanced fibrosis (3.51 cases per 100 persons-years, 95%CI: 3.33-3.67). Moreover, patients with LS without advanced fibrosis had higher rates of mortality and HCC compared to those with advanced fibrosis but without steatosis[42]. Kono *et al*[44] concluded in their study of 286 CHC patients that fatty liver along with advanced liver fibrosis is associated with sustained liver damage with abnormal alpha-feto protein (AFP) and ALT levels even after HCV cure. In a prospective study conducted by Noureddin *et al*[45], 47.5% of the HCV patients with SVR had evidence of LS. Long-term follow-up of these patients is critically important to monitor progressive liver disease.

Diabetes mellitus

Diabetes mellitus (DM) is identified as a significant risk factor for HCC in HCV patients after SVR but the mechanism remains unclear[46-48]. There is some evidence suggesting hyperinsulinemia and insulin-dependent signaling pathways are linked to the pathogenesis and progression of HCC. Insulin resistance increases the rate of fibrosis progression in HCV infected patients. Hyperinsulinemia and insulin resistance as a result of cirrhosis can further promote the development of HCC[49]. HCC risk after interferon-induced SVR in patients with DM and cirrhosis had been reported. Subsequently, this association was also noted after DAA therapy. A 3-year follow-up study including 565 CHC patients with cirrhosis treated with DAAs identified diabetes as an independent predictor of *de novo* HCC[50-54]. Degasperi *et al*[47] identified diabetes as a strong independent predictor for *de novo* HCC development and also HCC recurrence in a cohort of 546 HCV patients treated with DAA. On the multivariate analysis, diabetes [hazard ratio (HR): 2.52, 95%CI: 1.08-5.87, P = 0.03] predicted *de novo* HCC as well as HCC recurrence (HR: 4.12, 95%CI: 1.55-10.93, P = 0.004)[47]. Similarly, in another study, Lu *et al*[48] also found that DM had a significant effect on the risk of HCC [adjusted HR (aHR): 1.65, 95%CI: 1.09-2.49]. In contrast, DM was not associated with an increased risk of developing HCC after DAA-induced SVR in studies by Kanwal *et al*[41].

Alcohol

Alcohol is an important HCC risk factor regardless of the presence of HCV. The annual incidence of HCC is higher among patients with alcohol use compared to those

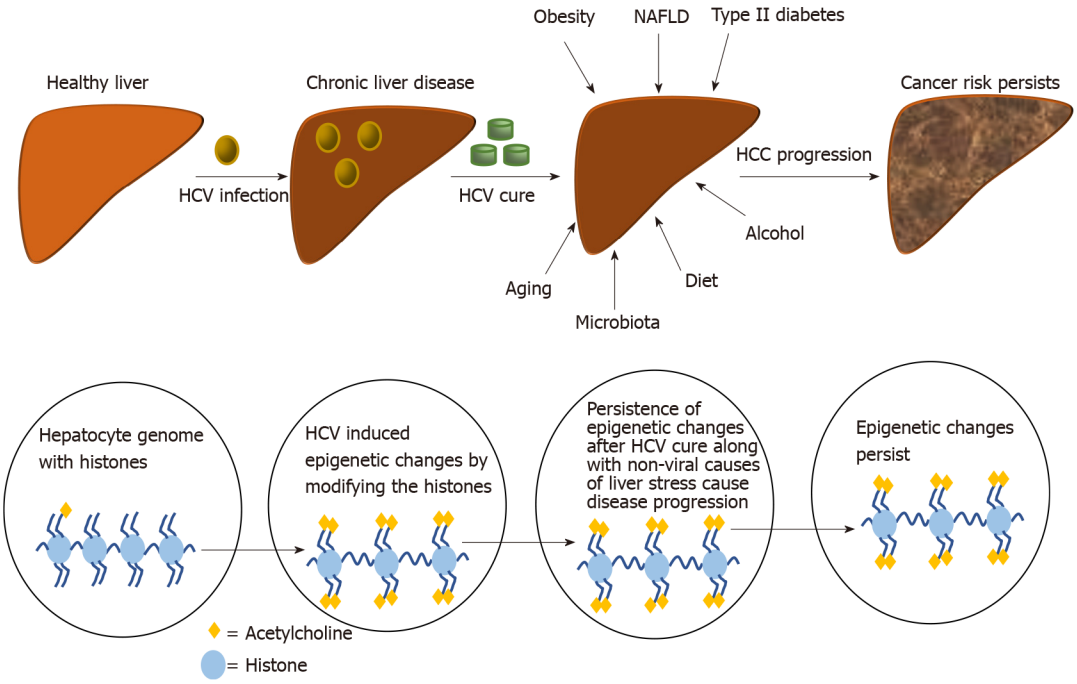

Figure 2 Pathogenesis of hepatocellular carcinoma. HCV: Hepatitis C virus; NAFLD: Non-alcoholic fatty liver disease; HCC: Hepatocellular carcinoma.

without (aHR: 4.73, 95%CI: 3.34-6.68)[41]. Alcohol-induced oxidative stress and the hepatic metabolism of ethanol could increase the conversion of pro-carcinogens to active carcinogens that results in HCC[42]. Caldwell *et al*[55] found that history of heavy alcohol consumption, defined as consumption of more than 2 drinks per day or 14 drinks per week for female; 3 drinks per day or 21 drinks per week for male, had a direct impact on FIB-4 score. It was significantly higher in the group with heavy alcohol abuse compared to no alcohol abuse. A daily intake of ≥ 80 g of ethanol for > 10 years is thought to increase the risk of HCC by approximately five-fold and women are more susceptible to alcohol toxicity than men[56,57]. Alcohol acts synergistically with HCV in accelerating the progression to cirrhosis and liver-related complications [58]. The ethanol's effects on hepatic fibrogenesis persist after HCV cure for those who continue to consume alcohol. A study by Kanwal *et al*[41] reported a higher annual incidence of HCC among patients with alcohol use (1.01%, 95%CI: 0.83-1.19) compared to those without (0.72%, 95%CI: 0.54%-0.91%; aHR: 1.56, 95%CI: 1.11-2.18) after achieving SVR post DAA therapy[41] (Figure 2).

PATHOGENESIS OF HCC AFTER HCV CURE

A number of key pathways are involved in the development of HCV-related HCC: (1) Fibrosis due to continuous necrosis; (2) Immune-surveillance failures attributable to persistent viral replication with immune system escape mechanisms; and (3) Direct carcinogenic effect of HCV proteins which deregulate host cell cycle checkpoints leading to DNA mutations in liver cells[59]. The pathogenesis of HCC after HCV cure remains elusive. A 186-gene expression signature in liver tissue of CHC patients with HCC suggested virus-induced transcriptional reprogramming in the liver leading to carcinogenesis[60,61]. Epigenetic modifications of histones, for example, can lead to chromatin opening and compacting which, in turn, affect gene regulation[62]. Hamdane *et al*[63] investigated HCV-induced epigenetic alterations that might increase HCC risk after DAA treatment in patients and mice with humanized livers. They found that chronic HCV infection induced specific genome-wide changes in H3K27ac. The 5318 modified genes associated with CHC correlated with changes in the expression of mRNAs and proteins. A number of the altered pathways resulting from epigenetic changes persisted after HCV cure with DAAs. Namely, molecular pathways involving tumor necrosis factor α signaling, inflammatory response, G2M checkpoint, epithelial-mesenchymal transition, phosphoinositide 3-kinase, Akt, and mammalian target of rapamycin[63]. This analysis showed that H3K27ac changes observed in HCV-infected patients were partly reversed after cure for those with stage

F2-3 fibrosis. This group shared only 42.5% of the HCV-modified genes. In contrast, in DAA-cured patients with cirrhosis (stage F4), 96.6% of the HCV-induced H3K27ac changes persisted[63]. By performing chromatin immunoprecipitation followed by next-generation sequencing of histone post-translational modifications that are epigenetic markers for active and repressed chromatin, Perez *et al*[64] also demonstrated that HCV infection induces genome-wide epigenetic changes. The "epigenetic signature" persisted after achieving DAA- related cure. Santangelo *et al*[65] examined the impact of DAAs on the ability of exosomal microRNAs (miRs) to modulate the innate immune response in patients with CHC. miR-122 was selectively studied as it is involved in HCV replication and its loss has been associated with HCC development. The study showed that miR-122-5p, miR-222-3p, miR146-5p, miR-150-5p, miR-30C-5p, miR-378a-3p, miR-20a5p were enriched in exosomes derived from the HCV-infected cells. The liver-specific miR-122 levels and the expression of the aforementioned miRs significantly decreased after DAAs therapy[65]. Human HCC cells express vascular endothelial growth factor (VEGF) that functions as a cytokine and affects cancer cell growth and survival[66]. The VEGF expression correlates with liver cancer angiogenesis and proliferative activity. Villani *et al*[66] studied the effect of DAA treatment-induced VEGF on HCC angiogenesis. In this study on 117 cirrhotic patients treated with DAA, a 4-fold increase in VEGF was observed compared to baseline. This significant increase in VEGF could potentially lead to an acceleration of cancer cell proliferation prior to HCV cure and the carcinogenesis remained after DAA even though the VEGF decreased to normal levels 12 wk after DAA treatment (Figure 2).

IDENTIFYING PATIENTS WITH HCC RISK AFTER HCV CURE

Although achieving SVR is the goal of HCV treatment, the risk of developing HCC remains high particularly in patients with advanced fibrosis and cirrhosis[67]. This risk ranges between 1.8% and 2.5% annually. The current guidelines suggest that these patients should undergo HCC surveillance every six months by ultrasound with or without alfa-fetoprotein indefinitely. On the contrary, patients with no or moderate fibrosis who achieved SVR and have no risk behavior could be discharged from specialty care[68]. Methods to identify patients with differential HCC risks can be challenging.

APRI and FIB-4 have been used to assess the HCC risks. These scores, however, were not developed specifically for HCC indication; thus, their accuracy is limited. Transient elastography, similarly, was not designed to detect HCC[68]. Specific score systems designed to predict HCC after HCV cure remain an unmet need.

A group in Japan developed a simple score to identify HCV patients at risk of HCC after achieving SVR[69]. The majority were HCV serotype 1 or 2 patients. They use multivariate analysis to identify predictive variables. They found that age (cutoff 75 years) and post-treatment AFP (cutoff 6 ng/mL) values were independent factors for HCC. Thus, they used a score with 0 and 1 point for each factor: < 75 and > 75 years were set as 0 and 1 point; < 6 and > 6 ng/mL were set as 0 and 1 points respectively. The sum of each factor was considered as the final score. HCC incidence increased significantly with higher scores. In the 0-point group, the incidence of HCC was 0% at 6 mo; 0.3% at 12, 18 and 24 mo; and only 1.26% at 36 mo. In contrast, the risk increased in the 2-point group: 2.88% at 6 mo; 4.92% at 12 mo; 11.61% at 18 mo; and up to 18.37% after 24 mo. This scoring system is simple to apply but needs to be validated prospectively in different patient populations.

In Egypt, Shiha *et al*[70] conducted a prospective study to develop an HCC risk model after SVR. Their model used clinical variables to create scores for low, intermediate and high HCC risk. Each variable was given a score according to its HR. This General Evaluation Score included age (< 54 = 0; > 54 = 1), gender (male = 3.5; female = 0), fibrosis stage (F3 = 1.5; F4 = 3), albumin (> 3.8 g/dL = 0; < 3.8 g/dL = 2) and alpha-fetoprotein levels (< 20 ng/mL = 0; > 20 ng/mL = 3). The score range was between 0 and 12.5. The low-risk group (score < 6) had a 1-year HCC incidence of 0.1%, 1.2% at 2 years and 1.9% at 3 years. The intermediate-risk group (score 6-7.5) had a 1-year incidence of 0.7%, 3.3% at 2 years and 5.8% at 3 years. Finally, the high-risk group (score > 7.5) had a 1-year HCC incidence of 1.2% which increased to 7.1% at 2 years and 9.5% at 3 years. The advantage of this tool is that it uses commonly available clinical variables that can be applied in different settings including low and medium-income populations. This study included only patients with HCV genotype 4. If it is validated in other HCV genotypes and populations, it can be a cost-effective tool for HCC surveillance.

Ioannou *et al*[71] developed different sets of models according to treatment modalities for CHC. For those with DAA-induced HCV cure, the regression model showed that age > 60, platelet count < 61 × 10^4, serum AST/ALT ratio > 8.8 in non-cirrhotic and > 11.01 in cirrhotic; and albumin < 2.9 were major predictive variables for the development of HCC. By applying these variables in the models, the cirrhotic/non-SVR group was predicted to have a 13.1% HCC risk at 2.6-year follow-up; the cirrhotic/SVR group had a 4.5% incidence at 2-year follow-up; the non-cirrhotic/non-SVR had a 4.2% incidence at 3.7-year follow-up; whereas the non-cirrhotic/SVR group had only a low 0.7% HCC risk at 2.3-year follow-up. Given the differential risks according to the clinical characteristics, the HCC screening guidelines could potentially be narrowed to specific risk groups. Although this model was internally validated and is easily available as a web-calculator tool, external validation would be necessary since it was performed using the Veterans Affairs healthcare data only and the majority of patients had HCV genotype 1.

Recently, a model using transient elastography was developed in Spain by Alonso L ópez *et al*[72], they built two dynamic models for patients with advanced fibrosis and cirrhosis who achieved SVR. Their objective was to identify very low HCC risk patients who may not require continued HCC surveillance despite the presence of advanced fibrosis prior to therapy. The first model included baseline albumin, baseline and 1-year follow-up elastography. Given that elastography may not be available in every setting, the second model included serological markers only: Baseline albumin, baseline and 1-year follow-up FIB-4 and 1-year gamma-glutamyl transferase. They found that both models were useful as predictors of HCC. Moreover, after stratification of risk assigned by scoring each variable in both models, the ones who scored 0 had 0%-0.4% risk of developing HCC. The ability to accurately identify those at very low HCC risk could effectively stratify patients for HCC surveillance.

Alpha-fetoprotein is the most available HCC biomarker. Its sensitivity and specificity are very variable[73]. Recent studies have shown sphingolipids as potential biomarkers to detect hepatic decompensation in cirrhotic patients[74]. Two types of sphingolipids - C16-ceramide and sphingosine-1-phosphate - have been applied as HCC biomarkers in cirrhotic patients. Mücke *et al*[75] in Germany evaluated sphingolipids as early predictive HCC biomarkers in HCV patients with cirrhosis who had achieved SVR. They identified C16Cer as an independent biomarker for early detection of *de novo* HCC in both AFP-positive or AFP-negative patients. Although this finding seems novel and promising, prospective studies are needed to clarify the association between sphingolipids and carcinogenesis.

In the area of deep learning, Ioannou *et al*[76] utilized recurrent neural network (RNN) models to identify patients at high risk of developing HCC for at least a 3-year follow-up period after HCV cure. They used two types of variables: Baseline and longitudinal ones to evaluate the risk progression. They compared three models: Cross-sectional logistic regression (LR), longitudinal LR and RNN. The area under the receiver operating characteristic curve for these groups was 0.67, 0.70 and 0.80 respectively. The RNN model was superior to the conventional LR models and could be a promising tool after computational refi-nement.

CONCLUSION

In the DAA era, the development of HCC remains a significant concern especially among those with advanced hepatic fibrosis. A number of factors including diabetes mellitus, underlying non-alcoholic fatty liver disease and alcohol consumption have been associated with progression to HCC after HCV cure. Promising HCC predictive models are being developed but most require validation and standardization. The pathogenesis of HCC after HCV cure remains poorly understood. The understanding of the molecular mechanisms leading to HCC could facilitate the identification of novel biomarkers for early HCC detection.

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MINIREVIEWS

Artificial intelligence in the diagnosis and management of colorectal cancer liver metastases

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Abstract

Colorectal cancer (CRC) is the third most common malignancy worldwide, with approximately 50% of patients developing colorectal cancer liver metastasis (CRLM) during the follow-up period. Management of CRLM is best achieved *via* a multidisciplinary approach and the diagnostic and therapeutic decision-making process is complex. In order to optimize patients' survival and quality of life, there are several unsolved challenges which must be overcome. These primarily include a timely diagnosis and the identification of reliable prognostic factors. Furthermore, to allow optimal treatment options, a precision-medicine, personalized approach is required. The widespread digitalization of healthcare generates a vast amount of data and together with accessible high-performance computing, artificial intelligence (AI) technologies can be applied. By increasing diagnostic accuracy, reducing timings and costs, the application of AI could help mitigate the current shortcomings in CRLM management. In this review we explore the available evidence of the possible role of AI in all phases of the CRLM natural history. Radiomics analysis and convolutional neural networks (CNN) which combine computed tomography (CT) images with clinical data have been developed to predict CRLM development in CRC patients. AI models have also proven themselves to perform similarly or better than expert radiologists in detecting CRLM on CT and magnetic resonance scans or identifying them from the noninvasive analysis of patients' exhaled air. The application of AI and machine learning (ML) in diagnosing CRLM has also been extended to histopathological examination in order to rapidly and accurately identify CRLM tissue and

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its different histopathological growth patterns. ML and CNN have shown good accuracy in predicting response to chemotherapy, early local tumor progression after ablation treatment, and patient survival after surgical treatment or chemotherapy. Despite the initial enthusiasm and the accumulating evidence, AI technologies' role in healthcare and CRLM management is not yet fully established. Its limitations mainly concern safety and the lack of regulation and ethical considerations. AI is unlikely to fully replace any human role but could be actively integrated to facilitate physicians in their everyday practice. Moving towards a personalized and evidence-based patient approach and management, further larger, prospective and rigorous studies evaluating AI technologies in patients at risk or affected by CRLM are needed.

Key Words: Colorectal cancer; Liver metastases; Artificial intelligence; Machine learning; Deep learning; Neural networks; Radiomics

Core tip: The digitalization of healthcare generating huge amount of data set the ground for the progressive ubiquitous application of artificial intelligence (AI) technologies in healthcare. AI analyses can assist clinicians in all phases of colorectal liver metastases natural history: From predicting their occurrence, to increasing diagnostic accuracy or estimating recurrence risk after treatment and patient outcome. The implementation of AI resources supports the contemporary paradigm shift that sees healthcare focus moving from a generalized, disease-oriented to an individual, patient-centered, precision medicine approach.

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INTRODUCTION

Colorectal cancer liver metastases

Colorectal cancer (CRC) is the most common gastrointestinal cancer, the third most frequently diagnosed malignancy (10.0%) overall, and the second highest cause of cancer-related deaths (9.4%), with incidences varying significantly worldwide[1,2]. CRC development is predominantly sporadic, with patient age, environmental and genetic factors associated with a significantly increased risk[3,4]. Over 20% of newly diagnosed CRC patients have distant metastases at presentation[5], with estimated 5-year survival dropping from 80%-90% in patients with local disease to a dismal 10%-15% in those with metastatic spread[6]. The liver is the preferential metastatic site, due to its anatomical proximity and the portal systemic circulation. This results in 25%-50% of CRC patients developing liver metastasis during the course of the disease[7,8]. In cases of synchronous resectable colorectal cancer liver metastasis (CRLM), the treatment options range from the traditional staged approach, where the primary tumor is resected prior to systemic chemotherapy and liver metastasis resection, to the combined approach of bowel and liver resection during the same procedure, or the "liver first" approach[9]. Irrespective of the timing of the surgical resection, surgery in combination with chemotherapy is the optimal treatment for CRLM, but only 25% of patients are suitable candidates for resection at diagnosis[8,10]. In patients not amenable to surgery, chemotherapy is the usual treatment of choice, with the potential to render 10%-30% of tumors technically resectable through a good response and downsizing[11]. CRLM management is multidisciplinary, with oncologists, surgeons, radiologists and pathologists playing pivotal roles in the complex diagnostic and therapeutic decision-making processes aimed to achieve the best possible outcome for the patient[12]. In such a complex oncological scenario, with unsolved challenges in

timely diagnosis, reliable prognostic factor identification and optimal treatment selection, there is a strong need for a precision-medicine, personalized approach in order to optimize patients' survival and quality of life. The recent progressive implementation of artificial intelligence (AI) in healthcare has been welcomed with enthusiasm by both healthcare professionals and the general public; however, there remain several issues which are yet to be solved. AI has the potential to overcome some of the current practice limitations, and to play a crucial role in all steps of the management of CRLM but its clinical benefits have yet to be clearly established and validated.

The aim of this review is to summarize and analyze the available evidence on the application of AI technologies in the diagnosis and management of patients affected by CRLM.

AI

The term AI encompasses all the possible applications of technologies in simulating and replicating human intelligence[13]. These endless applications range from everyday life to finance and economics[14] or various medical fields, thanks to the advances in computational power and the collection and storage of large amounts of data in healthcare. After being adequately programmed and trained, AI has the potential to outperform clinicians in some tasks in terms of accuracy, speed of execution and reduced biases[15]. AI has therefore progressively demonstrated its potential across all human lifespan; from the optimization of embryo selection during *in vitro* fertilization[16] to the prediction of all-cause mortality[17]. The revolutionary potential of these technologies in healthcare has generated great interest in researchers, professionals and industries, with currently over 450 AI-based medical devices approved in Europe or the United States[18]. Nevertheless, the surge of AI and its implemen-tation in clinical practice has been accompanied by several issues including legal considerations regarding security and data, software transparency, flawed algorithms and inherent bias in the input data[13,19].

Machine learning

The replication of human intelligence by AI with the utilization of data-driven algorithms that have been instructed and self-train through experience and data analysis is generally defined as machine learning (ML)[13]. After been programmed, ML can find recurrent patterns in large amount of appropriately engineered data and progressively learn and independently improve performance accuracy without human intervention. The ML algorithms are generally classified in supervised learning (the most frequent one, which utilizes classified data), unsupervised learning (where algorithms can independently identify patterns in data without previous classification), semi-supervised learning (can use a combination of both labelled and unlabeled data) and reinforcement learning (uses estimated errors as proportional rewards or penalties to teach algorithms). Deep learning (DL) is a class of ML techniques that has the ability to directly process raw data and perform detection or classification tasks automatically without the need for human intervention. The sets of algorithms utilized by DL are generally artificial neural networks (ANNs) constituted by several layers that elaborate inputs with weights, biases (or thresholds) and deliver an output. ML models can be combined with the large amount of qualitative and quantitative information mined from medical images (radiomics) and clinical data to assist clinicians in evidence-based decision making processes[20].

PREDICTIVE AI MODELS FOR THE DEVELOPMENT OF CRLM

A significant proportion of patients affected by CRC will develop CRLM during the follow-up period[21], but only about a quarter of them will be eligible for surgical resection and therefore potential cure[22]. Being able to identify the subgroup of patients at higher risk of CRLM development could allow the adoption of individualized and more intense screening protocols and adjuvant therapies.

The Radiomics Intelligent Analysis Toolkit-based analysis platform built by Li *et al* [23] allowed the construction of individualized nomograms able to combine maximum-level enhanced computed tomography (CT) images in the portal venous phase and patients' clinical information [age, sex, carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9] to predict the development of CRLM in patients with CRC. The area under the receiver operating characteristic (AUROC) score obtained from the analysis of 100 patients (50 with CRLM and 50 controls) was 0.899 [95% confidence

interval (CI): 0.761-1.000] on the test set in a total execution time of 270 s. The ML predictive models built by Taghavi[24] including radiomics and a com-bination of radiomics with clinical features (contrast-enhanced portal venous phase CT of the liver or abdomen with age, sex, primary tumor site, tumor stage, nodal stage, CEA at primary diagnosis, administration of adjuvant/neoadjuvant chemotherapy) of 91 patients (24 of which developed metachronous CRLM), both presented an area under the curve (AUC) in the validation cohort of 86% (95%CI: 85%-87%) in predicting the development of CRLM within 24 mo. The convolutional neural network (CNN) model developed by Lee *et al*[25] in their retrospective, cross-sectional study in 2019 patients who underwent curative colectomy for stage I-III CRC was able to predict 5-year metachronous liver metastasis occurrence with a mean AUC of 0.747 when combining the analysis of the abdominal CT scan taken before the colectomy for clinical staging and clinical features (age, sex, tumor stage, nodal stage).

AI MODELS FOR THE DIAGNOSIS OF CRLM

Prompt diagnosis of CRLM at an early stage gives patients the best chances of effective treatment and a superior outcome. One of the key steps in the diagnostic process is tumor segmentation, with nodule volume being a better predictor than diameter[26]. This process is usually done manually but requires a significant expertise, is operator-dependent and time-consuming. In this setting, semiautomatic tumor segmentation methods based on texture analysis have been developed[26] in order to take full advantage of AI's unique potential to increase sensitivity and specificity of metastatic tumor detection[27].

CT radiomics models

Starting with a manual tumor/nontumor class prediction voxel classification, a deformable surface model fitting the tumor boundaries is instigated[27]. A multilayer perceptron feed-forward neural network model concurrently learns per-voxel image features and classifications and, after being trained, it performs a semiautomatic per-tumor segmentation on CT scans. The accuracy of the model resulted in 0.88 ± 0.11, with a sensitivity of 0.84 ± 0.13 and a specificity of 0.92 ± 0.16. The same group in 2019 published the results of a retrospective analysis of a fully CNN for liver lesion detection and segmentation on CT scans with a sensitivity of 71% and 85% and a positive predictive value of 83% and 94% for lesions bigger than 10 mm and 20 mm in diameter, respectively[28]. CRLM is most commonly diagnosed in the venous phase of contrast-enhanced CT scan, as it appears hypodense, with or without peripheral rim enhancement and calcification. Portal-venous phase scans are most reliable in the detection of CRLM, with a sensitivity of approximately 85% for helical CT[29], and such diagnostic power lies in an optimal timing of image acquisition after a delay following contrast intravenous injection. Different equipment, protocols, patient's body habitus and cardiovascular system function result in high variability and impact on measurement accuracy in the absence of reliable automatic timing quantification. Ma *et al*[30] designed a fully automatic DL CNN that in a 3-s timespan can recognize the optimal portal venous phase acquisitions on CT scans with an AUC of 0.837 (95%CI: 0.765-0.890) in the validation set and an AUC of 0.844 (95%CI: 0.786-0.889) in the external validation set. This is aimed to improve image quality, which is crucial for the detection and characterization of liver lesions and the evaluation of parameters identified as predictors of treatment response and outcome, such as the tumor size, enhancement and vascularity[30]. The DL-based algorithm of Kim *et al*[31] aimed at detecting CRLM without human manipulation and fed by raw data from CT images, showed a sensitivity of 81.82%, comparable to that of radiologists (80.81%, $P = 0.80$), but with significantly more false positives per patient (1.330 *vs* 0.357, $P < 0.001$).

A challenging scenario that can occur in 16%-26% of patients with CRC is when the staging CT scan shows small hypoattenuating hepatic nodules defined as too small to characterize. Further imaging such as magnetic resonance (MR), repeat CT after a time interval, or performing a biopsy can delay treatment, increase costs, remain inconclusive, or have the risk of complications and tumor seeding. However, obtaining a diagnosis is of paramount importance given that 9%-14% of these nodules will prove to be malignant[32,33]. CNN could represent a useful adjunct in the characterization of small hypoattenuating liver lesions, and the model developed by Khalili *et al*[34] presents an AUROC similar to the one of expert radiologists, with better diagnostic confidence (significantly lower proportion of nodules rated in the low confidence zone, 19.6 *vs* 38.4%).

MR radiomics models

Despite CT imaging being the most widely used modality in detecting metastatic liver tumors, it can still miss up to 25% of CRLM[35] and MR has progressively gained an established role thanks to the high sensitivity and specificity and absence of ionizing radiations[36,37]. AI utilizing CNN for liver segmentation and CRLM detection could assist radiologists in this complex task and potentially reduce the manual liver lesion detection failure rate of 5%-13%[38]. The CRLM detection method developed by Jansen *et al*[38] is based on a fully CNN with an automatic liver segmentation and the analysis of both dynamic contrast-enhanced and diffusion-weighted MR images in 121 patients. It resulted in an impressive a high sensitivity of 99.8% and a low number of false positives.

Volatile-organic-compound-based models

Interestingly, a ML model has been used by Steenhuis *et al*[39] to analyze data from a retrospective cohort of 62 patients following curative CRC resection to detect CRLM development or local recurrence. The volatile organic compounds (VOCs) from patients' exhaled air are gaseous products of metabolism known to be altered by pathological processes, such as abnormal cell growth, necrosis or intestinal microbioma alteration, and have been evaluated by ML techniques for pattern recognition. This pilot study, despite the limitations due to the small sample size and lack of histological confirmation in about a quarter of patients, showed that the noninvasive, repeatable, and easily applicable eNose analysis was able to identify CRLM or local recurrence with a sensitivity of 0.88 (95%CI: 0.69-0.97), specificity of 0.75 (95%CI: 0.57-0.87), and an overall accuracy of 0.81. Miller-Atkins *et al*[40] combined VOC analysis and demographic data (age and sex) in a predictive model developed using random forest ML and cross-validation that was able to identify patients with CRLM from healthy controls with a classification accuracy of 0.86, specificity of 0.94 but a sensitivity limited to 0.51.

Histology-based models

The applications of AI and ML in diagnosing CRLM have been extended to histopathological examination in order to rapidly and accurately identify CRLM tissue. A probe electrospray ionization-mass spectrometry and ML model was able to distinguish CRLM (103 samples) from noncancer liver parenchyma (80 control samples) with an accuracy rate of 99.5% and a AUROC of 0.9999[41]. CRLM patients are a heterogeneous group with considerable variations, including histopathological growth patterns (HGPs) and corresponding microvasculature[42]. The two predominant types of HGPs are the desmoplastic and replacement, with the pushing and mixed types being far less common. Once accurately determined by analyzing the interface between the tumor cells and the nearby normal liver, HGPs can represent a useful prognostic and predictive biomarker for response to therapy and overall survival[43-46]. The MR-based radiomics model developed by Han *et al*[47] aims at preoperatively identifying HGP of CRLM with an AUC of 0.906 in the internal validation cohort when the analysis is performed on the tumor-liver interface zone.

AI MODELS FOR TREATED CRLM

Surgical resection offers patients presenting with synchronous or metachronous CRLM the only potential for cure and a superior long-term survival[48] but unfortunately only a fraction of newly diagnosed patients are suitable for surgery. Liver-directed ablative therapies have progressively gained a role in treating nonsurgical candidates with acceptable safety and efficacy profiles[49]. In spite of this, recurrence after CRLM treatment represents a major problem, with an overall risk of local or distant tumor development after surgical resection or ablation as high as 70%-80%, with early recurrences being associated with a poorer prognosis[50,51]. Chemotherapy is of paramount importance in determining outcome of patients with either resectable or unresectable CRLM[8] and can convert up to one third of initially unresectable patients to receive potentially curative treatment[52].

AI models predicting response to chemotherapy

A reliable assessment of response to chemotherapy is of paramount importance for the personalized treatment decision-making process to determine eligibility for surgery, or the need for second-line treatments[53]. Discriminating responsive from unresponsive

nodules or new lesions on the CT scan often represents a challenging task for radiologists, therefore Maaref *et al*[54] developed a fully automated framework based on DL CNN that achieved an accuracy of 0.91 (95%CI: 0.88-0.93) for differentiating treated and untreated lesions, and 0.78 (95%CI: 0.74-0.83) for predicting the response to a FOLFOX + bevacizumab-based chemotherapy regimen. Similarly, the DL ra-diomics model by Wei *et al*[55] was able to predict response to chemotherapy (CAPEOX, mFOLFOX6, FOLFIRI or XELIRI regimens) of CRLM based on contrast-enhanced CT according to the response evaluation criteria in solid tumors with an AUC in the validation cohort of 0.820 (95%CI: 0.681-0.959) that increases to 0.830 (95%CI: 0.688-0.973) combining the DL-based model with the CEA serum level. Human epidermal growth factor receptor 2 amplification or overexpression is found in 2%-6% of stage 2/3 CRC patients and treatment with trastuzumab and lapatinib has proven to be beneficial in the 70% of metastatic cases[56]. Giannini *et al*[57] published the results of an ML algorithm predicting the therapeutic response in such a subgroup of patients with an overall sensitivity of 92% (95%CI: 75%-99%) and specificity of 86% (95%CI: 42%-100%). The radiomics-based prediction model for the response of CRLM to oxaliplatin-based chemotherapy developed by Nakanishi *et al*[58] with radiomics features extracted from the pre-treatment CT scans, significantly discriminated good responders (AUC: 0.7792, 95%CI: 0.618-0.941).

AI models predicting recurrence after local ablative therapies

In order to predict early local tumor progression after ablation treatment of up to five nodules per patient with a maximum diameter of 30 mm, Taghavi *et al*[59] developed a ML-based radiomics analysis of the pretreatment CT scan combined with patients' clinical features that showed a concordance index in the validation cohort of 0.79 (95%CI: 0.78-0.80).

AI MODELS PREDICTING SURVIVAL IN CRLM PATIENTS

AI models predicting overall survival

The systematic comparative analysis of quantitative imaging biomarkers based on the geometric and radiomics analysis of the liver tumor burden by Mühlberg *et al*[60], performed on a retrospective cohort of 103 patients with CRLM with automated segmentation of baseline contrast-enhanced CT images, showed that the tumor burden score (TBS) had the best discriminative performance for 1-year survival (AUC: 0.70; 95%CI: 0.56-0.90). The TBS[61] is calculated combining tumor number and maximum diameter through the Pythagorean theorem [TBS^2 = (maximum tumor diameter)^2 + (number of liver lesions)^2]. An ML method has been used by Hao *et al*[62] to analyze whole-genome methylation data to predict cancer *versus* normal tissue of four common tumors (including 29 of 30 CRLMs) with > 95% accuracy and patient prognosis and survival through DNA methylation analysis.

AI models predicting survival after chemotherapy

Anti-epidermal growth factor receptor (EGFR) therapies are an effective option for RAS wild-type mutational status CRLM, but there is a need for reliable biomarkers that can estimate the balance between risks and clinical benefits of such therapies in individual patients[63]. Dercle *et al*[64] developed an AI model that through ML could create a signature that evaluated a change in tumor phenotype on interval CT scan images (baseline to 8 wk). The resultant model was able to successfully predict both sensitivity to anti-EGFR therapy (0.80; 95%CI: 0.69-0.94) and overall survival ($P < 0.05$).

AI models predicting survival after surgical resection of CRLM

The ANN model constructed by Spelt *et al*[65] retrospectively analyzed a single-center cohort of 241 patients who underwent liver resection for CRLM. Six of the 28 potential risk variables (age, preoperative chemotherapy, size of largest metastasis, hemorrhagic complications, preoperative CEA level and number of metastases) were selected by the ANN model to predict survival more accurately than the Cox regression model, with C-index of 0.72 *versus* 0.66. Paredes *et al*[66] in 2020 published the results of their ML recurrence-free prediction model for patients with CRLM undergoing curative-intent resection using clinical, pathological and morphological tumor characteristics with genetic Kirsten rat sarcoma 2 viral oncogene homolog information. The model, built on the analysis of 1406 multi-institutional patients undergoing liver resection, showed a discriminative ability to predict the recurrence risk at 1, 3 and 5 years (AUROC of

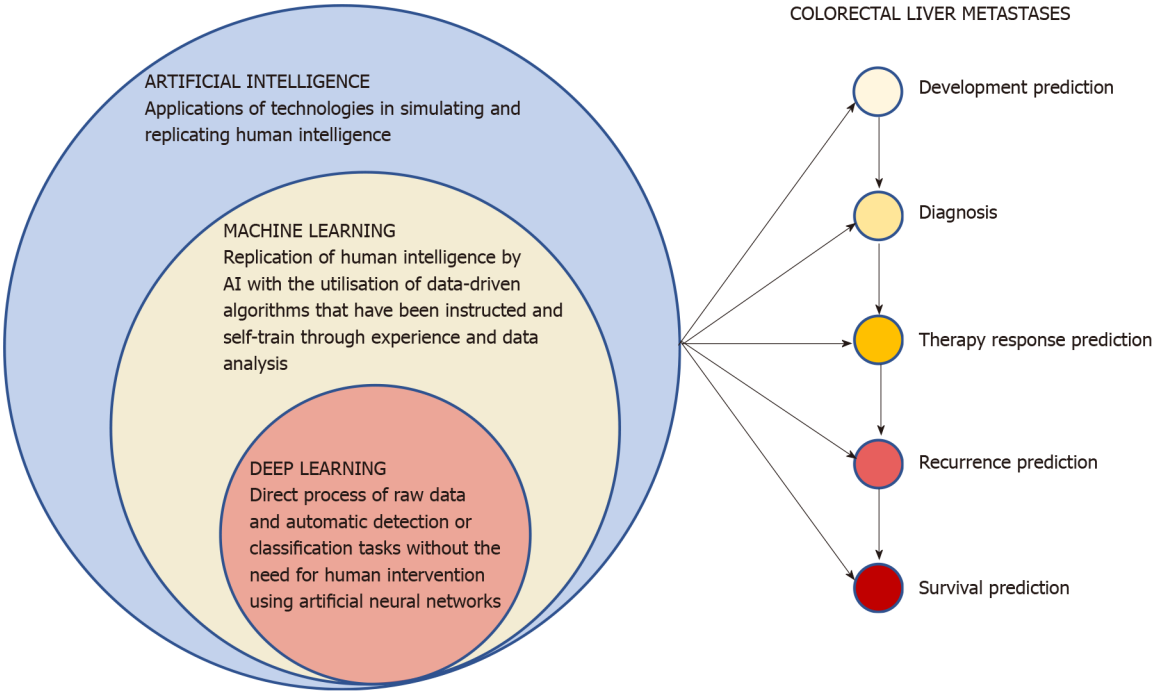

Figure 1 Possible applications of artificial intelligence technologies in the diagnosis and management of colorectal liver metastases. AI: Artificial intelligence.

0.693, 0.669 and 0.669, respectively) more accurate than the ones of Fong[67] and Vauthey[68] scores.

LIMITATIONS

In spite of AI's clear potential there remain several unresolved issues and limitations. These include the potential for artefacts in radiomics analyses to affect the results, the ethical and legal considerations, the definition of minimal accuracy rates and safeguards necessary to ensure public safety. Privacy, sensitive data protection and confidentiality need to remain the unmovable cornerstone of patient rights even in the digitalized era, but at the same time, some limitations on data utilization may affect the necessary linkages to prevent biases or errors in AI-driven analyses. There is a strong need from regulatory bodies for clear guidance during the AI-driven transformation of healthcare in order to take full advantage of the potential major improvements in individual and public health, while ensuring trust, safety and transparency. There is a significant variability in the algorithms investigated so far, as well as heterogeneity in the relatively small sample size of the population on which they have been trained and tested (Table 1). Analyses on large registries or national and international collaborations with data sharing could overcome part of the current limitations that limit the formal recognition of AI as a reliable and reproducible application in clinical scenarios.

CONCLUSION

The progressive widespread availability of high-performance computing, together with the accessibility to a large amount of data constantly generated as the result of the increase in the digitalization, set the ground for the ubiquitous implementation of AI technologies in contemporary healthcare. The fields of medical and surgical oncology have welcomed with enthusiasm the advent of augmented medicine with numerous studies investigating its potential, also given the high complexity and diversity of cancer patients. CRC makes no exception and still represents a leading cause of cancer-related death due to its high incidence, rapid progression potential and biological heterogeneity that advocate the need for reliable and individualized diagnostic, prognostic and treatment selection tools. Recent years have seen AI technologies tested

Table 1 Summary of the studies considered in this review

Author	Study design	AI model type	Data source	Total sample size/training cohort/validation cohort	AUC training/AUC validation	Sensitivity/specificity	PPV/NPV	Accuracy
CRLM development								
Li *et al*[23] (2020)	Retrospective; Single center	Radiomics/ML	CT images ± clinical data	100/NA/80	0.90/0.906	81%/84%	85%/79%	NA
Taghavi *et al*[24] (2021)	Retrospective; Multicenter	Radiomics/ML	CT images ± clinical data	91/70/21	0.95[2]-0.68[3]-0.95[4]/0.86[2]-0.71[3]-0.86[4]	NA/NA	NA/NA	NA
Lee *et al* [25] (2020)	Retrospective; Single center	Radiomics/CNN	CT images ± clinical data	2019/1413/606	NA/0.606[2]-0.709[3]-0.747[4]	NA/NA	NA/NA	NA
Diagnosis								
Vorontsov *et al*[26] (2017)	Retrospective; Single center	Radiomics/CNN	CT images	40/32/8	NA/NA	84%/92%	NA/NA	88%
Vorontsov *et al*[28] (2019)	Retrospective; Single center	Radiomics/CNN	CT images	156/115/15	NA/NA	59%[5]/NA	80%[5]/NA	NA
Ma *et al* [30] (2020)	Retrospective; Multicenter	CNN	CT images	909/479/202 (228[6])	NA/0.837-0.844[6]	82%[6]/74%[5]	75%[6]/81%[6]	NA
Kim *et al* [31] (2021)	Retrospective; Single center	DL	CT images	587/502/85	NA/0.631	81.82%/22.22%	NA/NA	NA
Khalili *et al*[34] (2020)	Retrospective; Single center	CNN	CT images ± liver metastatic status	199/150/49	NA/0.84-0.95[7]	(81.5%-81.5%[7])/(76.2%-96.4%[7])	NA/NA	78.3%; 90.6%[6]
Jansen *et al*[38] (2019)	Retrospective; Single center	CNN	MRI images	121/334[1]/86[1]	NA/NA	99.8%/NA	NA/NA	NA
Steenhuis *et al*[39] (2020)	Retrospective; Single center	ML	VOCs	62/NA/NA	NA/0.86	88%/75%	72%/90%	81%
Miller-Atkins *et al*[40] (2020)	Prospective; Single center	ML	VOCs	296/284/NA	NA/NA	51%/94%	NA/NA	86%
Kiritani *et al*[41] (2021)	Retrospective; Single center	ML	Histologic markers	183/NA/40	NA/0.999	100%/99%	NA/NA	99.5%
Han *et al* [47] (2020)	Retrospective; Single center	Radiomics/ML	MRI images ± clinical data	107/61[1]/31[1]	0.974[2]-0.659[3]-0.971[4]/0.912[2]-0.676[3]-0.909[4]	95.2%[2]-57.1%[3]-95.2%[4]/80.0%[2]-70.0%[3]-70.0%[4]	NA/NA	90.3%[2]; 61.3%[3]; 87.1%[4]
Chemotherapy response								
Maaref *et al*[54] (2020)	Retrospective; Single center	DL CNN	CT images	202/70%/10%	0.97/0.88	98%/54%	NA/NA	91%[8]; 78%[9]
Wei *et al* [55] (2021)	Retrospective; Single center	Radiomics/DL	CT images ± CEA	192/144/48	0.903[10]-0.935[11]/0.820[10]-0.830[11]	90.9%/73.3%	88.2%/78.6%	85.4%
Giannini *et al*[57] (2020)	Retrospective; Multicenter	Radiomics/ML	CT images	38/28/10	NA/NA	92%/86%	96%/75%	NA
Nakanishi *et al*[58] (2021)	Retrospective; Single center	Radiomics	CT images	42/94[1]/32[1]	0.8512/0.7792	NA/NA	NA/NA	NA
Local ablative therapies efficacy								
Taghavi *et*	Retrospective;	Radiomics/ML	CT images	90/63/27	NA/0.78[2]-	NA/NA	NA/NA	NA

al[59] (2021)	Single center				0.56[3]-0.79[4]			
Survival prediction								
Mühlberg *et al*[60] (2021)	Retrospective; Single center	Radiomics/ML	CT images ± WLTB ± TBS	103/NA/NA	NA/0.70[12] –0.73[13]-0.76[14]	NA/NA	NA/NA	NA
Hao *et al* [62] (2017)	Retrospective; Multicenter	ML	DNA methylation	1792[1]/NA/884[1] (718[1,6])	NA/NA	NA/NA	NA/NA	98.4%
Dercle *et al* [64] (2020)	Retrospective; Multicenter	ML	CT images	667/438/229	0.83/0.80	80%/78%	NA/NA	NA
Spelt *et al* [65] (2013)	Retrospective; Single center	ANN	Clinical variables	241/NA/NA	NA/NA	NA/NA	NA/NA	72%
Paredes *et al*[66] (2020)	Retrospective; Multicenter	ML	Clinical variables	1406/703/703	0.527[15]-0.525[16]-0.693[17]/0.524[15] -0.501[16]-0.642[17]	NA/NA	NA/NA	NA

[1]Number of lesions.
[2]Model based on radiomics data only.
[3]Model based on clinical data only.
[4]Model based on both radiomics and clinical data.
[5]Per patient values.
[6]Values calculated on the external validation set.
[7]Model based on both convolutional neural network and liver metastatic status.
[8]For differentiating treated and untreated lesions.
[9]For predicting the response to a FOLFOX + bevacizumab-based chemotherapy regimen.
[10]Model based on both deep learning and radiomics signature.
[11]Model based on deep learning and radiomics signature considering carcinogenic embryonic antigen values.
[12]Model based on tumor burden score.
[13]Model based on geometric metastatic spread of whole liver tumor burden.
[14]Model based on the Aerts radiomics prior model.
[15]Model based on Fong/Blumgart clinical risk score for predicting 1-year recurrence.
[16]Model based on Brudvik–Vauthey clinical risk score for predicting 1-year recurrence.
[17]Model based on Paredes–Pawlik clinical risk score for predicting 1-year recurrence.
AI: Artificial intelligence; ANN: Artificial neural network; AUC: Area under the curve; CEA: Carcinogenic embryonic antigen; CNN: Convolutional neural network; CRLM: Colorectal cancer liver metastases; DL: Deep learning; ML: Machine learning; NPV: Negative predictive value; PPV: Positive predictive value; TBS: Tumor burden score; VOCs: Volatile organic compounds; WLTB: Whole liver tumor burden.

by researchers in all phases of the CRLM natural history, aiming at overcoming the current difficulties and limitations faced by the multidisciplinary team responsible of the patients' care (Figure 1). The possibility of identifying the subgroup of patients at higher risk of CRLM development before the occurrence of the disease from the radiomics baseline CT scan analysis with high accuracy (AUC ≥ 0.75) and in less than 5 min could give such patients the best chances of an early diagnosis, more effective treatment, and therefore, a better outcome thanks to a personalized approach[23-25]. Radiomics has also demonstrated a great potential in assisting the radiologists in diagnosing CRLM from CT and MRI scans also by optimizing the identification of the optimal phases for lesions recognition and characterizing small nodules of uncertain nature[27-31,34,38]. A more efficient diagnostic process would help reduce timings and costs, resulting in a potential benefit for both patients and healthcare systems. AI application in order to rapidly and accurately identify CRLM tissue and its different histopathological growth patterns[41,47] could give a significant contribution towards a rapid oncological individualized approach and treatments. AI technologies have also shown potential as a prognostic and outcome tool, predicting with good accuracy response to chemotherapy[54,55,57,58], early local tumor progression after ablation treatment[59], and patient survival after surgery or chemotherapy[60,64-66].

The possibility of reducing human factors and error, increase accuracy and contain timings and costs while adopting a personalized medicine approach is undoubtedly fascinating and appealing, but despite showing promising results, the role of AI in CRLM patients has not yet been fully elucidated. The implementation of AI resources supports the contemporary paradigm shift that sees healthcare focus moving from a generalized, disease-oriented to an individual, patient-centered, precision medicine approach. The effectiveness of ML models lie on a rigid framework in which a well-defined problem and ground truth along with quantitative objective measures to train and validate the algorithm are needed, making the process efficient but rigid. There is

also a balance to be struck between the accuracy and artificial logic and the risk of AI becoming less intelligible and explainable. On the other hand, AI medical technologies could represent a way to enable patients to take ownership of their own care, increasing participation and autonomy for a more personalized approach.

AI will likely affect the immediate future of medicine and patients' management, but rather than replacing the human roles, it will probably be aimed to assist and facilitate physicians in their practice, while being supervised to ensure maximum safety. This could be in the context of diagnostic uncertainty or to assist in planning optimal treatment strategies. A possible future development would be to improve diagnosis and management through the AI analysis and integration of clinical information, radiomic and genetic data thanks to the recent developments in gene sequencing and liquid biopsies, that have showed great potential in gastrointestinal tumors including CRLM[69-72]. A personalized holistic approach providing reliable data for the diagnosis, management and outcome estimation of cancer patients would assist clinicians in the prevention as well as selecting the most appropriate individualized treatment that would grant the patient the best outcome as well as helping patients to make fully informed decisions.

In order to continue to pursue the ambitious goal of improving patients' care through AI healthcare technologies, further larger, prospective, randomized controlled and rigorous studies are needed.

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ORIGINAL ARTICLE

Basic Study

Focal adhesion kinase-related non-kinase ameliorates liver fibrosis by inhibiting aerobic glycolysis *via* the FAK/Ras/c-myc/ENO1 pathway

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Abstract

BACKGROUND

Hepatic stellate cell (HSC) hyperactivation is a central link in liver fibrosis development. HSCs perform aerobic glycolysis to provide energy for their activation. Focal adhesion kinase (FAK) promotes aerobic glycolysis in cancer cells or fibroblasts, while FAK-related non-kinase (FRNK) inhibits FAK phosphorylation and biological functions.

AIM

To elucidate the effect of FRNK on liver fibrosis at the level of aerobic glycolytic metabolism in HSCs.

METHODS

Mouse liver fibrosis models were established by administering CCl_4, and the effect of FRNK on the degree of liver fibrosis in the model was evaluated. Transforming growth factor-β1 was used to activate LX-2 cells. Tyrosine phosphorylation at position 397 (pY397-FAK) was detected to identify activated FAK, and the expression of the glycolysis-related proteins monocarboxylate transporter 1 (MCT-1) and enolase1 (ENO1) was assessed. Bioinformatics analysis was performed to predict putative binding sites for c-myc in the ENO1 promoter region, which were validated with chromatin immunoprecipitation (ChIP) and dual-

interest to declare.

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luciferase reporter assays.

RESULTS
The pY397-FAK level was increased in human fibrotic liver tissue. FRNK knockout promoted liver fibrosis in mouse models. It also increased the activation, migration, proliferation and aerobic glycolysis of primary hepatic stellate cells (pHSCs) but inhibited pHSC apoptosis. Nevertheless, opposite trends for these phenomena were observed after exogenous FRNK treatment in LX-2 cells. Mechanistically, the FAK/Ras/c-myc/ENO1 pathway promoted aerobic glycolysis, which was inhibited by exogenous FRNK.

CONCLUSION
FRNK inhibits aerobic glycolysis in HSCs by inhibiting the FAK/Ras/c-myc/ENO1 pathway, thereby improving liver fibrosis. FRNK might be a potential target for liver fibrosis treatment.

Key Words: Liver fibrosis; Hepatic stellate cells; Focal adhesion kinase; Focal adhesion kinase-related non-kinase; Aerobic glycolysis; Enolase1

Core Tip: We show that focal adhesion kinase-related non-kinase (FRNK) limits hepatic stellate cell (HSC) activation, proliferation, and migration and promotes HSC apoptosis by inhibiting aerobic glycolysis, thereby ameliorating liver fibrosis. FRNK may represent a potential therapeutic candidate for liver fibrosis treatment.

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INTRODUCTION

Long-term damage to liver function by hepatitis viruses, alcohol, and diet may cause chronic hepatic injuries leading to liver fibrosis and cirrhosis[1-4], which is characterized by the activation of hepatic stellate cells (HSCs) and their transformation into myofibroblasts[5]. This process continuously damages the liver and disrupts the balance of liver self-repair, causing increased cell proliferation and migration and a reduced apoptosis rate[6-8]. At present, the pathological changes associated with chronic liver injuries in individuals without cirrhosis can be reversed after removing the etiological agent, such as in a small proportion of patients with hepatitis B and alcoholic fatty liver disease and most patients with hepatitis C[1,9], but the remaining patients with hepatic fibrosis develop irreversible cirrhosis due to an inability to completely and effectively reverse the pathogenesis and a lack of effective antifibrotic drugs[10]. Therefore, studies of the treatment of liver fibrosis are particularly critical, among which the regulation of the relevant biological functions of HSCs is the most important antifibrotic approach[11,12].

Focal adhesion kinases (FAKs) are a class of nonreceptor cytosolic protein tyrosine kinases that belong to the protein tyrosine kinase superfamily[13,14]. FAK plays an important role in cellular signal transduction and enhances biological behaviors such as proliferation, migration, wound healing and angiogenesis in cells and tissues after integrating signals from integrins, growth factors and mechanical stimuli[15,16]. FAK binds to extracellular matrix (ECM) proteins through an accumulation of integrin receptors to form FAK dimers, which further induce tyrosine phosphorylation at position 397 (pY397-FAK); pY397-FAK regulates these biological functions in cells[17,18] and therefore plays an important role in a variety of malignant tumor cells[18,19]. FAK-related non-kinase (FRNK), which has a nucleotide sequence corresponding to

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the C-terminus of FAK but lacks the N-terminal functional site of FAK, is an independently expressed protein[20] with the main function of inhibiting FAK phosphorylation, thereby inversely regulating the function of FAK after cell activation [21,22]. FRNK negatively regulates FAK signaling axis function, thereby improving pulmonary fibrosis in an experimental mouse model[23].

FAK is also overexpressed in pancreatic ductal adenocarcinoma cells[24,25], promoting the conversion of pyruvate into lactate by increasing enolase1 (ENO1), pyruvate kinase 2, lactate dehydrogenase, and monocarboxylate transporter (MCT)-1 expression and lactate transport, enhancing aerobic glycolysis in cancer cells, and inhibiting mitochondrial oxidative phosphorylation in cancer cells[16]. This switch to aerobic glycolysis is an important mechanism by which tumor cells acquire energy[17,26], as shown by the fact that oxidative phosphorylation simultaneously provides energy to cells performing aerobic glycolysis even in the presence of sufficient oxygen and normal mitochondrial function[27,28]. HSCs also exhibit increased aerobic glycolysis, resulting in lactate accumulation and gluconeogenesis inhibition when they differentiate into myofibroblasts[8,28]. This phenomenon also occurs in individuals with congenital pulmonary fibrosis[29]. Therefore, the inhibition of FAK-related pathways by FRNK may reduce energy acquisition through aerobic glycolysis during HSC activation and could be used as a targeted therapy to ameliorate liver fibrosis. Nevertheless, few studies have focused on the physiological or pathological role of FRNK in obtaining energy during hepatic fibrosis, and its mechanism remains unclear.

In the present study, we first showed that FRNK was downregulated in human liver fibrotic tissues. Then, we verified that FRNK knockout *in vivo* and *in vitro* promoted aerobic glycolysis and hepatic fibrosis. Exogenous FRNK inhibited aerobic glycolysis by inhibiting the FAK/Ras/c-myc/ENO1 pathway, limiting HSC activation, migration, and proliferation and increasing apoptosis to ameliorate liver fibrosis. Together, these data provide a detailed mechanism through which FRNK functions and suggest that FRNK represents a potential target to inhibit aerobic glycolysis in HSCs and treat liver fibrosis.

MATERIALS AND METHODS

Human liver samples

Paraffin blocks of liver tissues from 15 patients with liver fibrosis were collected from the Department of Infectious Diseases, Affiliated Hospital of Guizhou Medical University (Guiyang, China) between March 2019 and September 2019; none of the patients had any other organ-specific or systemic diseases, and liver fibrosis was diagnosed by pathological biopsy. Fifteen healthy liver samples were obtained from distal hepatocarcinoma liver tissue without any abnormalities in specimens surgically resected from patients at the Department of Hepatobiliary Surgery, Affiliated Hospital of Guizhou Medical University. None of the aforementioned subjects had contraindications to liver biopsy, and the study was approved by the Ethics Committee of the Affiliated Hospital of Guizhou Medical University (Approval 2018 Ethics Review No. 032) and conducted in accordance with the ethical guidelines of the 1975 Declaration of Helsinki. Written informed consent was obtained from the patients.

Animals

FRNK knockout ($FRNK^{-/-}$) mice were a gift from the Respiratory and Critical Care Medicine Center, School of Medicine, University of Alabama at Birmingham, Birmingham, AL, United States. All mouse interventions were approved by the Animal Care Committee (IACUC) of Guizhou Medical University (No. 1801109), and the methods and experimental procedures were performed in accordance with the relevant guidelines and regulations. Wild-type (WT) mice of the same genotype were used as controls, and all experimental mice were on the C57BL/6 background.

Mice were maintained under pathogen-free conditions at a controlled temperature (22 ± 2 °C) with a consistent photoperiod (12:12 h light-dark cycle); five mice were housed in each cage, with cages containing soft bedding. The mice were habituated to these conditions for 2 d before inclusion in an experiment. Healthy male mice (aged 8-11 wk, weighing 20 ± 3 g) were selected and intraperitoneally injected with 1.5 μL/g of a 10% Carbon tetrachloride (CCl_4) in corn oil solution three times a week to establish a liver fibrosis model. Mice in the control group were injected with a 1.5 μL/g solution of corn oil three times a week. Livers were harvested at each time point, namely, 0, 2, 4 and 6 wk, for experiments. Six mice per group were used.

Reagents and antibodies

CCl_4, corn oil, and OptiPrep were purchased from Sigma-Aldrich (St. Louis, MO, United States). Transforming growth factor-β1 (TGF-β1) was purchased from R&D Systems (Minneapolis, MN, United States). Primary antibodies specific for the following proteins were purchased from Abcam (Cambridge, United Kingdom): Desmin rabbit monoclonal antibodies (ab32362), FAK rabbit monoclonal antibodies (ab40794), ENO1 mouse monoclonal antibodies (ab190365), alpha SMA rabbit polyclonal antibodies (ab5694), k-ras rabbit monoclonal antibodies (ab275876) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) rabbit polyclonal antibodies (ab9485). Anti-c-myc (13987) rabbit monoclonal antibodies were purchased from Cell Signaling Technology (Shanghai, China). MCT-1 rabbit polyclonal antibodies (20139-1-AP) were purchased from Proteintech (Wuhan, China). pY397-FAK rabbit polyclonal antibodies (AF3398) were purchased form Affinity Bioscience (Cincinnati, OH, United States). All other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, United States) and Fisher Scientific (Waltham, MA, United States).

Immunohistochemistry (IHC), hematoxylin & eosin (H&E), Masson's trichrome and Sirius Red staining and hydroxyproline assay

H&E staining kits, Masson's trichrome staining solution and Sirius Red staining solution were purchased from Solarbio Biotechnology Co., Ltd. (Beijing, China) and used according to the manufacturer's guidelines. A hydroxyproline assay was performed using a Nanjing Jiancheng Biotechnology (Nanjing, China) hydroxyproline kit. All kits were used according to the instructions for use. Liver samples were fixed with neutral buffered formalin and embedded in paraffin for IHC. Briefly, sections were incubated with the indicated antibodies. Horseradish peroxidase-conjugated antibodies were used as the secondary antibodies. Finally, a diaminobenzidine colorimetric reagent solution was applied, followed by hematoxylin counterstaining. The slides were then scanned, and representative images were acquired.

Cells and cell culture

LX-2 cells were purchased from Zhongqiao Xinzhou (Shanghai, China). Human HSCs (ZQ0026) and LX-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS; Biological Industries, Kibbutz Beit-Haemek, Israel). Primary hepatic stellate cells (pHSCs) were extracted from C57BL/6 WT or $FRNK^{-/-}$ mice aged 8-11 wk, as previously described[30,31]. Briefly, the abdominal cavity was opened with a "cross" incision, an 18-gauge trocar was inserted from the left ventricle to inject the preperfusate, and the blood in the liver was flushed by exsanguination until the tissue turned yellow. Then, the preperfusate was replaced with pronase and collagenase for 15-20 min, and the liver was removed and washed with normal saline. The liver capsule and connective tissue were removed, fully digested with a digestion solution at 37 °C with shaking and ground to generate a single-cell suspension. The supernatant was discarded after centrifugation at 1500 rpm for 5 min, and the pellet was resuspended in D-Hank's solution. The hepatocytes were removed by centrifugation, and a gradient lymphocyte separation solution was directly added. HSCs were isolated in one step using monolayer gradient centrifugation, and the cells were washed twice with DMEM and cultured with DMEM containing 10% FBS. Cell survival was evaluated by performing trypan blue staining, and cell purity was identified by desmin immunocytochemical staining. All cells were cultured in an incubator containing 5% CO_2 at 37 °C.

Recombinant FRNK adenoviral vector transfection and HSC activation

An adenovirus-mediated gene delivery system was used to effectively deliver the FRNK cDNA into HSCs. An adenoviral vector carrying the FRNK protein and green fluorescent protein (Ad-FRNK) as well as a green fluorescent protein-carrying adenovirus (Ad-GFP) were purchased from Jikai Gene (Shanghai, China). All transfections were performed according to the manufacturer's instructions, and cells in serum-free medium (DMEM with 1% BSA) were transfected with Ad-FRNK or the control vector (Ad-GFP) 24 h before TGF-β1 treatment. Twenty-four hours later, the cells were cultured with complete medium containing 2 ng/mL TGF-β1 and treated for 36 h[31].

Transwell, cell counting kit-8 (CCK-8) and flow cytometry assays

Transwell migration experiments used 8.0-μm pore size membranes (Corning, United States) according to the manufacturer's protocol. A total of 10^5 cells were seeded in the upper chamber of each well in 100 μL of serum-free medium, while 600 μL of complete

medium was added to the lower chamber as a chemoattractant. After a 6-h incubation at 37 °C, the cells remaining on the upper surface of the membrane were removed with a cotton swab, and the cells on the lower surface of the membrane were considered migrated cells. After fixation with 4% paraformaldehyde and staining with a 0.1% crystal violet solution, images were acquired under an inverted microscope. Cell Counting Kit-8 (CCK-8) was purchased from Dojindo (Shanghai, China), and 10^4 cells (100 μL/well) were seeded in 96-well plates. After placing the culture plate in an incubator for preincubation (37 °C, 5% CO_2), 10 μL of CCK-8 solution was added to each well, and then the culture plate was evaluated with a microplate reader to detect the absorbance at 450 nm. A total of 10^5 cells in each group were stained with an Annexin V-PE/7-AAD apoptosis kit (Hangzhou Lianke, Hangzhou, Zhejiang Province, China) according to the instructions for use, sorted with a flow cytometer (Beckman, United States) and analyzed using Flow Jo software (Tree Star); dead cells were excluded based on forward scatter and side scatter data.

Western blot analysis

Western blot analysis was performed as previously described[30]. Briefly, 1% NP-40-treated whole-liver tissue lysates or whole-cell lysates were used for Western blot analysis. Protein levels were quantified using a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA) after total protein extraction. Twenty milligrams of each protein sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). GAPDH was used as a loading control for all blots. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes, which were incubated with primary antibodies overnight at 4 °C. The next day, after an incubation with an appropriate secondary antibody, signals were generated with an electrochemiluminescence detection kit.

Glucose consumption, 2-NBDG uptake and lactate assays

The lactate level in culture medium was detected with the Lactate Colorimetric Assay Kit (BioVision, Milpitas, CA, United States) according to the manufacturer's instructions. The 2-NBDG Glucose Uptake Kit (BioVision, Milpitas, CA, United States) was used to detect the cellular uptake of glucose, and the Glucose Colorimetric Assay Kit (BioVision, Milpitas, CA, United States) was used to detect the glucose concentration in culture medium and thus measure the cellular consumption of glucose. The 2-NBDG Glucose Uptake Kit and the Glucose Colorimetric Assay Kit were used according to the manufacturer's protocol.

Chromatin immunoprecipitation (ChIP) assay

JASPAR (http://jaspar.genereg.net) and PROMO (http://alggen.lsi.upc.es) database analyses predicted two putative c-myc binding sites in the ENO1 promoter region. A total of 10^7 cells fixed with formaldehyde were collected in 500 μL of lysis buffer from the Magna ChIP HiSens Kit (Millipore, Bedford, Massachusetts, United States) according to the manufacturer's manual. Cells were then sonicated for 25 cycles with a 6-s power-on interval of 30 s and an intensity of 200 W. Afterward, the supernatant was diluted and thoroughly mixed with Protein A/G beads. Then, 5 μg of IgG or an anti-c-myc antibody was added and incubated with the mixture overnight at 4 °C. After washing the beads the next day, the mixture was incubated with elution buffer at 62 °C for 2 h and then 95 °C for 10 min. The eluted DNA was then purified and subjected to a PCR assay to assess the binding sequence. Specific primer sequences were used to perform PCR.

Dual-luciferase reporter assays

The effect of c-myc on the ENO1 promoter was determined by cotransfecting pcDNA-c-myc or pcDNA-vector (NC) into LX-2 cells with pGL3-based constructs containing an empty sequence (NC) or the WT or MT1/MT2 ENO1 promoter sequences, and Renilla luciferase reporter plasmids. Twenty-four hours after transfection, firefly and Renilla luciferase activities were measured with a luciferase reporter assay kit (Genomeditech, Shanghai, China). Fluorescence detection was performed according to the instructions of the instrument, the parameters were set, the measurement time was 10 s, and the measurement interval was 2 s. Each sample was added into a measuring tube in a total volume of 20 μL (the sample volume was consistent in each measurement), and then 20 μL of Firefly Luciferase Assay Reagent was added, mixed well 2-3 times (without vortexing), mixed well again and evaluated to determine relative light unit (RLU) 1. Cell lysis buffer was set as the blank control well. The tested samples were mixed with 20 μL of prepared Renilla Luciferase Assay working solution

2-3 times and mixed well before measuring RLU2. The measured RLU1 value was compared to the corresponding RLU2 value, and the resulting ratio determined the degree of reporter activation. The ratio of firefly luciferase activity to Renilla luciferase activity was calculated for each sample.

Statistical analysis

Data were statistically analyzed using GraphPad Prism 5.0 software, and a two-tailed Student's *t* test was used for comparisons between different groups. $P < 0.05$ was considered statistically significant. Data are presented as the mean ± SD.

RESULTS

The level of the pY397-FAK protein was increased while the level of the FRNK protein was decreased in human fibrotic liver tissue

We first investigated the level of pY397-FAK in fibrotic liver tissue to explore the role of pY397-FAK in liver fibrosis. Compared with normal liver tissue, liver tissue samples from patients in the liver fibrosis group showed typical pathological features, including significant steatosis, inflammatory necrosis, significant collagen deposition, hepatic fibrosis and hepatocyte loosening. Masson's trichrome staining showed less collagen deposition and a normal cell morphology in the normal group, while a large number of blue-stained collagen fibers was observed in the liver fibrosis group, and the tissue had accumulated a wide band of collagen fibers that extended into and was distributed in the hepatic lobules. Sirius Red staining showed less collagen deposition in normal subjects and a normal cell morphology but substantial red staining indicating collagen deposition in portal areas in fibrotic liver tissues. Notably, IHC showed higher α-smooth muscle actin (α-SMA) and pY397-FAK expression in fibrotic liver tissue than in normal liver tissues (Figure 1A and B). Western blot analysis showed higher levels of the pY397-FAK protein in fibrotic liver tissues than in normal liver tissues. Conversely, in fibrotic liver tissue, FRNK was expressed at lower levels than that in normal tissues ($P < 0.05$, Figure 1C and D). These results suggest that pY397-FAK protein expression is increased and FRNK protein expression is decreased in fibrotic liver tissue.

Exacerbation of liver fibrosis and aerobic glycolysis in mice after FRNK knockout in vivo

We established a fibrosis model by injecting CCl_4 into WT mice and FRNK$^{-/-}$ mice. After two fortnights, the expression of the pY397-FAK protein peaked, while the FRNK protein was expressed at a low level ($P < 0.05$, Figure 2A and B). Therefore, mouse models with four weeks of injection were used in subsequent experiments. By performing H&E, Masson's trichrome and Sirius Red staining, we found a greater liver fibrosis area and more extensive liver fibrosis in FRNK$^{-/-}$ mice than in WT mice after the CCl_4 intervention ($P < 0.05$, Figure 2C and D), while the hydroxyproline content in FRNK$^{-/-}$ mice with fibrosis was greater than that in WT mice with fibrosis ($P < 0.05$, Figure 2E). Western blot analysis revealed higher levels of the pY397-FAK, MCT-1, ENO1 and α-SMA proteins in the liver tissues from FRNK$^{-/-}$ mice treated with CCl_4 than in WT mice ($P < 0.05$, Figure 2F and G). Based on these results, FRNK$^{-/-}$ mice develop more severe liver fibrosis after the CCl_4 intervention, along with increased expression of the aerobic glycolysis-related proteins MCT-1 and ENO1. It suggests that there may be more active aerobic glycolysis in the liver.

FRNK knockout promotes liver fibrosis and aerobic glycolysis in vitro

We extracted pHSCs from WT mice and FRNK$^{-/-}$ mice for *in vitro* experiments (Supplementary Figure 1). After 36 h of TGF-β1 treatment, the migration of pHSCs from the FRNK$^{-/-}$ groups in a Transwell chamber was increased ($P < 0.05$, Figure 3A). As indicated by the level of cell proliferation, pHSCs from the FRNK$^{-/-}$ group exhibited increased cellular activity ($P < 0.05$, Figure 3B). After adding TGF-β1 to pHSCs from FRNK$^{-/-}$ mice for 36 h, the apoptosis rate was lower than that of pHSCs from the control mice ($P < 0.05$, Figure 3C). Moreover, pHSCs from FRNK$^{-/-}$ mice showed increased glucose uptake and consumption compared with control pHSCs. Additionally, the lactate level in the medium of pHSCs from FRNK$^{-/-}$ mice was increased compared with the lactate level in the medium of pHSCs from the control group ($P < 0.05$, Figure 3D and E). Western blot analysis showed higher levels of the MCT-1, ENO1 and α-SMA proteins in the pHSCs from FRNK$^{-/-}$ mice was higher than in the

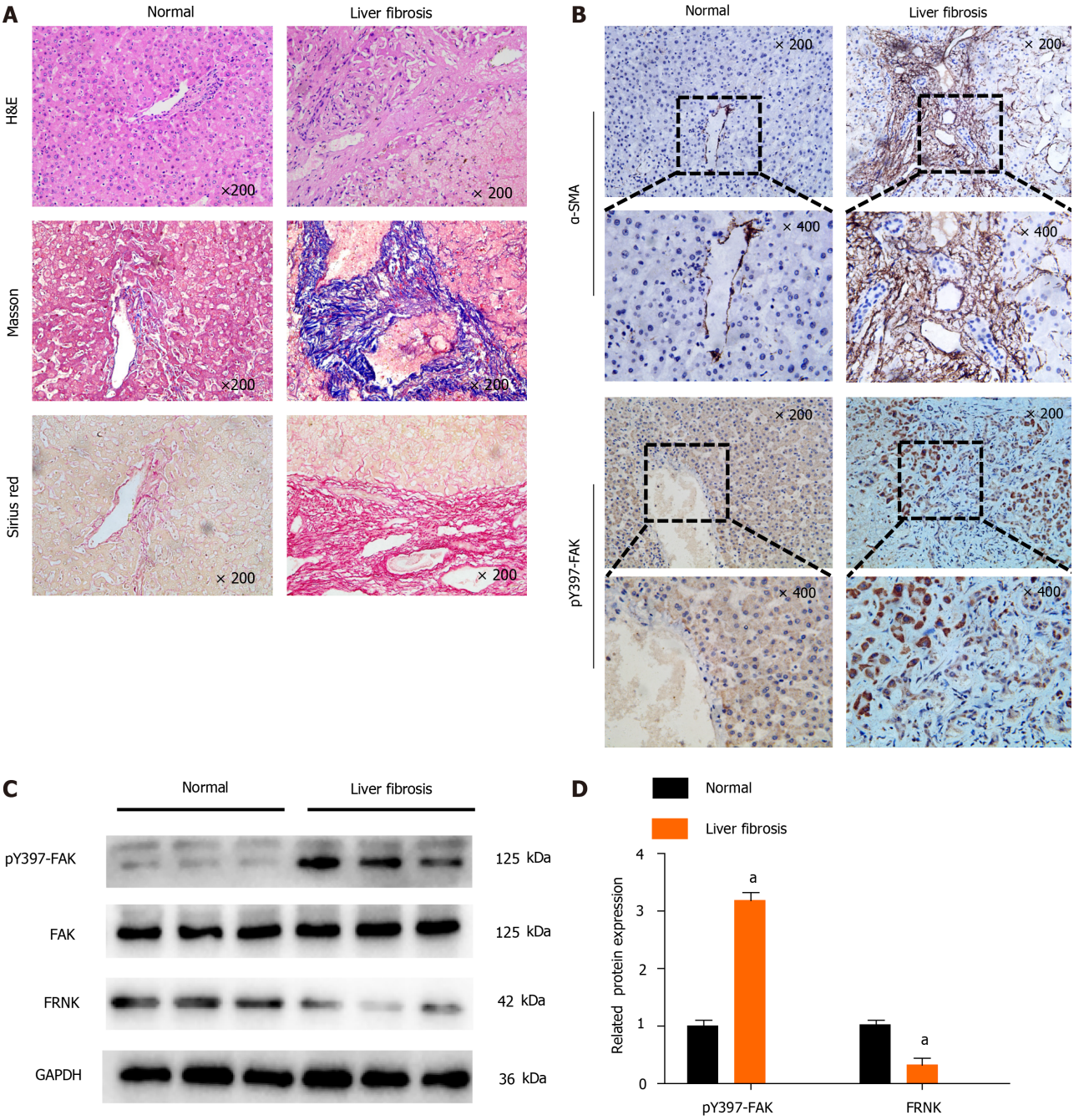
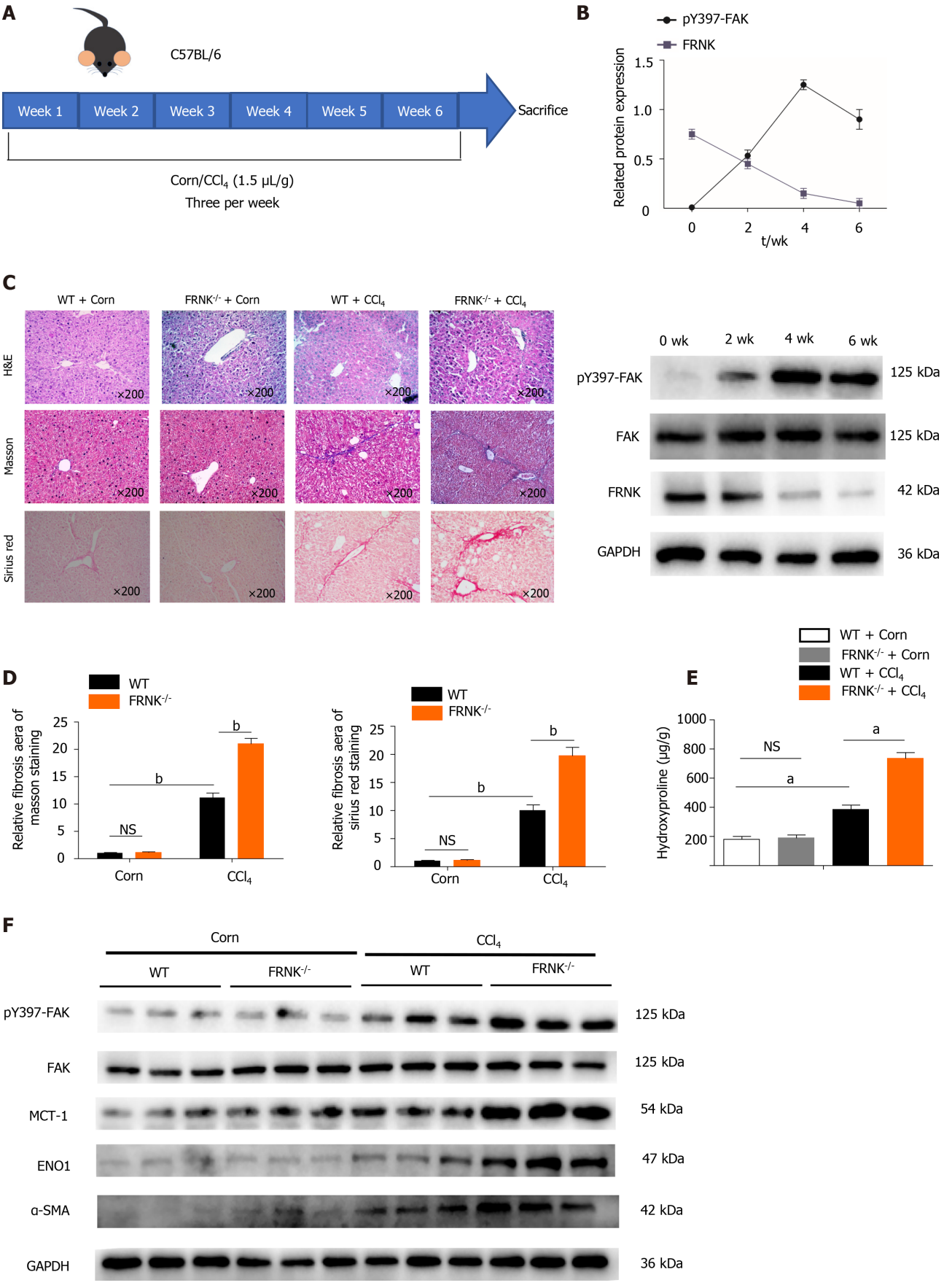


Figure 1 Tyrosine phosphorylation at position 397 of FAK is upregulated, while FRNK expression is downregulated in human fibrotic liver tissue. A: H&E, Masson's trichrome and Sirius Red staining were performed after liver biopsy to assess the tissues of normal subjects and patients with liver fibrosis under a light microscope at 200× magnification; B: Immunohistochemistry showed changes in the expression of α-smooth muscle actin (α-SMA) and tyrosine phosphorylation at position 397 of FAK(pY397-FAK) in the livers of normal subjects compared with patients with liver fibrosis under a light microscope at 200× or 400× magnification; C and D: Protein expression in biopsy tissues was analyzed using Western blotting. Representative results from three independent replicate assays are shown. $^{a}P < 0.05$. Data are presented as the mean ± SD. FAK: Focal adhesion kinase; FRNK: Focal adhesion kinase-related non-kinase; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

pHSCs from WT mice ($P < 0.05$, Figure 3F and G). The above results illustrate that FRNK knockout in mice increases the activation, migration, and proliferation of pHSCs and attenuates pHSC apoptosis while enhancing their aerobic glycolytic capacity *in vitro*.

Exogenous FRNK ameliorates experimental liver fibrosis and aerobic glycolysis in vitro

We transfected LX-2 cells with an adenovirus containing FRNK, induced the expression of the exogenous FRNK gene and incubated the cells with TGF-β1 for 36 h. We then performed Transwell, CCK-8 and flow cytometry assays with the transfected


A
C57BL/6
Week 1
Week 2
Week 3
Week 4
Week 5
Week 6
Sacrifice
Corn/CCl4 (1.5 μL/g)
Three per week
B
pY397-FAK
FRNK
Related protein expression
t/wk
C
WT + Corn
FRNK-/- + Corn
WT + CCl4
FRNK-/- + CCl4
H&E
Masson
Sirius red
×200
0 wk
2 wk
4 wk
6 wk
pY397-FAK
FAK
FRNK
GAPDH
125 kDa
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NS
b
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Hydroxyproline (μg/g)
NS
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CCl4
WT
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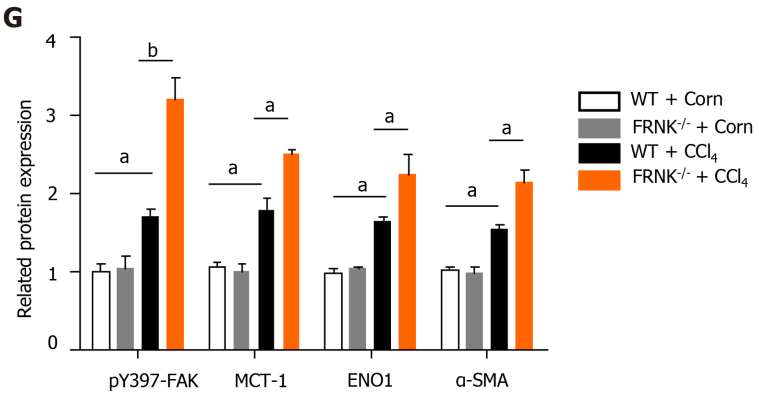

Figure 2 Liver fibrosis in mice was aggravated after FRNK knockout. A and B: WT mice were modeled for 6 wk, pY397-FAK and FRNK protein expression levels *in vivo* was measured using Western blotting every fortnight; C and D: FRNK$^{-/-}$ and WT mice were used to establish a liver fibrosis model by administering CCl_4 (1.5 μL/g), and liver tissues from these mice were stained with H&E, Masson's trichrome, and Sirius Red after 4 wk and observed under a light microscope × 200 magnification. The relative fibrotic areas were analyzed; E: The hydroxyproline content in liver tissues from the liver fibrosis model was also measured; F and G: Western blotting was used to detect the relative expression of proteins in the liver fibrosis model established with FRNK$^{-/-}$ mice and WT mice. Representative results from three independent replicate assays are shown (n = 6). $^{a}P < 0.05$ and $^{b}P < 0.01$. Data are presented as the mean ± SD. MCT-1: Monocarboxylate transporter-1; ENO1: Enolase1.

cells to evaluate migration, proliferation and apoptosis. The migration of LX-2 cells was inhibited and apoptosis was increased after the introduction of exogenous FRNK compared to the control treatment ($P < 0.05$, Figure 4A and C). In addition, proliferation was also inhibited ($P < 0.01$, Figure 4B). Based on these results, exogenous FRNK inhibits cell migration and proliferation and promotes apoptosis. The abilities of cells in the Ad-FRNK group to take up and consume glucose were reduced, and the lactate level in the cell culture medium was reduced ($P < 0.05$, Figure 4D and E). Subsequently, cellular proteins were extracted, and the relative levels of intracellular pY397-FAK, MCT-1, ENO1 and α-SMA proteins were detected by Western blotting. The relative expression of the aforementioned proteins in the Ad-FRNK group was lower than that in the control group ($P < 0.05$, Figure 4F and G). Thus, the introduction of exogenous FRNK into HSCs inhibits cell proliferation and migration and promotes apoptosis. It also inhibits cellular aerobic glycolysis and thus inhibits cellular energy generation *in vitro*.

FRNK does not directly target ENO1

To explore the precise molecular mechanism by which FRNK regulates the ENO1 protein, TGF-β1 was used to stimulate LX-2 cells to activate FAK. The level of pY397-FAK was increased after stimulation with TGF-β1. The expression of the K-Ras, c-myc and ENO1 proteins downstream of FAK was also examined ($P < 0.05$, Figure 5A and B). While examining whether FRNK directly inhibits ENO1 protein expression, increased ENO1 protein expression was observed after introducing exogenous c-myc into LX-2 cells, but ENO1 protein expression was not reduced after the continued introduction of exogenous FRNK ($P < 0.05$, Figure 5C and D), suggesting that exogenous FRNK does not directly inhibit ENO1 protein expression to exert its biological function. As a method to investigate whether c-myc directly regulates ENO1, a bioinformatics analysis of the ENO1 promoter was performed to predict the putative binding site for c-myc in the ENO1 promoter, followed by ChIP and dual-luciferase reporter assays to verify that c-myc transcriptionally activates the ENO1 promoter ($P < 0.05$, Figure 5E and F). The results suggested that TGF-β1 activates FAK by inducing FAK phosphorylation at position 397. Then, pY397-FAK increased the expression of downstream K-Ras and c-myc proteins, followed by transcriptional activation of ENO1 expression by c-myc, promoting aerobic glycolysis and activation in HSCs. pY397-FAK and its biological functions were inhibited by the introduction of exogenous FRNK, limiting aerobic glycolysis and activation in HSCs and ameliorating liver fibrosis (Figure 6).

DISCUSSION

The imbalance between liver injury and self-repair is the key to the development of liver fibrosis, and restoring the balance from an imbalanced state is a potential

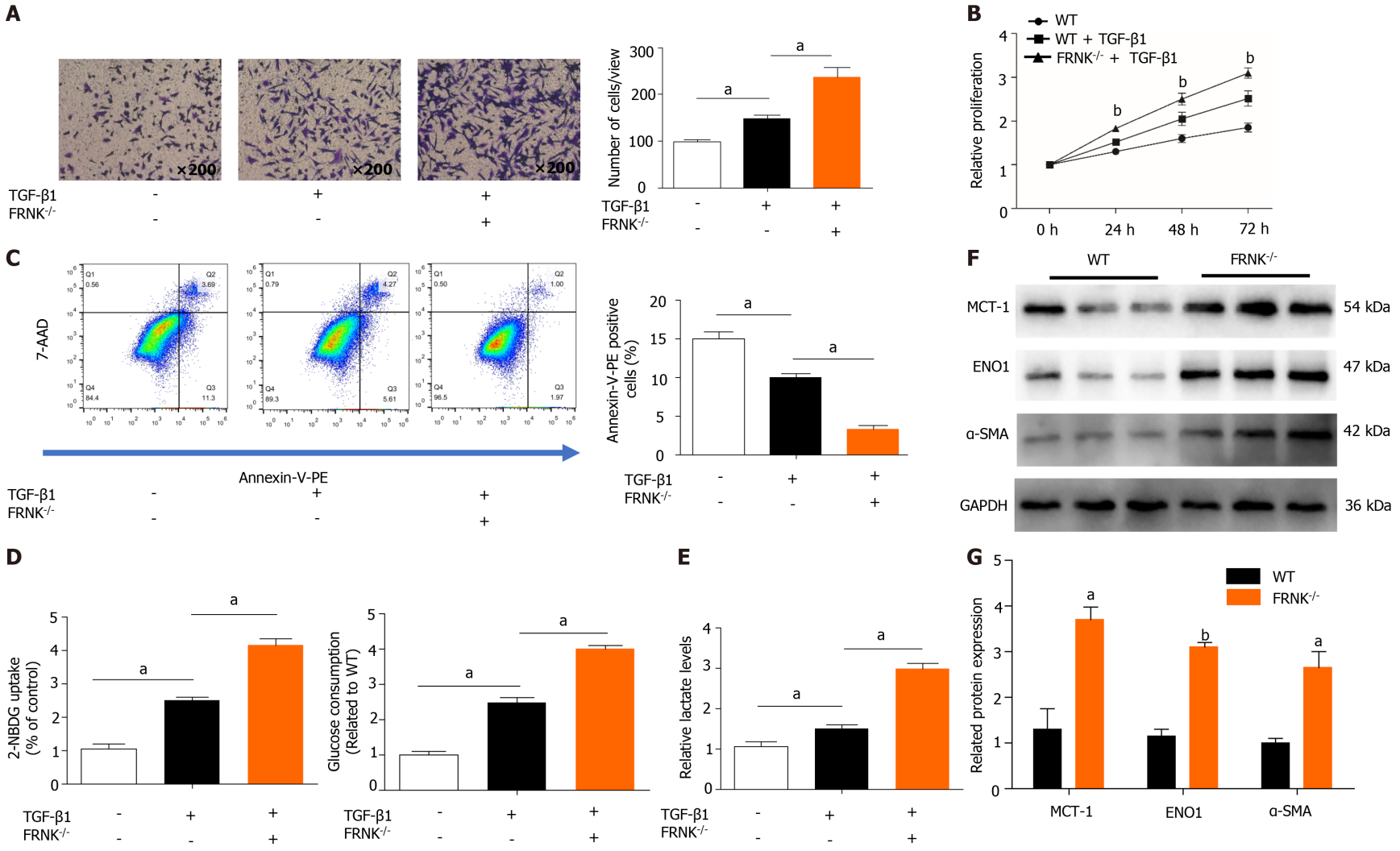

Figure 3 Knockout of FRNK promotes liver fibrosis and aerobic glycolysis *in vitro*. A: After 36 h of culture with TGF-β1 (2 ng/mL), the migratory ability of primary hepatic stellate cells(pHSCs) was measured under a light microscope at × 200 magnification (10^5 cells per well); B: The proliferation of pHSCs was assessed with a CCK-8 assay; C: The apoptosis of pHSCs was analyzed using flow cytometry after 36 h of intervention; D and E: pHSCs cultured under the same intervention conditions were examined for glucose uptake and consumption, and lactate levels in the cell culture medium were also assessed; F and G: MCT-1, ENO1 and α-SMA levels in pHSCs were assessed using Western blotting. Representative results from three independent replicate assays are shown. $^aP < 0.05$ and $^bP < 0.01$ Results are presented as the mean ± SD.

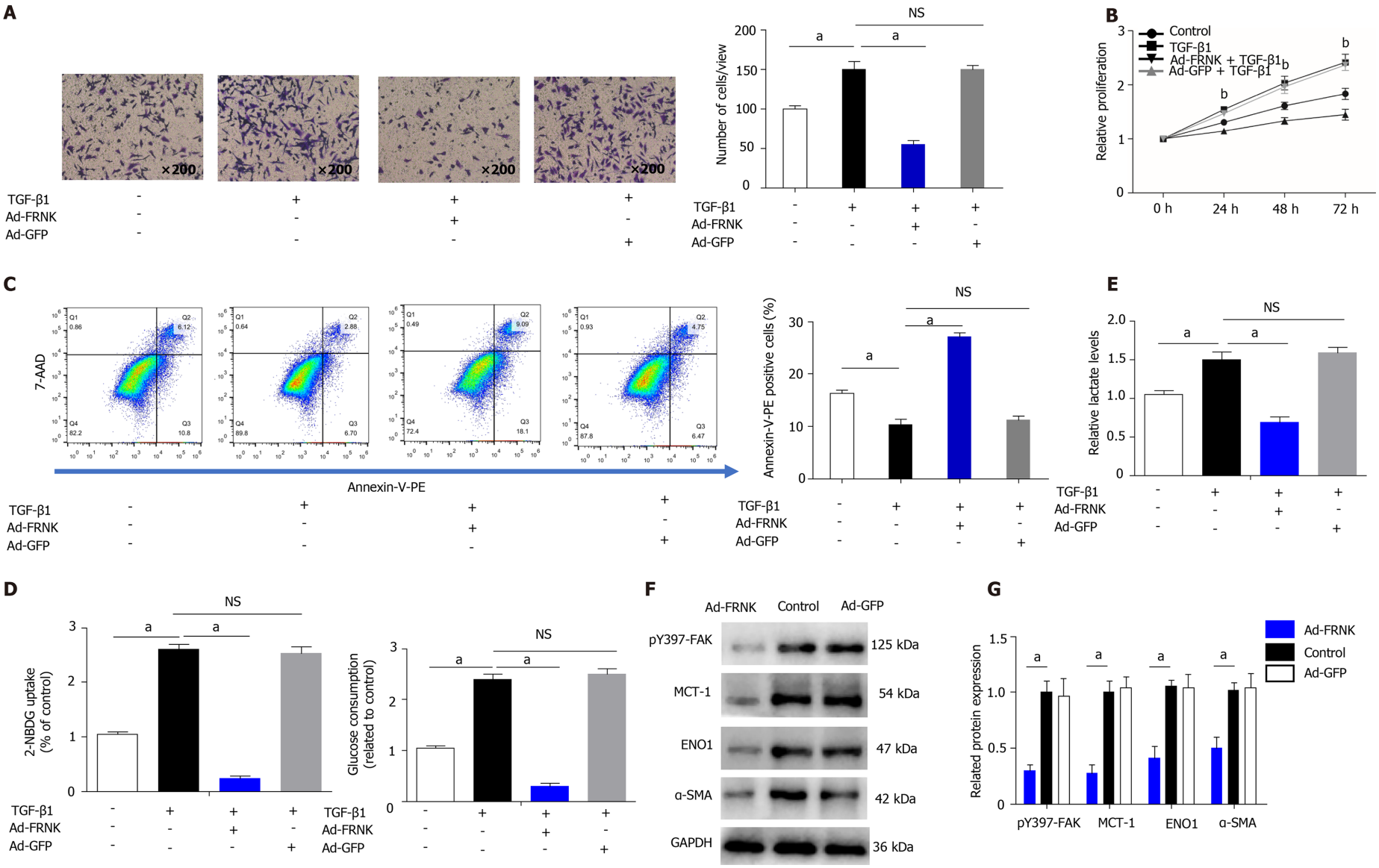

Figure 4 Exogenous FRNK ameliorates experimental liver fibrosis and aerobic glycolysis *in vitro*. LX-2 cells were transfected with a green fluorescent protein-carrying adenoviral vector (Ad-GFP) also encoding the FRNK gene. A: Cultured with TGF-β1 (2 ng/mL) for 36 h (10^5 cells per well). Migration was measured by analyzing cells under a light microscope × 200 magnification; B: The proliferation of LX-2 cells cultured under the intervention conditions is presented; C: The

apoptosis of LX-2 cells was analyzed after 36 h of intervention using flow cytometry; D and E: LX-2 cells cultured under the same intervention conditions were examined for glucose consumption and uptake abilities, and lactate levels in the cell culture medium were also assessed; F and G: Levels of pY397-FAK, MCT-1, ENO1 and α-SMA in LX-2 cells were detected using Western blotting. Representative results from three independent replicate assays are shown . $^{a}P < 0.05$ and $^{b}P < 0.01$. Results are presented as the mean ± SD.

treatment for liver diseases. In the present study, we verified that FRNK alters the activation, proliferation, migration and apoptosis of HSCs by regulating aerobic glycolysis during liver fibrosis. FRNK inhibits aerobic glycolysis in HSCs by suppressing ENO1 activation through the FAK/Ras/c-myc/ENO1 pathway.

Early studies by our group verified that FAK plays important roles in the activation of HSCs and the development of liver fibrosis and that inhibition of FAK gene expression inhibits liver fibrogenesis[30]. Ding *et al*[23] verified that FRNK negatively regulates pulmonary fibrosis induced by FAK phosphorylation during pulmonary fibrosis. If FRNK inhibits the biological function of FAK in pulmonary fibrosis and uses a similar mechanism to repress liver fibrosis, it may represent a potential therapeutic target in liver fibrosis. Previous studies on FRNK have focused on the inhibition of the migratory function of vascular smooth muscle[32,33], combined with the presence of extracellular lactate accumulation during HSC activation[28,34] and FAK activation of aerobic glycolytic function in tumor cells[35-37]. FRNK may improve liver fibrosis by inhibiting aerobic glycolysis and inhibiting FAK activation in HSCs, but the mechanism by which FRNK exerts this effect remains unclear.

In the current study, we first observed increased expression of the pY397-FAK protein and decreased expression of the FRNK protein in tissues from patients with liver fibrosis (Figure 1). Subsequent experiments using CCl_4 to replicate liver fibrosis in a mouse model yielded the same results (Figure 2B). Therefore, we speculated that a correlation between the occurrence of liver fibrosis and the downregulation of FRNK expression may exist and subsequently performed experiments in WT mice and FRNK$^{-/-}$ mice. After the CCl_4 intervention, the degree of liver fibrosis in WT mice was lower than that in FRNK$^{-/-}$ mice (Figure 2C and D). The expression of the aerobic glycolysis-related proteins MCT-1 and ENO1 in the liver tissue of FRNK$^{-/-}$ mice was increased (Figure 2F and G), suggesting that FRNK gene deletion may promote intrahepatic aerobic glycolysis and aggravate the occurrence and development of liver fibrosis. We extracted pHSCs from WT mice and FRNK$^{-/-}$ mice for *in vitro* experiments to further explore the effect of FRNK on the biological functions of HSCs. After treatment with TGF-β1, the biological functions of pHSCs from FRNK$^{-/-}$ mice were more active than those of pHSCs from WT mice, as evidenced by the increased migration and proliferation and reduced apoptosis rate (Figure 3A-C). Furthermore, the uptake and consumption of glucose and extracellular lactate levels of FRNK$^{-/-}$ pHSCs were increased (Figure 3D and E), suggesting that HSCs lacking FRNK exhibited more active aerobic glycolysis, which supplied energy for their biological functions, such as activation, proliferation and migration. On the other hand, we transfected LX-2 cells with an adenovirus carrying the FRNK gene to verify whether exogenous FRNK is a promising therapeutic target in liver fibrosis. After FRNK

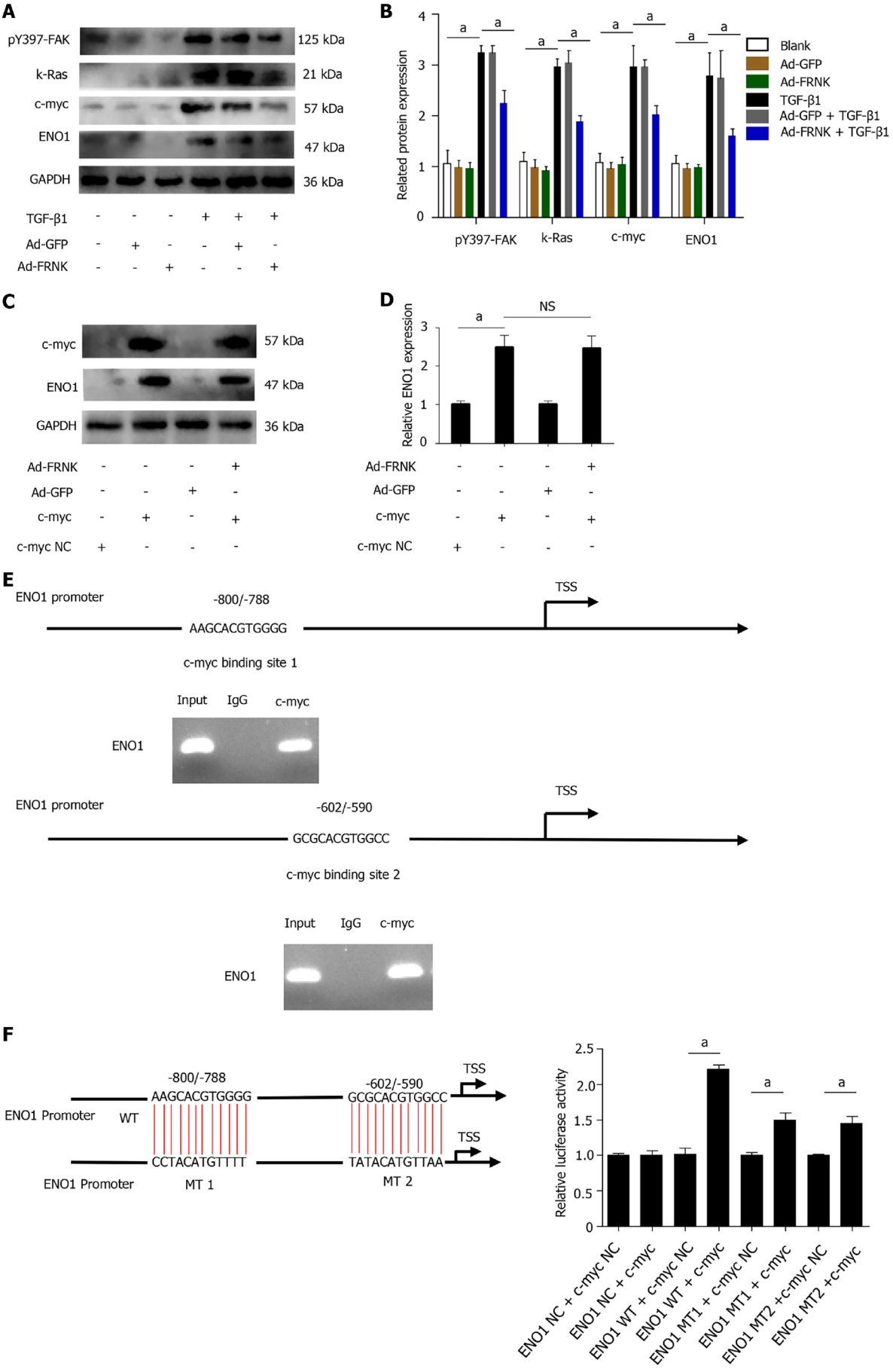

Figure 5 FRNK does not directly target ENO1. A and B: Proteins were extracted from LX-2 cells, and relative levels of the pY397-FAK, K-Ras, c-myc and

ENO1 proteins were determined; C and D: After transfection of LX-2 cells with pcDNA-c-myc or pcDNA-vector (NC), adenoviral vectors containing the FRNK gene (Ad-FRNK) or the negative control (Ad-GFP) were transfected, and the extracted protein was used to evaluate ENO1 expression through Western blotting; E: Schematic representation of the structure of the putative c-myc binding site in the human ENO1 promoter and chromatin immunoprecipitation (ChIP) assays with anti-c-myc or IgG; F: A dual-luciferase reporter assay showed the luciferase activity of WT, mutation (MT)1 and MT2 ENO1 promoters in LX-2 cells transfected with the c-myc or NC plasmid. Representative results from three independent replicate assays are shown. $^{a}P < 0.05$ and $^{b}P < 0.01$. Results are presented as the mean ± SD.

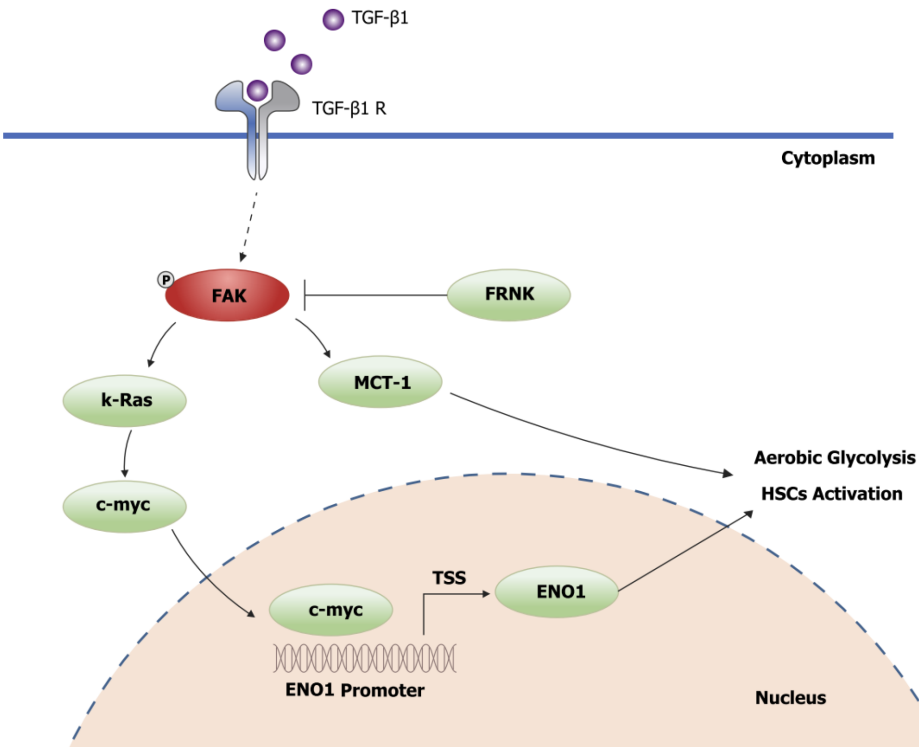

Figure 6 Schematic diagram of FRNK inhibition of the FAK/Ras/c-myc/ENO1 pathway to ameliorate liver fibrosis.

overexpression, cell proliferation and migration decreased, while the percentage of apoptotic cells increased (Figure 4A-C). The uptake and utilization of glucose and the extracellular lactate level in LX-2 cells were also decreased (Figure 4D and E), and pY397-FAK expression in these cells was decreased (Figure 4F and G). These results further indicated that increasing exogenous FRNK expression prevented HSCs from obtaining energy through aerobic glycolysis and reduced cell activation and the energy supply required for a series of biological functions after activation, thereby inhibiting liver fibrosis. While investigating the mechanism by which FRNK regulates aerobic glycolysis in HSCs, we found that FAK is phosphorylated in LX-2 cells stimulated with TGF-β1 and that the downstream proteins K-Ras, c-myc, and ENO1 are activated. Following the introduction of FRNK, ENO1 protein expression was reduced (Figure 5A and B). We transfected both the c-myc and FRNK genes into LX-2 cells to determine whether FRNK directly inhibited ENO1 expression and found that ENO1 protein expression was not reduced upon increased exogenous FRNK expression (Figure 5C and D), thus suggesting that FRNK does not directly inhibit ENO1 protein expression. Therefore, we further hypothesized that c-myc directly alters ENO1 protein expression. Early studies revealed that c-myc is involved in the regulation of various biological functions, including metabolism, cell growth, cell cycle regulation and apoptosis[38,39], consistent with the results of our study. An increasing number of studies have shown that c-myc is involved in the regulation of promoters as a transcription factor. Hence, we predicted the putative c-myc binding site in the ENO1 promoter region by performing a bioinformatics analysis, followed by confirmation of our hypothesis that c-myc transcriptionally activates ENO1 and subsequently promotes liver fibrosis in HSCs by performing dual-luciferase reporter and ChIP assays (Figure 5E and F). Therefore, our study revealed that FRNK alleviated hepatic fibrosis *via* the FAK/Ras/c-myc/ENO1 pathway. The molecular mechanism by which FRNK regulates ENO1 and MCT-1 expression should be confirmed by conducting more complicated investigations in the future, and our group will be

dedicated to studying this pathway.

CONCLUSION

In conclusion, this study is the first to reveal the effect of FRNK on liver fibrosis at the metabolic level. The experimental results suggest that the FAK/FRNK genes are potentially useful therapeutic targets in liver fibrosis and provide some rationale for the development of related drugs in the future.

ARTICLE HIGHLIGHTS

Research background

Hepatic stellate cell (HSC) hyperactivation is a central link in liver fibrosis development. HSCs perform aerobic glycolysis to provide energy for their activation.

Research motivation

Focal adhesion kinase (FAK) promotes aerobic glycolysis in cancer cells or fibroblasts, while FAK-related non-kinase (FRNK) inhibits FAK phosphorylation and biological functions.

Research objectives

To elucidate the effect of FRNK on liver fibrosis at the level of aerobic glycolytic metabolism in HSCs.

Research methods

Mouse liver fibrosis models were established by administering CCl_4, and the effect of FRNK on the degree of liver fibrosis in the model was evaluated. Transforming growth factor-β1 was used to activate LX-2 cells. Tyrosine phosphorylation at position 397 (pY397-FAK) was detected to identify activated FAK, and the expression of the glycolysis-related proteins monocarboxylate transporter 1 (MCT-1) and enolase1 (ENO1) was assessed. Bioinformatics analysis was performed to predict putative binding sites for c-myc in the ENO1 promoter region, which were validated with chromatin immunoprecipitation (ChIP) and dual-luciferase reporter assays.

Research results

The pY397-FAK level was increased in human fibrotic liver tissue. FRNK knockout promoted liver fibrosis in mouse models. It also increased the activation, migration, proliferation and aerobic glycolysis of primary hepatic stellate cells (pHSCs) but inhibited pHSC apoptosis. Nevertheless, opposite trends for these phenomena were observed after exogenous FRNK treatment in LX-2 cells. Mechanistically, the FAK/Ras/c-myc/ENO1 pathway promoted aerobic glycolysis, which was inhibited by exogenous FRNK.

Research conclusions

FRNK inhibits aerobic glycolysis in HSCs by inhibiting the FAK/Ras/c-myc/ENO1 pathway, thereby improving liver fibrosis. FRNK might be a potential target for liver fibrosis treatment.

Research perspectives

The molecular mechanism by which FRNK regulates ENO1 and MCT-1 expression should be confirmed by conducting more complicated investigations in the future, and our group will be dedicated to studying this pathway.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Dynamics of cytokines predicts risk of hepatocellular carcinoma among chronic hepatitis C patients after viral eradication

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Abstract

BACKGROUND

Chronic hepatitis C virus (HCV) infection induces profound alterations in the cytokine and chemokine signatures in peripheral blood. Clearance of HCV by antivirals results in host immune modification, which may interfere with immune-mediated cancer surveillance. Identifying HCV patients who remain at risk of hepatocellular carcinoma (HCC) following HCV eradication remains an unmet need. We hypothesized that antiviral therapy-induced immune reconstruc-

collected the clinical data; Yu ML and Chuang WL designed the study, interpreted data, and supervised the manuscript; all authors had read and approved the final manuscript.

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tion may be relevant to HCC development.

AIM
To investigate the impact of differential dynamics of cytokine expression on the development of HCC following successful antiviral therapy.

METHODS
One hundred treatment-naïve HCV patients with advanced fibrosis (F3/4) treated with direct-acting antivirals (DAAs) or peginterferon/ribavirin who achieved sustained virologic response [SVR, defined as undetectable HCV RNA throughout 12 wk (SVR12) for the DAA group or 24 wk (SVR24) for the interferon group after completion of antiviral therapy] were enrolled since 2003. The primary endpoint was the development of new-onset HCC. Standard HCC surveillance (abdominal ultrasound and α-fetoprotein) was performed every six months during the follow-up. Overall, 64 serum cytokines were detected by the multiplex immunoassay at baseline and 24 wk after end-of-treatment.

RESULTS
HCC developed in 12 of the 97 patients over 459 person-years after HCV eradication. In univariate analysis, the Fibrosis-4 index (FIB-4), hemoglobin A1c (HbA1c), the dynamics of tumor necrosis factor-α (TNF-α), and TNF-like weak inducer of apoptosis (TWEAK) after antiviral therapy were significant HCC predictors. The multivariate Cox regression model showed that ΔTNF-α (≤ -5.7 pg/mL) was the most important risk factor for HCC (HR = 11.54, 95%CI: 2.27-58.72, P = 0.003 in overall cases; HR = 9.98, 95%CI: 1.88-52.87, P = 0.007 in the interferon group). An HCC predictive model comprising FIB-4, HbA1c, ΔTNF-α, and ΔTWEAK had excellent performance, with 3-, 5-, 10-, and 13-year areas under the curve of 0.882, 0.864, 0.903, and 1.000, respectively. The 5-year accumulative risks of HCC were 0%, 16.9%, and 40.0% in the low-, intermediate-, and high-risk groups, respectively.

CONCLUSION
Downregulation of serum TNF-α significantly increases the risk of HCC after HCV eradication. A predictive model consisting of cytokine kinetics could ameliorate personalized HCC surveillance strategies for post-SVR HCV patients.

Key Words: Hepatitis C virus; Hepatocellular carcinoma; Sustained virologic response; Tumor necrosis factor-α; Tumor necrosis factor-like weak inducer of apoptosis; Cytokine

Core Tip: Successful hepatitis C virus (HCV) eradication does not eliminate hepatocellular carcinoma (HCC) development. Clearance of HCV by antiviral agents results in host immune modification, which might interfere with immune-mediated cancer surveillance. We attempted to identify immune biomarkers to predict HCC occurrence after antiviral therapy. The dynamics of serum tumor necrosis factor-α (TNF-α) and TNF-like weak inducer of apoptosis were associated with HCC occurrence after HCV clearance. We established a predictive model to assess the risk of HCC among HCV patients after HCV eradication. Our findings provide a clue for the pathogenesis of hepatocarcinogenesis and a strategy for HCC surveillance based on risk stratification.

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INTRODUCTION

Chronic hepatitis C virus (HCV) infection is a major cause of liver cirrhosis and hepatocellular carcinoma (HCC). As HCV treatment evolves from an interferon (IFN)-based regimen to a therapy based on direct-acting antiviral agents (DAAs), it yields a sustained virologic response (SVR) rate of more than 97% in chronic hepatitis C patients[1,2]. However, successful antiviral therapy does not eliminate HCC development. In a meta-analysis of observational studies, IFN therapy decreased the risk of HCC by 76% in patients with bridging fibrosis or cirrhosis who achieved SVR[3]. Recent studies have reported that HCC occurrence and recurrence rates are potentially increased in HCV patients treated with DAAs[4-6]. This concern remains controversial due to the heterogeneous cohorts, variations in the inclusion criteria, and short duration of follow-up.

Persistent inflammation is a hallmark of chronic hepatic injury. HCV infection induces endogenous type I and III IFN activation, which activates natural killer (NK) cells[7] and leads to the expression of IFN-stimulated genes (ISGs)[8]. It causes profound alterations in the cytokine and chemokine signature in peripheral blood. HCV-specific CD8+ T cells play a central role in viral clearance. Chronic HCV infection is characterized by impaired HCV-specific CD8+ T cell responses resulting from viral escape and T cell exhaustion[9]. IFN-based therapy failed to recover the function of HCV-specific CD8+ T cells. This result suggested that the damage to CD8+ T cells might be permanent even after virus elimination[10]. In contrast, the combination of deleobuvir and faldaprevir resulted in the downregulation of programmed death-1, which led to rapid restoration of HCV-specific CD8+ T cell functions[11]. DAA-mediated HCV clearance is correlated with mitigation of the IFN-α-induced immune response, followed by the downregulation of CXCL10 and CXCL11 and normalization of the phenotype and function of NK cells[12].

It is unclear whether host immunological modification after viral eradication influences the development of HCC. Although DAAs are the first choice for HCV clearance, they are not sufficient to abolish hepatic inflammation. Long-term inflammatory responses may change the liver microenvironment and cause irreversible hepatocyte damage. A rapid decline in HCV viral load induced by DAAs results in the reconstitution of immune surveillance[4]. HCV eradication during DAA treatment is accompanied by downregulation of type II and III IFN, their receptors, and downstream ISGs[13], which may affect the antitumor activity of immune cells. IFNs have immunomodulatory properties that regulate various immune cells to inhibit tumor proliferation and angiogenesis. Unlike IFNs, DAAs have neither antiproliferative nor antiangiogenic properties, which may allow the proliferation of malignant cells.

The identification of HCV patients who maintain a high risk of HCC following successful antiviral therapy remains an unmet need. Hepatocarcinogenesis despite HCV clearance is still unclear. First, this study aimed to investigate the impact of differential cytokine expression profiles on the development of HCC among chronic hepatitis C patients with advanced fibrosis who achieved SVR. Second, we attempted to identify immune biomarkers to predict the risk of HCC after successful antiviral therapy.

MATERIALS AND METHODS

Subjects

One hundred treatment-naïve chronic hepatitis C patients with advanced fibrosis treated with either pegylated IFN/ribavirin or IFN-free DAA who achieved SVR were recruited from Kaohsiung Medical University Hospital since 2003. Patients were required to satisfy any one of the following criteria to be diagnosed with advanced fibrosis (F3/4): Fibrosis-4 (FIB-4) index > 3.25[14], transient elastography (Fibroscan) > 9.1 kPa, or acoustic radiation force impulse elastography > 1.81 m/s. The exclusion criteria were coinfection with hepatitis B, hepatitis D or human immunodeficiency virus; history of liver transplantation; prior presence of HCC; decompensated liver cirrhosis; malignancy; alcoholism; primary biliary cholangitis; α1-antitrypsin deficiency; autoimmune hepatitis; renal function impairment; and psychiatric conditions.

Treatment

In the IFN group, the patients were subcutaneously administered peginterferon α-2a (180 μg/wk) plus weight-based ribavirin (1200 mg/d for weights ≥ 75 kg or 1000 mg/d for weights < 75 kg) for 24 to 48 wk depending on the HCV genotype. In the

DAA group, the physician selected IFN-free DAA regimens for 12 to 24 wk that were discreetly based on the HCV international treatment guidelines (The Asian Pacific Association for the Study of the Liver, European Association for the Study of the Liver and American Association for The Study of Liver Diseases).

Outcome assessment

SVR was defined as undetectable HCV RNA throughout 12 wk (SVR12) for the DAA group or 24 wk (SVR24) for the IFN group after completion of antiviral therapy[15,16]. The primary endpoint was the occurrence of new-onset HCC. Standard HCC surveillance [abdominal ultrasound and α-fetoprotein (AFP) every six months] was performed during the follow-up[17]. HCC development within six months of initiation of antiviral treatment was excluded. Proof of HCC was directly linked to the National Cancer Registration of Taiwan in Health and Welfare Data Science Center (Taiwan). This study was approved by the Institutional Review Board of Kaohsiung Medical University Hospital (No. KMUHIRB-E(I)-20180307 & KMUHIRB-G(II)-20170020). Written informed consent was acquired from all participants.

HCV genotyping and quantification

Anti-HCV antibodies were identified by a third-generation commercially available enzyme-linked immunosorbent assay (Abbott Laboratories, Chicago, IL, United States). HCV RNA was quantified by real-time polymerase chain reaction assay with a lower limit of detection of 12 IU/mL (RealTime HCV; Abbott Molecular, Des Plaines IL, United States)[18]. HCV genotypes were determined using a commercial kit (Abbott RealTime HCV Genotype II; Abbott Molecular, Des Plaines, IL, United States).

Cytokine measurement

Serum samples were collected from the participants at baseline and SVR24. In total, 64 serum cytokines and chemokines (Supplementary Tables 1-3) were measured by the magnetic bead multiplex immunoassay according to the manufacturer's instructions (Merck KGaA, Darmstadt, Germany)[19,20]. In brief, a calibration curve based on 1:3 dilutions of the highest standard was used for quantification. Beads were premixed and put into wells containing diluted serum and reagents. After fixation of the antigen on the capture antibody linked with the microspheres, a biotinylated detection antibody was added. The concentration of the analyte was quantified based on the bead color and the intensity of the fluorescent signal using the multiplex Luminex-200 (Luminex Corporation, Austin, TX, United States). All samples were analyzed in duplicate.

Statistical analysis

Student's *t* test and the Mann–Whitney *U* test were performed to compare the continuous variables. The chi-square (χ^2) test with Yates correction or Fisher's exact test was used to assess the categorical variables. Differences in the cumulative incidence of HCC between groups were analyzed by Kaplan-Meier survival analysis and the log-rank test. The risk factors for HCC were evaluated using multivariate Cox regression analysis. In conjunction with receiver operating characteristic area (ROC) analysis[21], the optimum cutoff value to distinguish between the risk strata was calculated by the Youden index[22]. The performance of biomarkers to predict the risk of HCC was calculated by time-dependent ROC curve analysis. The area under the ROC area (AUROC) was assessed by the timeROC package of R software (http://www.r-project.org). The statistical power for the comparison of survival curves between two groups under the Cox proportional hazards model was calculated by the powerSurvEpi package of R software. A two-tailed P value < 0.05 was considered statistically significant. The statistical analysis was conducted by the Statistic Packages for Social Science Program (SPSS v19.0 for Windows, SPSS Inc., United States). The statistical methods of this study were reviewed by Dr. Tsai PC from Kaohsiung Medical University.

RESULTS

Baseline demographics

The baseline demographics of the study subjects are shown in Table 1. There were no significant differences in age, sex, HCV genotype, FIB-4 index, or AFP levels between the DAA and IFN groups. HCV RNA was significantly higher in the DAA group than

Table 1 Baseline demographics of study subjects

Group	Total	DAA	IFN	*P* value (DAA *vs* IFN)
n	100	50	50	
Age (yr)	63.8 ± 7.2	64.9 ± 7.9	62.6 ± 6.3	0.100
Sex, *n* (%)				
Female	66 (66.0)	38 (76.0)	28 (56.0)	0.057
Male	34 (34.0)	12 (24.0)	22 (44.0)	
HCV genotype, *n* (%)				
1	65 (65.0)	37 (74.0)	28 (56.0)	0.098
2	23 (23.0)	10 (20.0)	13 (26.0)	
Mixed	12 (12.0)	3 (6.0)	9 (18.0)	
HCV RNA (log IU/mL)	2.47 ± 0.89	2.67 ± 0.84	2.28 ± 0.91	0.027
FIB-4	6.14 ± 3.28	6.55 ± 3.69	5.73 ± 2.80	0.213
AFP (ng/mL)	26.3 ± 56.7	28.8 ± 74.0	23.6 ± 29.3	0.662
Platelet (k/μL)	119.8 ± 34.7	115.2 ± 35.9	124.4 ± 33.2	0.186
AST (IU/L)	136.9 ± 79.9	115.2 ± 64.7	158.7 ± 87.9	0.006
ALT (IU/L)	177.5 ± 138.4	127.9 ± 83.9	227.0 ± 163.2	2.7×10^{-4}
γ-GT (IU/L)	67.6 ± 48.8	57.2 ± 42.6	76.7 ± 52.4	0.053
Cholesterol (mg/dL)	161.6 ± 34.8	158.6 ± 37.1	165.2 ± 32.0	0.388
Triglyceride (mg/dL)	97.7 ± 41.4	99.2 ± 40.5	96.0 ± 42.8	0.722
HDL (mg/dL)	47.0 ± 13.3	49.0 ± 11.6	44.1 ± 15.1	0.107
LDL (mg/dL)	90.6 ± 26.4	86.0 ± 24.4	97.2 ± 28.1	0.068
Cr (mg/dL)	0.79 ± 0.24	0.77 ± 0.27	0.82 ± 0.20	0.222
HbA1c (%)	5.8 ± 1.2	5.5 ± 0.7	6.0 ± 1.5	0.016
BMI (kg/m^2)	24.4 ± 4.7	24.0 ± 6.0	24.8 ± 2.9	0.381

DAA: Direct-acting antiviral agent; IFN: Interferon; HCV: Hepatitis C virus; FIB-4: Fibrosis-4 index; AFP: Alpha-fetoprotein; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; γ-GT: γ-glutamyltransferase; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; Cr: Creatinine; HbA1c: Hemoglobin A1c; BMI: Body mass index.

in the IFN group. Aspartate aminotransferase, alanine aminotransferase and hemoglobin A1c (HbA1c) were significantly elevated in the IFN group compared to the DAA group.

Cumulative probability of HCC development

HCC developed in 12 (IFN group *n* = 11, DAA group *n* = 1) of the 97 patients over 459 person-years of follow-up. Three patients were excluded because HCC occurred within six months of initiation of the antiviral treatment. The mean follow-up time was 7.46 years [interquartile range (IQR) = 3.65-12.23] in the IFN group and 1.84 years (IQR = 1.19-2.43) in the DAA group. The annual incidence of HCC was 2.95% in the IFN group and 1.16% in the DAA group. The Kaplan-Meier survival analysis showed no statistical significance in the accumulative probability of HCC between the IFN and DAA groups (log-rank *P* value = 0.712) (Figure 1).

Cytokines associated with HCC development

In total, 64 cytokines were used to analyze the relationship with HCC (Supplementary Table 1). Seven of the sixty-four cytokines were excluded from subsequent analysis because more than 80% of the samples were below the limit of detection. Members of the tumor necrosis factor (TNF) superfamily, including TNF-α and TNF-like weak inducer of apoptosis (TWEAK), were associated with the development of HCC. The baseline TNF-α level was significantly elevated in the HCC group compared

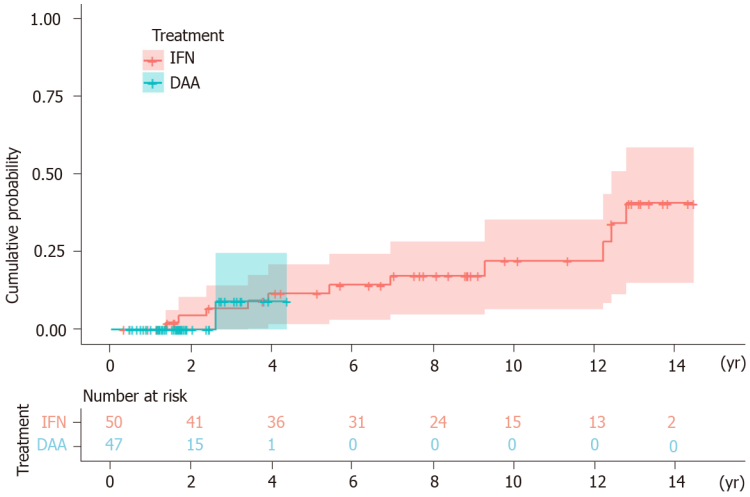

Figure 1 Kaplan-Meier survival analysis. HCC: Hepatocellular carcinoma; IFN: Interferon; DAA: Direct-acting antiviral agent.

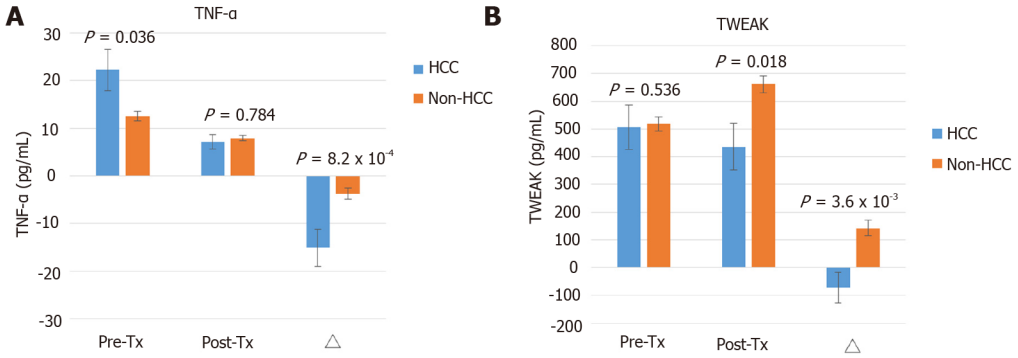

Figure 2 Cytokine expression between the hepatocellular carcinoma and non-hepatocellular carcinoma groups among chronic hepatitis C patients with advanced fibrosis. A: Tumor necrosis factor-α; B: Tumor necrosis factor-like weak inducer of apoptosis. Δ = (posttreatment cytokine level) - (pretreatment cytokine level). The bar represents the means ± SE. The *P* value was tested by the Mann–Whitney *U* test. HCC: Hepatocellular carcinoma; TNF-α: Tumor necrosis factor-α; TWEAK: TNF-like weak inducer of apoptosis; IFN: Interferon; Pre-Tx: Pretreatment; Post-Tx: Posttreatment.

to the non-HCC group (22.22 ± 4.33 *vs* 12.53 ± 1.03 pg/mL, $P = 0.036$). There was no significant difference in the posttreatment TNF-α levels between the HCC and non-HCC groups. The change in TNF-α levels (ΔTNF-α) before and after treatment significantly differed between the HCC and non-HCC groups (-15.11 ± 3.93 *vs* -3.78 ± 1.16 pg/mL, $P = 8.2 \times 10^{-4}$) (Figure 2A). The baseline TWEAK expression was similar in both groups. The posttreatment TWEAK level was significantly lower in the HCC group than in the non-HCC group (434.82 ± 84.18 *vs* 660.65 ± 30.34 pg/mL, $P = 0.018$). ΔTWEAK showed a reciprocal change between the HCC and non-HCC groups and achieved statistical significance (-71.78 ± 54.56 *vs* 142.81 ± 27.69 pg/mL, $P = 3.6 \times 10^{-3}$) (Figure 2B).

Among the HCV patients treated with pegIFN/ribavirin, the baseline TNF-α level was significantly higher in the HCC group than in the non-HCC group (22.55 ± 4.72 *vs* 9.13 ± 7.79 pg/mL, $P = 0.017$). The posttreatment TNF-α concentration was comparable between the HCC and non-HCC groups. ΔTNF-α levels significantly declined in HCC compared to non-HCC patients (-15.86 ± 4.22 *vs* -4.56 ± 1.85 pg/mL, $P = 0.007$) (Supplementary Figure 1A). The dynamic change in TWEAK did not show significant variations between the HCC and non-HCC groups (Supplementary Figure 1B).

Cox regression analysis of the relationship between the differentially expressed cytokines and HCC

In univariate Cox regression analysis, FIB-4 (≥ 9 *vs* < 9, crude HR = 4.04, 95%CI: 1.27-12.86, $P = 0.018$), HbA1c (≥ 7 *vs* < 7%, crude HR = 5.38, 95%CI: 1.38-20.99, $P = 0.015$), pretreatment TNF-α (≥ 18 *vs* < 18 pg/mL, crude HR = 5.15, 95%CI: 1.57-16.87, $P = 0.007$), ΔTNF-α (≤ -5.7 *vs* > -5.7 pg/mL, crude HR = 11.07, 95%CI: 2.27-53.87, $P = 0.003$), and ΔTWEAK (≤ -70 *vs* > -70 pg/mL, crude HR = 4.01, 95%CI: 1.20-13.40, $P = 0.024$)

were significant predictors of HCC. Multivariate stepwise Cox regression analysis revealed that ΔTNF-α was the only independent risk factor for HCC (HR = 11.54, 95%CI: 2.27-58.72, P = 0.003) (Table 2).

Among the HCV patients treated with pegIFN/ribavirin, univariate Cox regression showed that the significant predictors of HCC included sex, FIB-4 (≥ 9 *vs* < 9), HbA1c, baseline TNF-α (≥ 18 *vs* < 18 pg/mL) and ΔTNF-α (≥ -5.7 *vs* < -5.7 pg/mL). The association between ΔTWEAK (≤ -70 *vs* > -70 pg/mL) and HCC was borderline statistically significant. Stepwise multivariate Cox regression revealed that the ΔTNF-α level was the only independent risk factor for HCC in the IFN group (HR = 9.98, 95%CI: 1.88-52.87, P = 0.007 (Supplementary Table 2).

Subgroup analysis for the association between TNF-α and HCC

Since age and diabetes mellitus were important risk factors for HCC, the subjects were further stratified by age and HbA1c. The high- and low-risk groups were dichotomized based on ΔTNF-α with a cutoff value of -5.7 pg/mL. The multivariate Cox regression analysis revealed that the high-risk group (ΔTNF-α ≤ -5.7 pg/mL) had an 11-fold cumulative probability of HCC compared to that of the low-risk group (HR = 11.02, 95%CI: 1.86-65.17, P = 0.008) among HCV patients with HbA1c less than 7%. In the younger population (age < 65 years old), the HCC risk was borderline significant between the high- and low-risk groups (HR = 8.51, 95%CI: 0.78-92.86, P = 0.079). Among patients with both HbA1c < 7% and age below 65 years old, the high-risk group had a 20-fold cumulative probability of HCC in comparison with the low-risk group (HR = 19.99, 95%CI: 0.90-443.91, P = 0.058) (Figure 3). The level of ΔTNF-α did not influence the development of HCC in either the patients aged ≥ 65 years old or with HbA1c ≥ 7% (Supplementary Figure 2).

HCC predictive model

Based on previous analyses, the FIB-4 index, HbA1c, ΔTNF-α, and ΔTWEAK were selected as parameters to predict the risk of HCC. The HCC predictive model was as follows: Score = 4 × FIB-4 (≥ 9, yes = 1, no = 0) + 5 × HbA1c (≥ 7, yes = 1, no = 0) + 11 × ΔTNF (≤ -5.7, yes = 1, no = 0) + 4 × ΔTWEAK (≤ -70, yes = 1, no = 0).

The weighting coefficient for each parameter was derived from the crude hazard ratio of the univariate Cox proportional hazards model. The performance of this HCC predictive model was assessed by time-dependent ROC curve analysis. In overall cases, the 3-year, 5-year, 10-year, and 13-year areas under the ROC curve (AUCs) were 0.882, 0.864, 0.903, and 1.000, respectively (Figure 4A). In the IFN group, the 3-year, 5-year, 10-year, and 13-year areas under the ROC curve (AUCs) were 0.782, 0.802, 0.870, and 1.000, respectively (Figure 4B).

Kaplan-Meier analysis for HCV patients stratified by risk scores

To classify the predictive score according to the risk of HCC, the patients were further stratified into low- (score = 0-7), intermediate- (score = 8-15), and high-risk groups (score > 15). In the high-risk group, the 3-year, 5-year, and 10-year cumulative risks of HCC were 20.0%, 40.0%, and 60.0%, respectively. In the intermediate-risk group, the 3-year, 5-year, and 10-year cumulative probabilities of HCC were 11.4%, 16.9%, and 31.0%, respectively. In contrast, none of the low-risk patients had HCC within 14 years of follow-up after successful viral eradication among the overall cases (log-rank P value = 6.8×10^{-6}) (Figure 5A). Likewise, the Kaplan-Meier survival analysis revealed a significant difference in the cumulative probability of HCC among the IFN group stratified by the risk scores (log-rank P value = 9.6×10^{-5}) (Figure 5B).

DISCUSSION

This study revealed that there was no significant difference in the risk of HCC between the DAA and IFN groups after successful antiviral therapy. Downregulation of TNF-α and TWEAK increased the risk of hepatic carcinogenesis. ΔTNF-α was identified as an independent predictor of new-onset HCC among HCV patients with SVR. The effect of TNF-α was more prominent in young adults with normoglycemia. An HCC predictive model comprising FIB-4, HbA1c, ΔTNF-α, and ΔTWEAK had excellent performance, with 3-, 5-, 10-, and 13-year areas under the curve of 0.882, 0.864, 0.903, and 1.000, respectively. The 5-year accumulative risk of HCC was 0.0%, 16.9%, and 40.0% in the low-, intermediate-, and high-risk groups, respectively. These findings remained statistically significant among the HCV patients treated with pegIFN/ribavirin. Because there was only one HCC in the DAA group, the role of TNF-α in HCC should

Table 2 Factors associated with the onset of hepatocellular carcinoma: Univariate and multivariate Cox regression models

Variables	Univariate Cox regression		Multivariate Cox regression	
	Crude HR (95%CI)	*P* value	Adjusted HR (95%CI)	Adjusted *P* value
Age (yr)	1.07 (0.98-1.18)	0.149	-	-
Sex (male *vs* female)	3.19 (0.96-10.65)	0.059	-	-
HCV genotype	1.00 (0.47-2.13)	0.995	-	-
HCV RNA (log IU/mL)	0.68 (0.37-1.25)	0.213	-	-
FIB-4	1.13 (0.98-1.31)	0.089	-	-
FIB-4 (≥ 9 *vs* < 9)	4.04 (1.27-12.86)	0.018	-	-
Platelet (k/μL)	0.99 (0.98-1.01)	0.408	-	-
AFP (ng/mL)	1.00 (1.00-1.01)	0.308	-	-
HbA1c (%)	1.28 (1.01-1.62)	0.041	-	-
HbA1c (≥ 7 *vs* < 7%)	5.38 (1.38-20.99)	0.015	-	-
BMI (kg/m^2)	1.00 (0.83-1.20)	0.993	-	-
Treatment (DAA *vs* IFN)	0.66 (0.07-6.15)	0.713	-	-
TNF-α (pg/mL)				
Pre-Tx TNF-α ≥ 18	5.15 (1.57-16.87)	0.007	-	-
Post-Tx TNF-α ≥ 6	0.79 (0.25-2.46)	0.683	-	-
ΔTNF-α ≤ -5.7	11.07 (2.27-53.87)	0.003	11.54 (2.27-58.72)	0.003
TWEAK (pg/mL)				
Pre-Tx TWEAK ≥ 500	2.18 (0.64-7.39)	0.213	-	-
Post-Tx TWEAK ≥ 600	0.80 (0.20-3.11)	0.744	-	-
ΔTWEAK ≤ -70	4.01 (1.20-13.40)	0.024	-	-

The forward stepwise multivariate Cox regression model was adjusted by age, sex, hepatitis C virus (HCV) genotypes, HCV RNA, Fibrosis-4 index (FIB-4), platelet, alpha-fetoprotein, hemoglobin A1c, body mass index, treatment, tumor necrosis factor-α and tumor necrosis factor-like weak inducer of apoptosis (pretreatment, posttreatment, Δ). The cut-off value for each cytokine and FIB-4 was determined by Youden index of receiver operating characteristic curve. Δ = (posttreatment cytokine level) - (pretreatment cytokine level). DAA: Direct-acting antiviral agent; IFN: Interferon; HCV: Hepatitis C virus; FIB-4: Fibrosis-4 index; AFP: Alpha-fetoprotein; HbA1c: Hemoglobin A1c; BMI: Body mass index; HCV: Hepatitis C virus; TNF-α: tumor necrosis factor-α; TWEAK: TNF-like weak inducer of apoptosis; Pre-Tx: Pretreatment; Post-Tx: Posttreatment; HR: Hazard ratio; CI: Confidence interval.

be further verified in this population. The HCC risk could be modified by the pre-existing host background and adjusted by the immune signatures after viral eradication. This predictive model helps clinicians adopt appropriate surveillance strategies for chronic hepatitis C patients following successful antiviral therapy according to the risk of HCC.

Our study showed that elevation of pretreatment TNF-α levels raised the possibility of new-onset HCC. Consistent with our study, Tarhuni *et al*[23] found that HCV-related cirrhotic patients carrying TNF-α 308 G>A had higher basal TNF-α production and exhibited a higher risk of HCC. Elevated basal TNF-α indicates sustained hepatic inflammation accompanied by persistent liver damage, which is susceptible to carcinogenesis. A systematic review showed that TNF-α was one of the strongest host genetic predictors for HCC in HCV-infected patients[24]. These findings suggested that the immune background was affected before antiviral therapy.

Interestingly, the abrupt decline in TNF-α levels after successful antiviral therapy increased the risk of HCC in our study. This implies a potential modification of the immune milieu by antiviral therapy that may trigger HCC development. Stimulation of the immune system effectively protects tissues from malignant cell invasion. Both cytotoxic T lymphocytes and NK cells are potent effectors in immune surveillance. TNF-α mediates the immune response against tumor cells by creating a microenvironment toward immunogenic activation rather than suppression[25]. Suppression of TNF signaling enables tumor cells to evade attack by cytotoxic T lymphocytes and attenuate *in vivo* antitumor responses[26]. Antiviral therapy may disrupt the balance

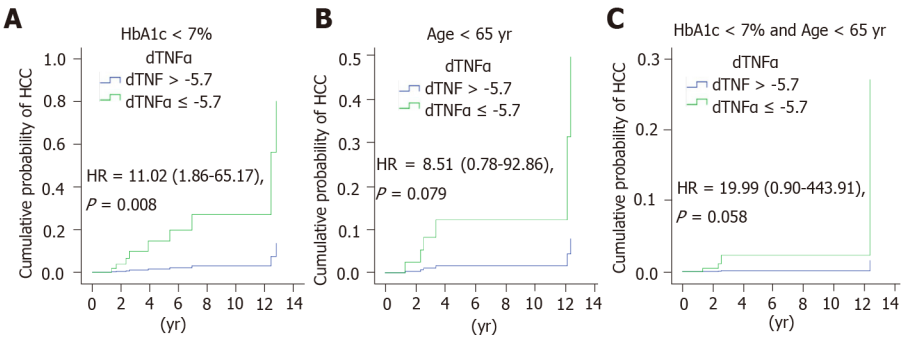

Figure 3 Multivariate Cox regression analysis of tumor necrosis factor-α associated with hepatocellular carcinoma in subgroups. Comparison of the cumulative probability of hepatocellular carcinoma development divided by Δtumor necrosis factor-α with a cutoff value of -5.7 pg/mL in patients with (A) hemoglobin A1c (HbA1c) < 7%, (B) age < 65 years old and (C) HbA1c < 7% and age < 65 years old. The *P* value was adjusted by age, sex, Fibrosis-4 index, and HbA1c. HR: Hazard ratio; CI: Confidence interval; HCC: Hepatocellular carcinoma; TNF-α: Tumor necrosis factor-α; HbA1c: Hemoglobin A1c.

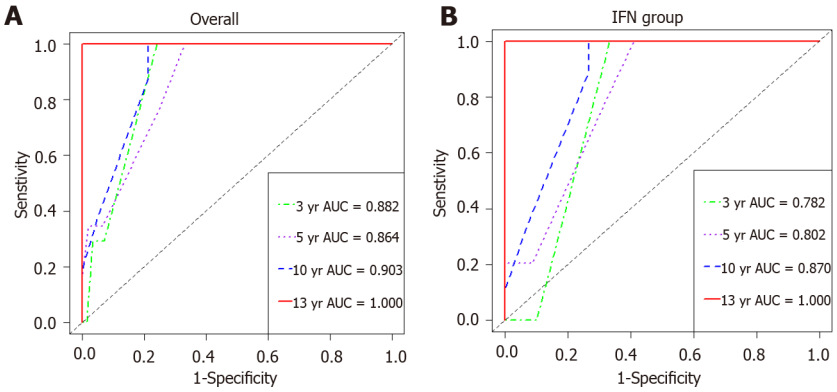

Figure 4 Time-dependent receiver operating characteristic curve analysis for the hepatocellular carcinoma predictive model. IFN: Interferon; AUC: Area under the curve.

from TNF-α activation to inhibition in immune surveillance. Alternatively, Debes *et al* [27] found that HCV patients with early-onset or recurrent HCC within 18 mo maintained stable or even higher TNF-α levels after DAA therapy. This implied that those patients might exhibit precarcinogenic or ongoing carcinogenic activity induced by TNF-α in response to occult HCC.

Both TNF-α and TWEAK belong to members of the TNF superfamily. These cytokines are mainly produced by macrophages, monocytes, and lymphocytes. TWEAK is a multifunctional cytokine that regulates a variety of cellular activities, including cell proliferation, differentiation, apoptosis, inflammation, and angiogenesis, *via* the fibroblast growth factor-inducible 14 receptor[28]. TWEAK appears to attenuate the innate response switch to adaptive immunity[29]. In addition, TWEAK is a weak inducer of apoptosis and also participates in tissue repair[30]. In chronic liver injury and repair, TWEAK appears to initiate liver progenitor cell expansion and ductal proliferation[31]. Hyperstimulation of inflammatory cells simultaneously results in excessive matrix deposition by activated hepatic stellate cells and myofibroblasts *via* the lymphotoxin-β signaling pathway[32]. Our study showed that posttreatment TWEAK expression was upregulated in the non-HCC group. Viral clearance alleviates the inflammatory status in the liver. It provides a microenvironment to facilitate the reconstruction of hepatocytes aided by TWEAK, which may further delay HCC development.

Our study confirms the consensus that DAA treatment does not markedly increase the risk of HCC compared to IFN treatment[33]. Most evidence has shown a decline in HCC risk regardless of whether SVR was achieved by IFN alone, DAA-only, or combined regimens[34]. However, successful antiviral therapy cannot eliminate the risk of HCC. The standard surveillance strategy (ultrasound and AFP every six months) advocates for all HCV patients. However, interindividual variations in HCC risk raise the question of whether the recommendations for HCC screening should be adjusted. Age, male sex, diabetes mellitus, and advanced fibrosis are well-known independent predictors of HCC after viral eradication[35-37]. In the absence of

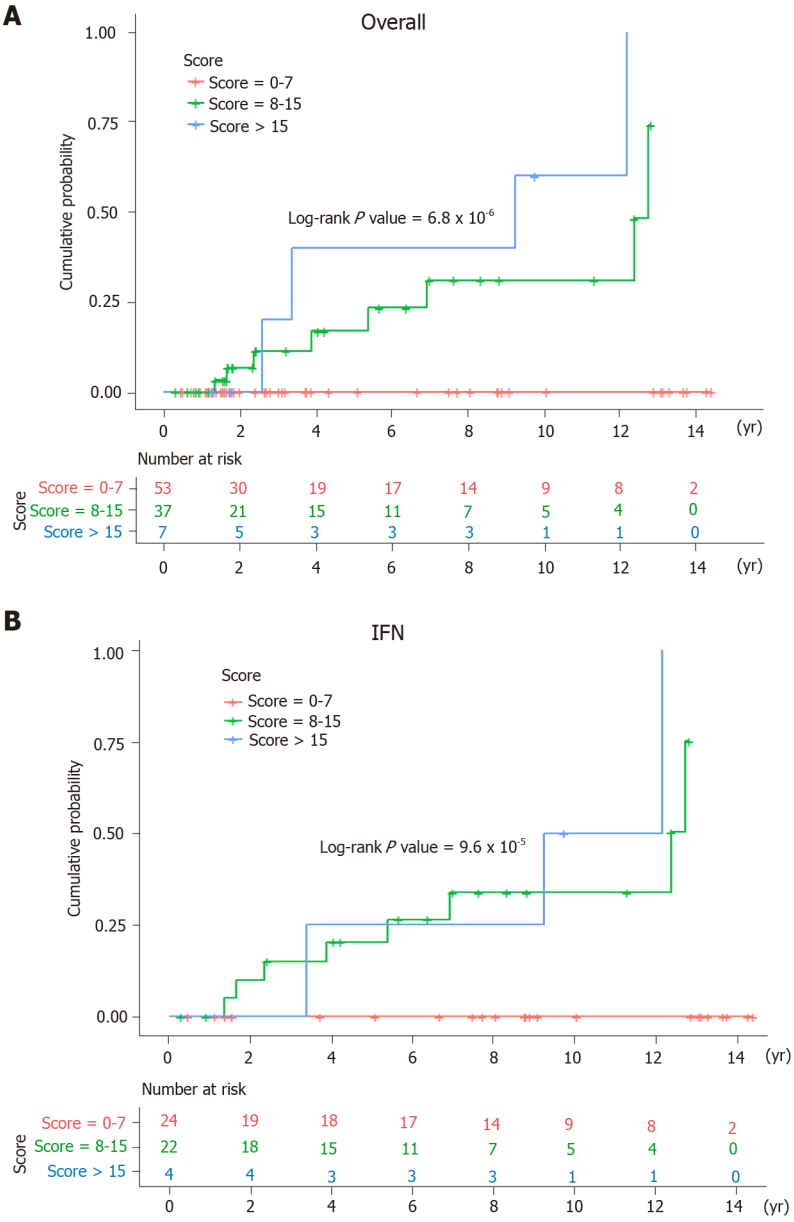

Figure 5 Kaplan-Meier survival analysis for chronic hepatitis C patients stratified by the risk scores. IFN: Interferon.

diabetes mellitus and old age (> 65 years old), the presence of ΔTNF-α ≤ -5.7 pg/mL increased the risk of HCC in patients with advanced fibrosis by 20-fold after HCV clearance. According to this HCC predictive model, patients with scores exceeding 15 should be closely monitored, since the 5-year cumulative risk of HCC reaches up to 40.0%. Nevertheless, none of the HCC cases had been identified over 14 years of follow-up in patients with a score of less than 7. The surveillance intervals may be extended among HCV patients achieving SVR in the absence of concurrent risk factors. In the post-DAA era, the risk model-based algorithm provides a cost-effective surveillance strategy for HCC.

The advancement of high-throughput technology makes early HCC detection more feasible. Currently, integrating multiomics data for HCC screening is also frequently observed[38]. The GALAD score consists of clinical factors (sex, age) and biomarkers (AFP, AFP-L3, and Des-carboxyprothrombin) that have an excellent performance to predict HCC, with an AUROC up to 0.97[39]. Using a miRNA panel (miR-22, miR-199a-3p) with AFP provided high diagnostic accuracy (AUROC = 0.988) for the early detection of HCC in HCV patients[39]. The methylation pattern of circulating cell-free DNA (APC, SFRP1, GSTP1, and RASSF1A) has demonstrated sufficient detection value to distinguish HCC patients from healthy controls[40]. Nevertheless, a majority of studies collected a cohort with a small sample size, and the analytical methods varied even in the same testing platform. These factors have limited the clinical application of these biomarkers.

Even though the sample size was limited in this pilot study, the statistical power was sufficient to be reliable. In overall cases, the statistical power of the association between ΔTNF-α and HCC was 99.9% to reject the null hypothesis at a *P* value < 0.05 under a hazard ratio of 11.54. In the IFN group, the statistical power of ΔTNF-α on HCC risk was 94.6% to reject the null hypothesis at a *P* value < 0.05 under a hazard ratio of 9.98. There are several limitations to this study. Although expensive IFN-free DAAs have been on the market since 2014, the National Health Insurance in Taiwan has reimbursed DAAs for HCV patients with advanced fibrosis since 2017. The follow-up time in most HCV patients treated with DAAs was less than 3 years. Owing to the small sample size and short follow-up time of the DAA group, a larger study cohort is necessary to validate the performance of this predictive model. IFN may induce distinct host immune alterations in comparison with DAA. As only one HCC case was identified in the DAA group throughout the follow-up period, it was unable to compare the diversity of cytokine profiles regarding HCC between the IFN and DAA groups. This predictive model was restricted to HCV patients with advanced fibrosis following successful antiviral therapy. Additionally, the optimal cutoff value should be further verified in other populations. The parameters of this predictive model were composed of serum cytokines involving the TNF superfamily. Host inflammation elicited by other etiologies may interfere with the predictive accuracy. Serum cytokines may not reflect the microenvironment within hepatocytes. To interpret this HCC predictive model, more care should be given to HCV patients presenting coinfection with other viruses, inflammatory disease, or malignancies.

CONCLUSION

This study revealed that downregulation of TNF-α increases the risk of HCC among HCV patients following successful antiviral therapy. Inhibition of TNF-α may attenuate host immune surveillance against tumor cells. Our findings provide a clue for the pathogenesis of hepatocarcinogenesis and a strategy for HCC surveillance based on risk stratification. With the development of high-throughput molecular technology, it is believed that more novel biomarkers will be applied in the early detection of HCC in the future.

ARTICLE HIGHLIGHTS

Research background

Successful hepatitis C virus (HCV) eradication cannot eliminate hepatocellular carcinoma (HCC) development. Chronic HCV infection induces profound alterations in cytokine and chemokine signatures. Clearance of HCV results in host immune modification, which may interfere with immune-mediated cancer surveillance.

Research motivation

The mechanism of hepatocarcinogenesis despite HCV clearance is still unclear.

Research objectives

To investigate the impact of differential cytokine expression on the development of HCC following HCV eradication.

Research methods

One hundred treatment-naïve HCV patients with advanced fibrosis who received antiviral therapy and achieved sustained virologic response (SVR) were enrolled. The primary endpoint was the development of new-onset HCC. In total, 64 serum cytokines were detected by the multiplex immunoassay at baseline and 24 wk after end-of-treatment.

Research results

The dynamics of serum tumor necrosis factor-α (TNF-α) and TNF-like weak inducer of apoptosis (TWEAK) were associated with HCC occurrence after HCV clearance. Multivariate Cox regression analysis showed that ΔTNF-α ≤ -5.7 pg/mL was an independent risk factor for HCC. An HCC predictive model comprising the Fibrosis-4 index, hemoglobin A1c, ΔTNF-α, and ΔTWEAK had excellent performance in stra-

tifying the risk of HCC among HCV patients with SVR.

Research conclusions

Downregulation of serum TNF-α significantly increased the risk of HCC after HCV eradication.

Research perspectives

Our findings provide a clue for the pathogenesis of hepatocarcinogenesis and a strategy for HCC surveillance based on risk stratification.

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SYSTEMATIC REVIEWS

Current guidelines for the management of celiac disease: A systematic review with comparative analysis

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Abstract

BACKGROUND

Wheat and other gluten-containing grains are widely consumed, providing approximately 50% of the caloric intake in both industrialised and developing countries. The widespread diffusion of gluten-containing diets has rapidly led to a sharp increase in celiac disease prevalence. This condition was thought to be very rare outside Europe and relatively ignored by health professionals and the global media. However, in recent years, the discovery of important diagnostic and pathogenic milestones has led to the emergence of celiac disease (CD) from obscurity to global prominence. These modifications have prompted experts worldwide to identify effective strategies for the diagnosis and follow-up of CD. Different scientific societies, mainly from Europe and America, have proposed guidelines based on CD's most recent evidence.

AIM

To identify the most recent scientific guidelines on CD, aiming to find and critically analyse the main differences.

METHODS

We performed a database search on PubMed selecting papers published between January 2010 and January 2021 in the English language. PubMed was lastly accessed on 1 March 2021.

RESULTS

We distinguished guidelines from 7 different scientific societies whose reputation is worldwide recognized and representative of the clinical practice in different geographical regions. Differences were noted in the possibility of a no-biopsy diagnosis, HLA testing, follow-up protocols, and procedures.

CONCLUSION

Grade A (Excellent): 0
Grade B (Very good): B, B, B, B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

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We found a relatively high concordance between the guidelines for CD. Important modifications have occurred in the last years, especially about the possibility of a no-biopsy diagnosis in children. Other modifications are expected in the next future and will probably involve the extension of the non-invasive diagnosis to the adult population and the follow-up modalities.

Key Words: Celiac disease; Gluten; Gluten-free diet; Gluten sensitivity; Clinical guidelines; Non-invasive diagnosis; Histopathological findings; Serological markers; Genetics

Core Tip: Once considered a rare condition, celiac disease (CD) is becoming a significant health issue globally. An increasing number of studies have investigated this condition. International scientific societies have proposed guidelines for the management of CD to translate this evidence into clinical practice. In this review, we critically analyse both the converging and diverging points in the current clinical guidelines of CD, focusing on the diagnostic aspects and follow-up procedures.

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INTRODUCTION

Celiac disease (CD) is an immune-mediated reaction to gluten characterised by an inflammatory injury to the small bowel in genetically predisposed subjects as a result of an inappropriate T cell-mediated immune response[1].The epidemiology of CD is well known, with an estimated worldwide prevalence of 0.6%-1% of the general population[2]. However, CD remains largely underdiagnosed in developing countries and has a higher impact on children[3,4]. Simultaneously, the misdiagnosis of CD is becoming an emergent problem worldwide[5].

An evidence-based approach is needed to optimise diagnostic accuracy to avoid life-threatening complications (including small bowel carcinoma and lymphoma)[6] resulting from unrecognised CD on the one hand, and unnecessary cost burden and impact on the quality of life due to incorrect prescription of a life-long gluten-free diet (GFD) on the other hand.

Simultaneously, follow-up of patients with CD who are on a GFD is of critical importance to assess the responsiveness to the GFD, detect complicated CD, find associated autoimmune diseases, and identify metabolic alterations induced by the GFD[7].

Thus, an increasing number of scientific societies have proposed guidelines for diagnosing and managing CD. In our systematic review, we identified the most recent and significant national and international guidelines and compared their recommendations. We also underlined the most apparent differences among these guidelines to identify 'hot topics' on CD and possible future developments.

MATERIALS AND METHODS

The primary aim of this review was to identify the most recent national and international guidelines for CD by means of a systematic review and to compare their main recommendations.

We performed a database search on PubMed and selected papers published between January 2010 and January 2021 in the English language. PubMed was last accessed on 1 March 2021. The following keywords and terms were used: (1) *Coeliac Diseaseor Celiac Disease*; (2) *Guideline*; and (3) *Management*. The following string was used: (("coeliac disease"[All Fields] OR "celiac disease"[MeSH Terms] OR ("celiac"[All

Fields] AND "disease"[All Fields]) OR "celiac disease"[All Fields] OR ("coeliac disease"[All Fields] OR "celiac disease"[MeSH Terms] OR ("celiac"[All Fields] AND "disease"[All Fields]) OR "celiac disease"[All Fields])) AND ("guideline"[Publication Type] OR "guidelines as topic"[MeSH Terms] OR "guideline"[All Fields] OR ("manage"[All Fields] OR "managed"[All Fields] OR "managements"[All Fields] OR "managements"[All Fields] OR "manager"[All Fields] OR "manager s"[All Fields] OR "managers"[All Fields] OR "manages"[All Fields] OR "managing"[All Fields] OR "management"[All Fields] OR "organization and administration"[MeSH Terms] OR ("organization"[All Fields] AND "administration"[All Fields]) OR "organization and administration"[All Fields] OR "management"[All Fields] OR "disease management"[MeSH Terms] OR ("disease"[All Fields] AND "management"[All Fields]) OR "disease management"[All Fields]))).

A total of 415 papers were identified with no duplicates, and, as a first step, no papers were excluded for other reasons (PRISMA flow diagram reported in Figure 1). However, twenty-one records were unavailable, leaving 396 papers for further evaluation. As a second step, we excluded papers that were not pertinent to any of the following criteria: (1) Clinical guidelines related to diagnosis and management of CD; and (2) Clinical guidelines published by governmental agencies and scientific associations. We included only the last version of the guidelines, excluding the previous updated versions.

According to the selection criteria, out of the 396 results of PubMed research assessed for eligibility, seven guidelines were finally included in this analysis. These guidelines strictly focus on the diagnosis and management of CD. These papers are presented in order of publication (newest to oldest): (1) European Society Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) 2020[8]; (2) European Society for the Study of Coeliac Disease (ECD) 2019[9]; (3) World Gastroenterology Organization (WGO) 2017[10]; (4) Central Research Institute of Gastroenterology, Russia, 2016[11]; (5) National Institute for Health and Care Excellence (NICE), 2015 [12]; (6) British Society of Gastroenterology (BSG), 2014[13]; and (7) America College of Gastroenterology (ACG), 2013[14].

The recommendations provided by each selected guideline were systematically explored and classified into five categories: patients to be tested for CD, diagnostic tests (serology, duodenal biopsy, genetic test, no-biopsy diagnosis), potential/silent/seronegative CD, refractory/complicated CD, and follow-up. These categories represent the most discussed topics of CD.

The results are reported in different paragraphs, containing both a brief introduction to the specific topic (with references derived from the supporting evidence used by the guidelines and other relevant papers according to a narrative approach) and a comparative analysis of the guidelines' recommendations (collected using a strictly systematic approach).

RESULTS

Clinical presentation and risk factors: who should be tested for CD?

CD is a diagnostic challenge as it may develop at any age (even in older adults) and with a polymorphic clinical presentation[15]. The clinical spectrum of CD includes both symptomatic and silent forms revealed only by serological screening[16,17]. CD-related symptoms can be both intestinal and extraintestinal, reflecting the systemic nature of the disease. These manifestations are classified as 'classical' and 'non-classical' according to the historical presentation of first described cases. Table 1 reports the main manifestations of CD according to their categorization[1,17-26].

Some guidelines draw specific attention to some extraintestinal symptoms (Figure 2). In particular, the ESsCD 2019 guidelines focus on oral-dental and neuropsychiatric manifestations[9]. CD testing is advised in cases of dental enamel defects and recurrent oral aphthae. Special attention to neurological manifestations has also been drawn by the Russian Central Research Institute of Gastroenterology[11]. These guidelines also focus on reproductive disorders, such as delayed sexual development, amenorrhea, infertility, and miscarriage[11].

Despite these premises, all the guidelines agree on testing for CD in children, adolescents, and adults showing classical and non-classical symptoms of CD[7-13]. There is also a consensus on considering iron-deficiency anaemia and hypertransaminasemia as the most common laboratory abnormalities[8-14].

Table 1 Most frequent clinical manifestions of celiac disease

	Intestinal	Extraintestinal
Classical	Diarroea	Iron deficiency anaemia
	Failure to thrive	Muscle waisting
	Weight loss	Oedema
	Bloating	
Non classical	Chronic abdominal pain	Short stature
	Abdominal distension	Delayed puberty
	Constipation	Amenorrhea
	Vomiting	Irritability, unhappiness
		Chronic fatigue
		Epilepsy
		Peripheral neuropathy
		Joint/muscle pain
		Elevated aminotransferases
		Aphtous stomatitis
		Recurrent miscarriages
		Reduced bone mineral density

The high-risk group of patients did not change over time. These groups include first-degree relatives of patients with CD, patients with autoimmune conditions (such as type 1 diabetes mellitus and thyroid diseases) or genetic disorders such as IgA deficiency, Down syndrome, Turner syndrome, and Williams-Beuren syndrome[8-14].

Diagnosis.

There is no 'gold standard' for the diagnosis of CD. Clinical features, serology, or histology alone cannot provide a definitive diagnosis. Instead, the final diagnosis of CD relies on a combination of these elements. All the guidelines agree on a sequential approach to diagnosis, consisting of serology as a first-line test in high-risk patients, followed by duodenal biopsy in cases of positive serology or persistent suspicion of malabsorption (Figure 3). A positive serology paired with evidence of duodenal villous atrophy indicate a definite CD diagnosis, whereas cases with discordant findings should undergo HLA testing. All the guidelines also agree that patients with dis-cordance between serology, histology, and HLA DQ2/DQ8 positivity should be evaluated on a patient-by-patient basis in expert centres. The so-called 'four-out-of-five rule' has long been advocated as a standard of care[27]. According to this rule, four of the following criteria are sufficient to establish CD diagnosis: (1) Typical signs and symptoms (diarrhoea and malabsorption), (2) Antibody positivity, (3) HLA-DQ2 or HLA-DQ8 positivity, (4) Intestinal damage (*i.e.*, villous atrophy and minor lesions); and (5) Clinical response to GFD. This rule also helps physicians to identify various subtypes of CD, that is, non-classic CD (absence of point 1), seronegative CD (absence of point 2), potential CD (absence of point 4), and non-responsive CD (absence of point 5). However, the 'four-out-of-five rule' is yet to be recognised by any guideline.

We will report the guidelines' detailed suggestions for obtaining key diagnostic elements from serology, histology, and genetic testing in the following paragraphs.

Serology

All diagnostic serological testing should be performed in patients on a gluten-containing diet[28]. Serum immunoglobulin A(IgA) anti-tissue transglutaminase antibody (anti-tTG-IgA) is widely accepted as the most sensitive test for CD diagnosis, although it suffers from low specificity, especially at low titres[29-33]. In contrast, IgA anti-endomysial antibodies (EMA-IgA) are nearly 100% specific for CD but are less sensitive, more expensive, and more operator-dependent than anti-tTG-IgA. Therefore, these characteristics make EMA-IgA an ideal second-line test[34]. The diagnostic performance of both anti-tTG-IgA and EMA-IgA is limited in patients with concurrent

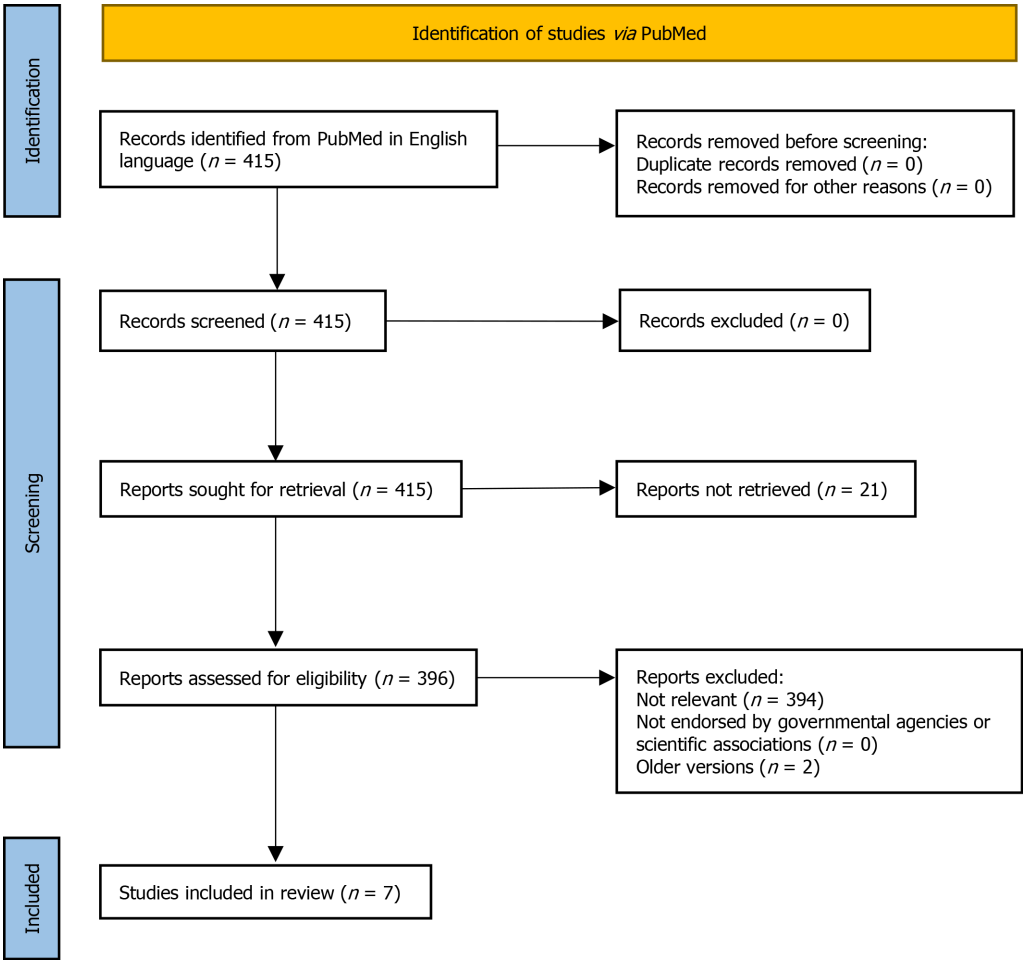

Figure 1 PRISMA flow diagram.

IgA deficiency. Antibodies to deamidated gliadin peptides (DGP) of the IgG class are advantageous in this setting and for younger children[35,36]. Even with the most recent advancements in CD serology, up to 2% of patients with CD have no circulating markers of gluten sensitivity, defining a condition of seronegative CD[37].

Currently, the guidelines are concordant and suggest anti-tTG-IgA as the initial serological test, complemented by a determination of total IgA levels to rule out concurrent IgA deficiency (Figure 4)[8-14]. This initial approach was suggested for both children and adults.TheACG2013 guidelines suggest a combination of different IgA and IgG antibodies in children younger than two years of age (for instance, anti-tTG IgA and DGP-IgG)[14]. This approach is still accepted only by the WGO2017 guidelines[10]. The remaining guidelines advise against this strategy, as a combination of antibodies implies a higher sensitivity at the expense of a reduced specificity, often leading to the necessity of histological confirmation. This scenario represents an obstacle in the pursuit of a no-biopsy approach in children, for whom the anti-tTG-IgA + total IgA strategy fits better[8]. Alternatively, DGP-IgG (together with anti-tTG-IgG) maintained the unanimous recommendation as the test of choice in patients with IgA deficiency[8-14].

Further, EMA-IgA is considered a confirmatory test, particularly when TG2 has a low titre, *i.e.*,< 2x the upper normal limit (UNL)[9,10,12]. A positive result is also required for a no-biopsy CD diagnosis in children with anti-tTG IgA > 10x[8]. However, the use of paired anti-tTG and EMA-IgA as the first diagnostic test is not supported by any guideline.

Currently, all of the guidelines strongly discourage urine, stool, and saliva tests in clinical practice due to their low-performances[8-14] and the consequent risk of initiating a GFD without a firm diagnosis, impacting the final diagnosis[13].

Biopsy

For a long time considered the 'gold standard' for diagnosing CD (ambiguously suggesting that other tests were of lesser importance), duodenal biopsies remain the

Recommendations		Change over time
In children, classical symptoms of malabsorption are more specific. Some non-classical symptoms are more specific than others (including iron deficiency anaemia, diarrhoea IBS-like, chronic constipation, and enamel defects)		No major changes over time
In adults, sensitivity and specificity of classical and non-classical symptoms are moderate. Testing for CD among individuals with only subtle and non-classical symptoms is advised		No major changes over time
Consider testing for CD in high-risk groups such as CD first-degree relatives, patients with autoimmune conditions such as type 1 Diabetes Mellitus, thyroid disease, liver disease, patients with genetic conditions such as Down syndrome, Turner syndrome, Williams-Beuren syndrome and IgA deficiency		No major changes over time

European Society Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) 2020 (8).

European Society for the Study of Coeliac Disease (ECD) 2019 (9).

World Gastroenterology Organization (WGO) 2017 (10).

Central Research Institute of Gastroenterology, Russia, 2016 (11).

National Institute for Health and Care Excellence (NICE), 2015 (12).

British Society of Gastroenterology (BSG), 2014 (13).

America College of Gastroenterology (ACG), 2013 (14).

Figure 2 Recommendations about case finding.

mainstay of CD diagnosis, and all guidelines unanimously recognise this role. The presence of positive histology, however, was not considered CD-specific. Thus, clinical, and serological correlations are mandatory (Figure 5) [8-14].

Duodenal biopsies should be obtained from all patients with suspected CD. In high-risk symptomatic patients, duodenal biopsies should be performed irrespective of serology results for CD[9,13,14]. Some authors also suggested that duodenal biopsies should be considered in any individual undergoing endoscopy because of the relatively high prevalence of CD in the general population and its polymorphic presentation[13].

Histology samples should be collected from multiple sites, given the possible patchy distribution of CD lesions. Current evidence suggests collecting four biopsies from the second duodenal portion and two biopsies from the bulb[38]. Biopsy sample orientation using cellulose acetate Millipore filters is of paramount importance to avoid artefacts, potentially leading to a false diagnosis of villous atrophy[39].

The histological findings are currently categorised according to the classification proposed by Marsh and subsequently modified by Oberhuber[40]. Pathology findings are reported as Marsh-Oberhuber 0 (normal histology), 1, 2, or 3 (subdivided into 3a, 3b, and 3c).

An increase in intraepithelial lymphocytes (IELs) without villous atrophy defines Marsh 1 Lesion. In most cases, Marsh 1 Lesions (also called minimal lesions) are attributable to other causes, including lymphocytic colitis, bacterial and parasitic intestinal infections (especially *Helicobacter pylori* and *Giardia lamblia*), small intestinal bacterial overgrowth, Crohn's disease, common variable immunodeficiency, and non-steroidal anti-inflammatory drugs[41]. While a Marsh 1 Lesion is not considered sufficient to diagnose CD, the BSG 2014 guidelines state that minimal lesions combined with positive serology could represent a probable CD. A trial with a GFD could be considered to support the diagnosis of CD[13]. When the increase in IELs is paired with hyperplasia of the duodenal crypts, the lesion is classified as Marsh 2.

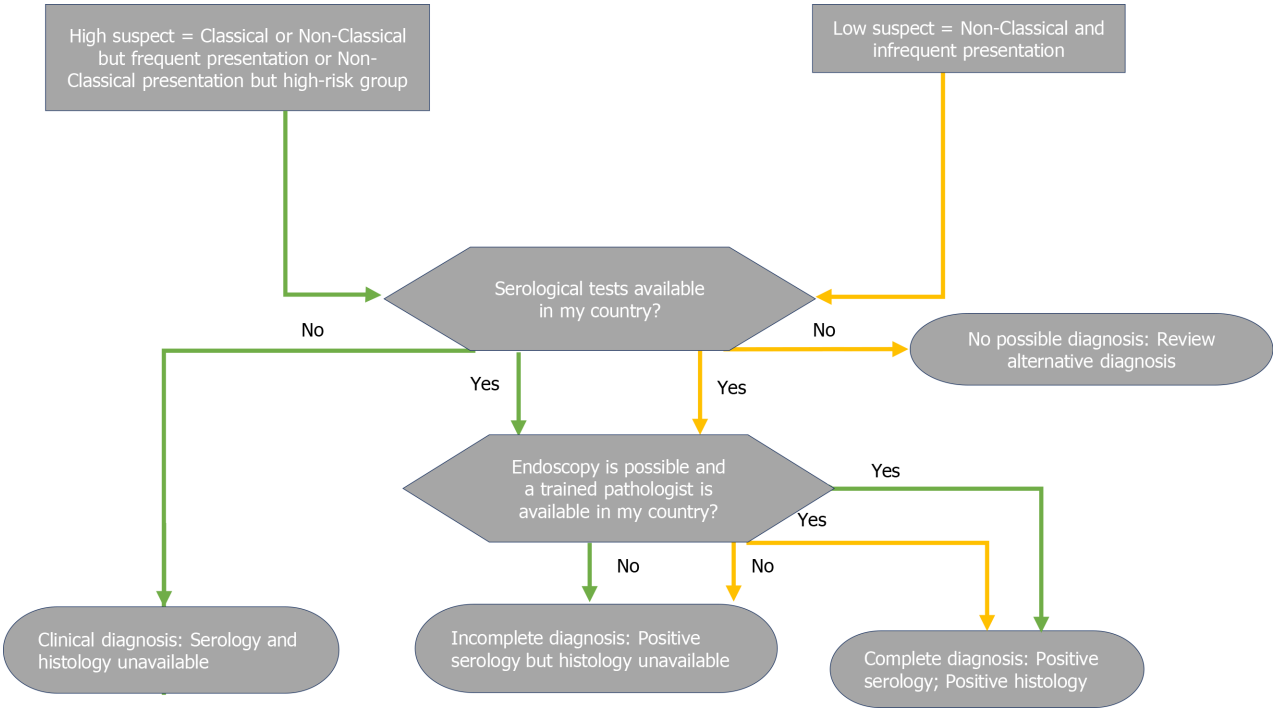

Figure 3 Worldwide adapted decision-making process for diagnosing celiac disease. Highly suspicious celiac disease (CD) comprises "classical presentation" (*i.e.*, classical symptoms in children include failure to thrive, weight loss, growth failure, vomiting, chronic diarrhea, bloating, Iron-deficiency anemia, muscle wasting, oedema due to hypoproteinemia, irritability and unhappiness; in adults, classical symptoms include chronic diarrhea, weight loss, iron-deficiency anemia, malaise and fatigue, oedema due to hypoproteinemia, and osteoporosis), frequent "non-classical presentation" (*i.e.*, iron deficiency and hypertransaminasemia) and "non-classical presentation" but high risk group (*i.e.*, CD first-degree relatives, autoimmune conditions such as type 1 Diabetes Mellitus, and thyroid disease, genetic conditions such as IgA deficiency, Down syndrome, Turner syndrome and Williams-Beuren syndrome).

Conversely, increased IELs in combination with villous atrophy define the typical CD lesion (Marsh 3), subclassified as mild (3a), moderate (3b), or subtotal (3c)[40]. Some authors proposed a simplified histopathological grading, reducing the possible grades from five to three, thus reducing the possible inter-operator variability in the histological interpretation[42].This simplified classification is yet to be adopted by the international guidelines, which currently recommend the Marsh-Oberhuber classification[8-14].

At present, there is no alternative to duodenal biopsy for examining mucosal damage[8-14]. For instance, in children, video-capsule endoscopy (VCE) gives no indications[8], although in adults, VCE could support the diagnosis in cases of discordance between serology and biopsy[13] or if the patient is unwilling or unable to undergo traditional endoscopy[14]. VCE could also play a role in detecting CD complications (*i.e.*, lymphoma, adenocarcinoma, ulcerative jejunitis)[9] and in helping to differentiate extended diseases (*e.g.*, CD *vs* proximal Crohn's disease)[11]. Anti-actin IgA antibodies have been shown to be predictive of severe villous atrophy in CD patients at the time of diagnosis[43]. Theoretically, they may also provide indirect information about villous recovery following the introduction of the GFD; however, data are still lacking in this setting. The available information about faecal and salivary microbiome, at present, is not sufficient to allow a reliable conclusion for the diagnosis of CD[44,45]. Intestinal fatty-acid binding protein (I-FABP) are higher in dietary non-adherence and unintentional gluten intake and could be used as a sensible blood marker of mucosal damage[46,47].This exam was first mentioned in the ESsCD guidelines[9].

A repeated small intestinal biopsy, including biopsies from the jejunum, could be considered in adults with discordance between histopathology and anti-tTG-IgA results[13]. In children, re-cutting biopsies and/or a second opinion from an experienced pathologist is preferred over endoscopic repetition[8].

In adults, a gluten challenge should be proposed for patients with uncertain CD diagnosis, who have been started on a GFD[9-14]. In children, gluten challenge is discouraged before the age of 5 years and during puberty, and in general, it should be reserved for unusual cases[8].

Recommendation		Change over time
Anti-tissue Transglutaminase 2 IgA (TGA-IgA) should be used as the initial serological test, complemented by total IgA value in children of any age and adults		Changes over time
In patients with low total IgA concentrations, an IgG-based test, preferably TGA-IgG or DGP-IgG, should be performed as a second step		No major changes over time
A strategy based on a combination of antibodies addressing the same target (*i.e.*, TGA-IgA and EMA-IgA) as a first approach is not recommended		No major changes over time

European Society Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) 2020 (8).

European Society for the Study of Coeliac Disease (ECD) 2019 (9).

World Gastroenterology Organization (WGO) 2017 (10).

Central Research Institute of Gastroenterology, Russia, 2016 (11).

National Institute for Health and Care Excellence (NICE), 2015 (12).

British Society of Gastroenterology (BSG), 2014 (13).

America College of Gastroenterology (ACG), 2013 (14).

Figure 4 Recommendations about serology. IgA: Immunoglobulin A; IgG: Immunoglobulin G; DGP: Deamidated gliadin peptides; EMA: Anti-endomysium antibodies.

Gluten challenge protocols are not homogeneous. A diet containing at least 10 g of gluten per day for 6-8 wk seems to be the most effective way to achieve disease relapse; however, the evidence is weak[28]. In shorter protocols, a diet containing at least 3 g of gluten per day for at least 2 wk seems to be sufficient for most patients[10,13,14]. Certainly, a shorter and lighter approach would fit better for highly symptomatic patients. A strategy for optimising the result would be to undergo a serology test after two weeks and, if negative, to extend the challenge to 8 wk[13].

After reintroducing gluten, physical symptoms should not be used for diagnosis in the absence of other supportive evidence[8,9,11-14]. A diagnosis based only on the disappearance of symptoms on GFD and relapse during gluten re-introduction can be relevant in geographic areas where serology tests are not available, as the only way to confirm the diagnosis and treat the disease[10].

Human Leukocyte Antigen testing

The strong genetic component of CD is testified by its high familial recurrence and high disease concordance among monozygotic twins (75%-80%)[48]. The presence of human leukocyte antigen (HLA) -DQ2/DQ8 is a pathogenic requisite for the development of the typical immune alterations found in CD. Simultaneously, HLA DQ2/DQ8 can be found in up to 30%-40% of the general population, so its specificity is remarkably poor[49]. In contrast, the absence of HLA DQ2/DQ8 virtually excludes CD diagnosis[48,49].Restricting this observation to the sole HLA DQ2 alleles, a recent systematic review of the literature confirmed that only 5.06% of patients with CD were completely lacking the HLA-DQB1*02 allelic variant[50].

Consequently, all the guidelines advise against using HLA testing as a first-line tool for the diagnosis of CD (Figure 6)[8-14]. They are also concordant in allocating this resource for: (1) Patients with uncertain diagnosis of CD, already on a GFD; (2) Patients with a flat intestinal mucosa but negative serology; and (3) In patients already on a GFD, serology and histology can be inconclusive. In this context, before embarking on a so-called 'gluten-challenge', it is advisable to verify the presence of HLA-DQ2/DQ8[8-14].

Recommendation		Changes over time
Adult patients with a positive serology must undergo endoscopy with duodenal biopsies to achieve a final diagnosis		With exceptions
In children, in precise conditions, diagnosis can be achieved without a duodenal biopsy		Major changes over time
Duodenal biopsy should be performed, irrespective of positive serology for CD, in case of high clinical suspicion of CD		No major changes over time
At least 4 biopsies from the distal duodenum and at least 1 from the duodenal bulb should be taken for histology assessment during a gluten-containing diet		No major changes over time
The diagnosis is confirmed in the presence of Marsh ≥ 2 lesions. Marsh 1 is not sufficient to diagnose CD		No major changes over time. Minor exceptions
A gluten challenge should be proposed to patients who have been started on a GFD but have a doubtful diagnosis		Minor changes over time and guidelines
A diagnosis based only on the disappearance of symptoms on GFD and relapse during gluten re-introduction is absolutely discouraged.		With exceptions
No exams can surrogate mucosal damage without biopsy		No major changes over time. Minor exceptions

European Society Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) 2020 (8).

European Society for the Study of Coeliac Disease (ECD) 2019 (9).

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British Society of Gastroenterology (BSG), 2014 (13).

America College of Gastroenterology (ACG), 2013 (14).

Figure 5 Recommendations about serology.

HLA tests would be useless for patients with positive serology before a gluten-challenge because virtually 100% of those patients would be positive. Therefore, HLA typing is no longer a criterion for the 'no-biopsy' approach of diagnosis in children with a TGA-IgA > 10x UNL[8]. In patients with positive histology (*i.e.,* villous atrophy, though occasionally detected on esophagogastroduodenoscopy), and negative or questionable serology, HLA testing can exclude the diagnosis of CD[9]. In contrast, a positive result cannot confirm the diagnosis, which should be carefully evaluated on a patient-by-patient basis in expert centres.

The use of HLA typing in high-risk populations is controversial. HLA-DQ2/DQ8 can be found in more than 50% of first-degree relatives of patients with CD and in patients with other autoimmune or genetic disorders related to CD[14,49]. Most

Recommendation		Changes over time
HLA -DQ2/DQ8 testing has only a high negative predicting value and is recommended in selected patients to rule out coeliac disease in patients: Already on a gluten-free diet With a negative or questionable serology but positive histology		No major changes over time
HLA -DQ2/DQ8 testing is not recommended alone or combined with serology tests to confirm the diagnosis		No major changes over time
HLA) -DQ2/DQ8 testing in high-risk populations can indefinitely exclude these patients from a periodic screening		Minor changes over time

 European Society Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) 2020 (8).

 European Society for the Study of Coeliac Disease (ECD) 2019 (9).

 World Gastroenterology Organization (WGO) 2017 (10).

 Central Research Institute of Gastroenterology, Russia, 2016 (11).

 National Institute for Health and Care Excellence (NICE), 2015 (12).

 British Society of Gastroenterology (BSG), 2014 (13).

 America College of Gastroenterology (ACG), 2013 (14).

Figure 6 Recommendations about Human Leukocyte Antigen testing.

guidelines suggest excluding HLA-DQ2/DQ8 in CD first-degree relatives and high-risk patients, even if asymptomatic, to avoid periodic monitoring[9,10,13,14]. This strategy can be questioned in terms of resources and costs[10,11,14]. Some authors hav esuggested screening high-risk patients only if they complain of gastrointestinal or extraintestinal symptoms or have laboratory abnormalities[11]. In addition, a two-step genetic screening procedure starting with HLA-DQ β chains has been proposed[51]. Thus, the choice of screening for symptomatic or asymptomatic first-degree relatives or high-risk patients, with or without a preliminary determination of HLA-type, remains debated, needing to take local resources and cost-benefit rates into account.

No-biopsy diagnosis

While most guidelines allow a no-biopsy diagnosis in children under strict conditions, endoscopy with duodenal biopsies is still mandatory to achieve a final diagnosis of CD in adults[9-14]. As the only exception, the WGO guidelines allow a diagnosis based on serology and clinical response to the GFD (Figure 7) in developing countries where endoscopy may not possible or trained pathologists may not be available[10].

The ESPGHAN2012 guidelines endorsed the possibility of a no-biopsy approach in children for the first time. This possibility was limited to certain conditions, which included the presence of classic symptoms, with tTG-IgA > 10x UNL, EMA-IgA positivity, and presence of permissive HLA[8].

This approach was subsequently adopted by a plurality of international guidelines [9-12]. although, the ACG2013 and BSG 2014 guidelines did not include this approach [13,14].

The 2020 update of the ESPGHAN guidelines removed classic symptoms, EMA-IgA positivity, and HLA DQ-2 or DQ-8 as crucial criteria for a diagnosis not based on biopsy[7]. However, EMA-IgA positivity is not discouraged[8,10]. The increasing confidence in diagnosing CD without biopsy in children has increased so rapidly that many recent studies consider tTGA > 10x as a new possible cut-off to further reduce the need for biopsies[52].

Recommendation		Changes over time
In children with classic symptoms, TGA-IgA titre > 10x, EMA-IgA positivity, and HLA DQ2/DQ8, the diagnosis can be achieved without a duodenal biopsy		Major changes over time
In children, classic symptoms, EMA-IgA positivity and HLA DQ2 /DQ-8 are not mandatory to diagnose CD if TGA-IgA titre is > 10x		Major changes over time
In adults, a diagnosis of CD without a positive biopsy is still discouraged		Exception Probable future changes over time 

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 European Society for the Study of Coeliac Disease (ECD) 2019 (9).

 World Gastroenterology Organization (WGO) 2017 (10).

 Central Research Institute of Gastroenterology, Russia, 2016 (11).

 National Institute for Health and Care Excellence (NICE), 2015 (12).

 British Society of Gastroenterology (BSG), 2014 (13).

 America College of Gastroenterology (ACG), 2013 (14).

Figure 7 Recommendations about the possibility of a no-biopsy diagnosis. TGA: Anti-transglutaminase antibodies; IgA: Immunoglobulin A; EMA: Anti-endomysium antibodies; HLA: Human leukocytes antigen; CD: Celiac disease.

CD diagnosis without a positive duodenal biopsy has always been discouraged in adults[9-14]. This choice was not dictated by the reduced reliability of the serological tests in adults. In fact, large population studies concluded that tTG-IgA>10x could accurately predict villous atrophy[53]. Rather, other considerations currently prevent the extension of paediatric criteria into the adult population. First, CD at onset can be associated with complications. In the case of primary or secondary resistance, or slow response to the GFD, the absence of baseline histology may make the diagnosis of complications difficult[9]. Index histology may also predict the risk of future complications, such as lymphoma[54]. Moreover, endoscopy may help diagnose other treatable disorders associated with CD, such as eosinophilic esophagitis, autoimmune gastritis, and lymphocytic gastritis[9].

Both complicated CD and possible differential diagnoses of CD are virtually absent in children. However, they represent a serious concern in adults, thus justifying different diagnostic algorithms according to the age of presentation of the first symptoms.

Potential, silent and seronegative CD

Potential CD is characterised by a positive serology for CD in the absence of mucosal damage at biopsy[1]. As stated above, Marsh 1 Lesions (*i.e.*, an increased IELs count) are not suggestive of an active CD but may increase the risk of developing villous atrophy[41].

It is widely accepted that symptomatic potential CD may benefit from a GFD, and a direct challenge would be run[8-14]. In adult patients with both positive TGA-IgA and EMA-IgA CD is likely, and a GFD may be initiated irrespective of symptoms[9]. A serological response after a period of approximately 12 mo confirms the diagnosis of CD[9]. In EMA-IgA negativity, HLA-typing may exclude the diagnosis before embarking on follow-up[9]. If a follow-up is started, potential CD patients should be

retested after consuming a gluten-containing diet for 3-6 mo to confirm persistent seropositivity before referral for a new endoscopy (Figure 8)[9,10].

Silent CD is characterised by the presence of both positive serology and histology for CD in the absence of classical or non-classical symptoms[1]. It is widely recommended to start a GFD in patients with silent CD because it is considered an active form of the disease[8-14].

Seronegative CD is characterised by the presence of active enteropathy and negative serology for CD, with no other causes, and with clinical and histological responses to a GFD[1,37]. In these cases, other causes of enteropathy should be excluded before embarking on the direct challenge of a GFD[37,55]. HLA-typing can also rule out the diagnosis of CD in seronegative enteropathies[9,14,37]. Finally, the direct challenge of a GFD is advised only in patients with seronegative enteropathy, positive HLA typing with no other causes. A documented histological response after 1-3 years of GFD is needed to confirm the diagnosis[9,14,37]. No major changes occurred over time in the management of seronegative CD[9,14].

Refractory and complicated CD

CD can be complicated by a persistent active form of the disease, independent of gluten intake, known as refractory CD (RCD)[1]. Other rare complications of CD can be neoplastic. Primarily, enteropathy-associated T-cell lymphoma (EATL) is a rare T-cell lymphoma associated with untreated CD. EATL has an abysmal prognosis and can occur primarily at diagnosis or as an evolution of RCD type 2[56]. Duodenal adenocarcinoma is possible, albeit less frequent in the CD population[57].

Refractory CD (RCD) is characterised by the persistence or recurrence of symptoms and signs of malabsorption, with documented villous atrophy, despite a strict GFD for more than 12 mo and in the absence of other causes[9-14]. No major changes occurred in this definition over time (Figure 9).

RCD can be primary (refractory at the time of the first diagnosis), or secondary (occurring after a period of response to the GFD)[1]. The first step in evaluating suspected RCD is to re-evaluate the initial diagnosis of CD by reviewing biopsies and serology tests obtained at the time of diagnosis[58]. The most common cause of GFD failure is inadvertent gluten ingestion[59].Therefore, evaluation by an expert dietitian should always be included[9,10,13,14]. Other associated or concomitant pathological conditions should be excluded before RCD diagnosis. These include lactose and fructose intolerance, small intestinal bacterial overgrowth, microscopic colitis, pancreatic insufficiency, and inflammatory bowel diseases[59,60]. All guidelines recommend this strategy[9,10,13,14].

RCD is further classified into type 1 (RCD-1) and type 2 (RCD-2)[1]. T-cell flow cytometry is the most reliable method for classifying RCDs. Aberrant T cells lose the normal surface markers CD3 and CD8 with preserved expression of intracytoplasmic CD3. In RCD-1, the percentage of aberrant T cells is below 20%, whereas in RCD-2, they represent more than 20% of the total IELs[58]. RCD-2 can be considered a pre-lymphoma or low-grade lymphoma[54]. T-cell receptor (TCR) g chain clonality analysis lacks sensitivity and specificity, and is of limited value in separating RCD-1 from RCD-2[54]. TCR analysis has been formerly indicated as a criterion for differentiating RCD-1 from RCD-2[11,13,14]. The latest ESsCD guidelines exclude TCR analysis in the RCD classification[9].

RCD-1 has an extremely high 5-year survival rate (> 90%)[54,59,60]. In RCD-1, the first-line therapy should be 'open-capsule' budesonide (OCB), 3 mg, 3 times a day[61]. Budesonide (open capsule or not) has been progressively accepted as the first-line therapy for RCD-1[9,11,13,14]. In the ACG 2013 guidelines, systemic steroids are considered the first-line therapy for RCD-1[14]. Second-line treatment for RCD-1 includes immunosuppressive drugs such as steroids (prednisone 0.5-1 mg/kg/day) and azathioprine (2-2.5 mg/kg/day)[60]. Most guidelines agree with this strategy[9,11,12]. Systemic steroids can also be considered as first-line treatment while waiting for a specialist's advice[12]. Infliximab may be the preferred biological therapy for second-line treatment of RCD-1[62]. Evidence is still weak, and only one guideline includes infliximab as an RCD-1 treatment[9].Withdrawing of immunosuppressive therapy after 2-3 years of complete response may be considered[9,54].

RCD-2 is rarer than RCD-1, has a much higher mortality rate, and treatment is less well defined. Systemic steroids or open-capsule budesonide should be the first choice for milder presentations. In severe cases, cytoreductive therapies such as cladribine and fludarabine or autologous hematopoietic stem cell transplantation should be chosen[59,60]. Guidelines are mostly aligned with this strategy[9,13,14]. Some guidelines also report azathioprine, 6-mercaptopurine, methotrexate, cyclosporine, and anti-TNF antibodies as possible therapies, but the data are weaker[11,13,14]. Not every

Recommendation		Changes over time
Children and adults with symptomatic potential CD responding to GFD may be considered CD patients, despite the absence of villous atrophy		Minor changes over time. Extensions
Children and adults with silent CD are considered CD patients and must be treated		No major changes over time
In adults, a seronegative CD diagnosis can be achieved with a direct challenge of a GFD in patients with villous atrophy with no other causes, negative serology tests and positive HLA typing. A follow-up biopsy of confirmation is required		No major changes over time

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Central Research Institute of Gastroenterology, Russia, 2016 (11).

National Institute for Health and Care Excellence (NICE), 2015 (12).

British Society of Gastroenterology (BSG), 2014 (13).

America College of Gastroenterology (ACG), 2013 (14).

Figure 8 Recommendations about potential, silent, and seronegative celiac disease. GFD: Gluten-free diet; HLA: Human leukocytes antigen.

guideline has raised the topic of RCD-2 treatment[10-14].

Transformation to enteropathy-associated T-cell lymphoma (EATL) is likely in RCD-2[59]. VCE, positron-emission tomography (PET), and magnetic resonance (MR) enterography can be useful in cases of suspected progression to EATL to assess the extent of the disease[63]. All guidelines advise the use of these tools in RCD-2 staging [9-14]. Severe RCD-2 and EATL may require surgery, chemotherapy, or bone marrow transplantation[64]. The former therapeutic strategies are mostly based on case reports, and only one guideline extensively discusses them[9].

Follow-up

Since CD is the only autoimmune disease with a known environmental trigger (*i.e.*, gluten), a periodical assessment of compliance to a GFD is essential[65]. Poor GFD compliance is not infrequent, and mucosal damage can persist despite negative serology and the absence of symptoms[66]. Follow-up is also essential for evaluating possible complications[54]. Osteoporosis and metabolic complications of GFD should also be evaluated during follow-up[67-69]. Suggested follow-up schedules are based on the frequency of complications, risk of GFD non-compliance, and reported quality of life[70].

Therefore, there is universal agreement on the necessity of long-term monitoring of patients with CD to assess the compliance and responsiveness to the GFD and allow early detection of complicated CD (Figure 10)[8-14]. Follow-up evaluations should be scheduled every 3-6 mo during the first year and then every 1-2 years[9-14]. In children, follow-up should continue until they reach their final height[9-11,14], focusing on normal growth and development[9,10,14].

There is disagreement about who should oversee follow-up. While most guidelines show no preference between primary care physicians, specialists, or dietitians[9-11,13,14], the NICE 2015 guidelines suggest that dietitians with expertise in CD may be best suited to carry out an annual follow-up[12]. However, on a general principle, all guidelines agree that newly diagnosed patients should be referred to a dietitian[9-14]. Some guidelines suggest that nutritionist counselling should coincide with medical visits during follow-up[10,13]. The inclusion of a dietitian assessment at diagnosis and

Recommendation		Change over time
Slow-responder CD is defined as the persistence of symptoms, signs and laboratory abnormalities despite at least 6–12 months of GFD. This term replaces the former "non-responsive CD"		Major change over time
Refractory CD is defined as the persistence/recurrence of malabsorption, with documented villous atrophy, despite a strict GFD for > 12 mo and absence of other causes		No major changes over time
T-cell flow cytometry is the most reliable method for classification refractory CD		Major changes over time
TCR-gamma chain clonality analysis lacks sensitivity and specificity, and it is of limited value		Major changes over time
Budesonide is recommended as first-line therapy for refractory CD type 1		Major changes over time
Second-line treatments for refractory CD type 1 includes steroids, azathioprine and infliximab		Major changes over time
Therapy for refractory CD type 2 is not supported by strong clinical data		Major changes over time

European Society Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) 2020 (8).

European Society for the Study of Coeliac Disease (ECD) 2019 (9).

World Gastroenterology Organization (WGO) 2017 (10).

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America College of Gastroenterology (ACG), 2013 (14).

Figure 9 Recommendations about refractory and complicated celiac disease. GFD: Gluten-free diet; TCR: T-cell receptor.

during follow-up was supported by clinical data[71]. Indeed, nutritional counselling could also help manage metabolic alterations, which frequently appear during the first years of the GFD[67].

All guidelines also provide information about the essential information that should be collected during follow-up evaluations. These evaluations should include a dietary interview, serology (TTG-IgA if normal IgA), and laboratory tests[9-14]. Laboratory tests should evaluate the presence of micronutrients malabsorption, including complete blood count, iron status, folate, vitamin B12, calcium, phosphate, vitamin D, and should monitor associated autoimmune conditions (thyroid-stimulating hormone and serum glucose) and liver disorders (aspartate aminotransferase/alanine amino-transferase)[9-11,13,14]. Normalisation of tTG-IgA levels do not predict full recovery of villous atrophy. In contrast, persistently positive serology 12 mo after GFD initiation is a strong indicator of gluten ingestion[72]. All guidelines were aligned with the interpretation of tTG-IgA levels during follow-up[8-14].

The inability of serology alone to predict mucosal healing automatically leads to consider the opportunity of repeating duodenal biopsies after the start of the GFD. While the general agreement is that follow-up biopsies are not mandatory in

Recommendation		Changes over time
In adults, follow-up should be scheduled every 3-6 month during the first year and then every 1-2 yr		No major changes over time
A normal TGA level at the follow-up does not predict recovery of villous atrophy		No major changes over time
On the contrary, persistently positive serology 12 mo after starting a GFD strongly suggests gluten contamination		No major changes over time
The follow-up should include at least a dietary interview, serology, and laboratory tests evaluating absorption.		No major changes over time
Follow-up biopsy is not universally recommended but may be reasonable after 2 yr of GFD in high-risk patients		Minor changes over time
In children, follow-up should be scheduled every 3-6 mo during the first year and then every year until the end of development		No major changes over time
Newly diagnosed patients should be referred to a dietitian for management		Minor differences
Primary care physicians or dietitians with experience in dealing with CD may take responsibility for the follow-up		Some differences
Follow-up should also include periodical bone densitometry, vaccinations and psychological support		Some differences

European Society Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) 2020 (8).

European Society for the Study of Coeliac Disease (ECD) 2019 (9).

World Gastroenterology Organization (WGO) 2017 (10).

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British Society of Gastroenterology (BSG), 2014 (13).

America College of Gastroenterology (ACG), 2013 (14).

Figure 10 Recommendations about follow-up of celiac disease. TGA: Anti-transglutaminase antibodies; GFD: Gluten-free diet.

asymptomatic patients on a GFD and without an increased risk of complications[9-14], the guidelines diverge regarding other points. Many guidelines consider it reasonable to repeat biopsy after 2 years of GFD to assess mucosal healing[9,11,14]. Other guidelines suggest repeating biopsies only for persistent symptoms or serological abnormalities after 12 mo of GFD[10,12,13]. A growing body of literature suggests that the risk of a complicated CD is higher in patients >40 years of age at the time of diagnosis or those with a classical presentation[54]. Some guidelines agree that repeating biopsies should be of interest in these selected populations[13,14].

Some guidelines also provide suggestions for further examinations to be performed during follow-up. According to the ECD and Russian guidelines, bone densitometry should be offered to every patient at the time of diagnosis and should be repeated after 3 years if abnormal, or 5 years if normal[9,11]. Other guidelines suggest performing

bone densitometry only in patients with a high risk of osteoporosis or those older than 55 years[12,13].

While there is a general agreement in recommending a pneumococcal vaccine[8-10, 12], the WGO2017 guidelines also recommend vaccinations against *Haemophilus influenzae typeB*, and *Meningococcus*, while other guidelines state that these vaccines have a less clear indication to be given to every patient with CD[9,11-13].

Mood disorders are another common problem in patients with dietary restrictions. Anxiety, depression, and fatigue may be associated with CD before and after diagnosis and can affect the quality of life[73]. In this context, most guidelines agree on advising patients to join CD support groups and associations[9,10,12,13]. Some of them also suggest that psychological support provided by a specialist may be offered[12,13].

Gluten-free diet

Gluten is a protein with high proline and glutamine content, primarily found in wheat. Rye and barley belong to the same tribe as wheat and are known to contain gluten. In contrast, oats are derived from a different tribe and do not contain pure gluten[1].

Uncontaminated oats are safe for almost all patients with CD, but a small percentage of patients may be sensitive to some oat cultivars[74] and should be monitored[9,10,12-14]. Some guidelines advise the initiation of a Gluten-free diet (GFD), excluding oats, and recently introduced them[10,13,14]. The Russian guidelines (2016)are against oat consumption in patients with CD because of the high risk of contamination[11]. Even if not stated, oat consumption would be safe in many countries, though it may be discouraged in developing countries where contamination could be widespread (Figure 11).

WHO guidelines on 'Standard for Foods for Special Dietary Use for Persons Intolerant to Gluten' state that foods labelled as 'gluten free' should contain ≤ 20 parts per million (ppm) of gluten[75].

Patients should be instructed to avoid contaminating their gluten-free food by using separate cooking utensils and cooking surfaces[9,10]. At present, shared items can be safely used if thoroughly cleaned with soap and water between use[9,76].

The duration of breastfeeding and the timing of gluten introduction to the infant seem to have no impact on the risk of developing CD, even in those at high risk[77]. Therefore, there are no strict indications for gluten introduction in infant diets[9]. Formerly, it was advised to avoid either early or late gluten introduction in children at risk of CD[13].

Dermatitis herpetiformis (DH) is a bullous cutaneous disease triggered by gluten consumption like CD[1]. DH and CD often coexist and share the same treatment, GFD [9,10,13,14]. Interestingly, the ESsCD guidelines suggest that psoriasis could also benefit from GFD in the case of documented CD serology, even in the absence of mucosal damage[9].

DISCUSSION

Our comparative analysis of the currently adopted CD guidelines underlined differences in diagnostic aspects and the management of the follow-up. These differences mirror some relevant clinical points in both developing and developed countries.

First, the differences in the diagnostic process of CD are important. The possibility of a no-biopsy diagnosis has relevant repercussions in developing countries. Most guidelines are still cautious in this regard, with the WGO2017 guidelines being the only ones contemplating this possibility in geographical areas with a paucity of resources. As correctly underlined by these guidelines, some absolute recommendations may not be valid for developing countries where the availability of serology or endoscopy may be lacking[10]. CD seems to have a non-negligible prevalence in Asia and sub-Saharan Africa[77,78]. Especially in Russia and Central Asia, the prevalence of CD is very likely to be underestimated due to poor disease awareness among physicians and/or patients, limited access to diagnostic resources, inappropriate use or interpretation of the serological tests, absence of standardised diagnostic and endoscopic protocols, and insufficient expertise in histopathological interpretation[3]. Specific guidelines are lacking in these geographical areas[79]. In addition, the incidence of undiagnosed CD in children can be extremely high[80]. Knowing the high mortality and disability related to untreated CD in childhood, it would be advisable to develop specific protocols for specific geographical areas.

Recommendation		Change over time
The mainstay for treatment of CD is a strict GFD. which usually resolves both classical and non-classical manifestations		No major changes over time
Uncontaminated oat is safe for almost every patient. A small percentage of patients may be sensitive to oats and should be monitored		Minor changes over time
GFD should be initiated also in psoriatic patients with positive CD serology		Major changes over time
The duration of breastfeeding and the timing of gluten introduction have no impact on the risk of developing CD		Major changes over time

European Society Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) 2020 (8).

European Society for the Study of Coeliac Disease (ECD) 2019 (9).

World Gastroenterology Organization (WGO) 2017 (10).

Central Research Institute of Gastroenterology, Russia, 2016 (11).

National Institute for Health and Care Excellence (NICE), 2015 (12).

British Society of Gastroenterology (BSG), 2014 (13).

America College of Gastroenterology (ACG), 2013 (14).

Figure 11 Recommendations about the gluten-free diet for celiac disease.

The no-biopsy approach has been discouraged for a long time, especially in adults [13,14]. In contrast, most recent guidelines have incorporated the ESPGHAN 2012 recommendations for a no-biopsy approach in children[9,10]. The possibility of an outright extension of these criteria into the adult population still meets key obstacles. However, in an era during which the COVID-19 pandemic has caused a staggering drop in new CD diagnoses even in industrialised countries[81], ESPGHAN released the advice to lower the TGA-IgA threshold for diagnosing CD without biopsy[52]. Moreover, retrospective data on a possible no-biopsy approach in adults are increasing [53]. Prospective data will probably lead to the integration of such an approach to future guidelines over the next decade.

Second, the differences in follow-up recommendations reflect a relatively low interest in this topic in the past. Arguably, the search for more reliable diagnostic tools was the right priority in an era characterised by a severe under-diagnosis of CD .Nowadays, significant diagnostic delays can still occur in a minority of Central European children[82], with socioeconomically deprived children being more likely to be underdiagnosed despite improved and easily available serological testing[4].

Nonetheless, the current physicians' awareness of CD has reached fairly high levels, and the case-detection strategy has significantly contributed to the increased number of diagnoses. Consequently, the correct management of follow-up is crucial. This topic is of special interest in developed countries, in which metabolic problems possibly caused by an unbalanced GFD are particularly prevalent. Uncontrolled weight gain, metabolic syndrome, and non-alcoholic fatty liver disease are epidemic in these countries and can also be facilitated by the GFD[67,69,83-85]. In addition, quick detection of associated autoimmune conditions can prove highly beneficial, especially in autoimmune liver diseases[86]. Finally, early detection of complicated CD requires particular attention, as both neoplastic and non-neoplastic complications may arise years after the diagnosis[6].

CONCLUSION

We found a relatively high concordance between CD guidelines. Important modifications have occurred in recent years, especially regarding the possibility of a no-biopsy diagnosis in children. Other modifications are expected in the future and will probably involve the extension of the non-invasive diagnosis to the adult population and the follow-up modalities.

ARTICLE HIGHLIGHTS

Research background

Celiac disease (CD) has risen from obscurity to global prominence in a few decades. These modifications have prompted experts from all over the world to identify effective strategies for the diagnosis and follow-up of CD. Different scientific societies, mainly from Europe and America regions, have proposed different guidelines.

Research motivation

CD guidelines are consistent when they deal key points in the diagnosis and follow-up of this condition. However, they differ in a number of other points.

Research objectives

To identify all of the existing guidelines across the globe and perform a comparative analysis to verify similarities and differences and, thus, discuss the most debated topics and the possible innovations in the next future.

Research methods

We searched PubMed for a complex string containing the terms "celiac disease", "management", and "guidelines". The results were subsequently explored to identify the most recent versions of existing guidelines of governmental agencies and scientific societies. The recommendations provided by each selected guideline were systematically explored and classified under five categories: Patients to be tested for CD, diagnostic tests (serology, duodenal biopsy, genetic test, no-biopsy diagnosis), potential/silent/seronegative CD, refractory/complicated CD, follow-up.

Research results

We identified 7 different guidelines [European Society Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) 2020; European Society for the Study of Coeliac Disease (ECD) 2019; World Gastroenterology Organization (WGO) 2017; Central Research Institute of Gastroenterology, Russia, 2016; National Institute for Health and Care Excellence (NICE), 2015; British Society of Gastroenterology (BSG), 2014; and America College of Gastroenterology (ACG), 2013]. These guidelines were mostly concordant but differed under certain recommendation for no-biopsy diagnosis, refractory CD, and follow-up.

Research conclusions

We found a relatively high concordance between the guidelines for CD. Important modifications have occurred in the last years, especially about the possibility of a no-biopsy diagnosis in children.

Research perspectives

Modifications of the current guidelines are expected in the near future. These modification will probably regard the possibility of a no-biopsy diagnosis (especially in developing countries) and the modalities of follow-up.

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