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Editorial Board Member of *World Journal of Gastroenterology*, Haruhiko Sugimura, PhD, MD, Vice President, Hamamatsu University School of Medicine; Professor and Chairman of the Department of Tumor Pathology, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-ku, Hamamatsu 431-3192, Japan. hsugimur@hama-med.ac.jp

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RESPONSIBLE EDITORS FOR THIS ISSUE

Induced pluripotent stem cells as an innovative model to study drug induced pancreatitis

Elena Genova, Gabriele Stocco, Giuliana Decorti

ORCID number: Elena Genova 0000-0002-6692-6573; Gabriele Stocco 0000-0003-0964-5879; Giuliana Decorti 0000-0002-9714-6246.

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Elena Genova, Giuliana Decorti, Institute for Maternal and Child Health, IRCCS Burlo Garofolo, Trieste 34137, Italy

Gabriele Stocco, Department of Life Sciences, University of Trieste, Trieste 34127, Italy

Giuliana Decorti, Department of Medical, Surgical and Health Sciences, University of Trieste, Trieste 34127, Italy

Corresponding author: Giuliana Decorti, MD, Full Professor, Institute for Maternal and Child Health, IRCCS Burlo Garofolo, Via dell'Istria 65, Trieste 34137, Italy. decorti@units.it

Abstract

Drug-induced pancreatitis is a gastrointestinal adverse effect concerning about 2% of drugs. The majority of cases are mild to moderate but severe episodes can also occur, leading to hospitalization or even death. Unfortunately, the mechanisms of this adverse reaction are still not clear, hindering its prevention, and the majority of data available of this potentially life-threatening adverse effect are limited to case reports leading to a probable underestimation of this event. In particular, in this editorial, special attention is given to thiopurine-induced pancreatitis (TIP), an idiosyncratic adverse reaction affecting around 5% of inflammatory bowel disease (IBD) patients taking thiopurines as immunosuppressants, with a higher incidence in the pediatric population. Validated biomarkers are not available to assist clinicians in the prevention of TIP, also because of the inaccessibility of the pancreatic tissue, which limits the possibility to perform dedicated cellular and molecular studies. In this regard, induced pluripotent stem cells (iPSCs) and the exocrine pancreatic differentiated counterpart could be a great tool to investigate the cellular and molecular mechanisms underlying the development of this undesirable event. This particular type of stem cells is obtained by reprogramming adult cells, including fibroblasts and leukocytes, with a set of transcription factors known as the Yamanaka's factors. Maintaining unaltered the donors' genetic heritage, iPSCs represent an innovative model to study the mechanisms of adverse drug reactions in individual patients' tissues not easily obtainable from human probands. Indeed, iPSCs can differentiate under adequate stimuli into almost any somatic lineage, opening a new world of opportunities for researchers. Several works are already available in the literature studying liver, central nervous system and cardiac cells derived from iPSCs and adverse drug effects. However, to our knowledge no studies have been performed on exocrine pancreas differentiated from iPSCs and drug-induced pancreatitis, so far. Hence, in

this editorial we focus specifically on the description of the study of the mechanisms of TIP by using IBD patient-specific iPSCs and exocrine pancreatic differentiated cells as innovative *in vitro* models.

Key Words: Induced pluripotent stem cells; Therapy personalization; Patient-specific cells; Drug-induced pancreatitis; Thiopurines; Inflammatory bowel disease

Core Tip: About 5% of inflammatory bowel disease patients develop pancreatitis after thiopurine administration. The mechanism of this adverse effect is still not clear making it difficult to prevent. By differentiating induced pluripotent stem cells into their pancreatic exocrine counterpart, it is possible to set up innovative personalized *in vitro* models to study this adverse effect in a more effective way.

INTRODUCTION

Gastrointestinal adverse effects are common especially with orally absorbed drugs and may result in undesirable consequences leading to the reduction of treatment efficacy and, in the most serious cases, to therapy interruption with associated healthcare costs. To better study and prevent these adverse events there is the need for dedicated clinical investigation[1]. Over the past years, adverse drug reactions (ADRs) have been widely studied also for their negative effect on the development of new drugs[2,3].

Among the different ADRs, drug-induced pancreatitis has become increasingly recognized as an important cause of acute pancreatitis with a wide range of drug classes involved in its development[4]. Unfortunately, the majority of data available of this potentially life-threatening ADR are principally limited to case reports, leading to a probably underestimated incidence, reported to be around 2%[4]. Furthermore, the mechanisms of drug-induced pancreatitis of many drugs are still not clear, making it difficult to determine a definitive association of causality between specific medications and acute pancreatitis, and in only less than 10% of cases the real cause has been determined. Drugs known to induce pancreatitis have been classified considering the number of case reports, the recurrence of pancreatitis with a re-challenge with the drug, consistent latency between the drug assumption and the onset of acute pancreatitis and the exclusion of alternative causes such as alcohol assumption or gallstones [4,5] (Table 1).

Interestingly, certain types of ADRs are reported to be more frequent in patients affected by specific diseases. An important example is thiopurine-induced pancreatitis (TIP), an idiosyncratic ADR affecting more frequently inflammatory bowel disease (IBD) patients taking thiopurines, such as azathioprine and mercaptopurine[6]. In the vast majority of cases, TIP is manageable, however patients have to stop the treatment and to be sometimes hospitalized until the symptoms are resolved[7]. The higher incidence of this adverse event in IBD patients, especially in the pediatric population, suggests that molecular mechanisms involved in the disease may contribute to TIP predisposition[6]. However, mechanisms determining TIP predisposition are still unknown and only hypotheses have been postulated. In particular, the mechanisms proposed can be divided into three different groups: genetic predisposition[8,9], alteration in thiopurine biotransformation[7] and abnormalities in innate or adaptative immunity[10].

The thiopurines azathioprine, mercaptopurine and thioguanine undergo an extensive biotransformation catalyzed by several enzymes[11]. Regarding genetic predisposition, TIP seems unrelated to candidate variants on important genes of the thiopurine biotransformation pathway, such as *TPMT, ITPA* and *NUDT15*, well-known to induce severe ADRs, including myelosuppression and hematologic toxicity[12,13].

Table 1 Classification system of drugs related to pancreatitis development[4,5]

Class	
Class Ia	At least one case report with positive rechallenge, excluding other possible causes such as alcohol, gallstones and other drugs
Class Ib	At least one case report with positive rechallenge but not excluding other possible causes
Class II	At least four cases in the literature without rechallenge but with consistent latency in greater than 75% of cases
Class III	At least two cases in the literature without rechallenge and consistent latency
Class IV	Single case reported in the literature not fitting the previous described classed without rechallenge

Recently, two different research groups have found a strong association between the Class II *HLA* gene region polymorphism rs2647087 and TIP[8,9], but more efforts are needed to translate these variants into clinical practice. TIP development may be also related to direct damage to the exocrine pancreatic cells or to an accumulation of toxic metabolites (biotransformation hypothesis). However, pancreatitis frequently occurs early after thiopurine administration, making the accumulation of toxic metabolites unlikely, while more probably immunological reactions are involved. However, direct toxicity of thiopurines or their metabolites on patients' pancreatic cells cannot be completely excluded[7,10].

To study and discover TIP mechanisms and predisposition, innovative patient-specific *in vitro* models could be helpful and decisive. In this regard, induced pluripotent stem cells (iPSCs) and their differentiated counterpart are widely used to set up groundbreaking personalized *in vitro* models representative of patients' genetic background. The peculiar characteristics of these cells allow to set up *in vitro* models to study disease mechanisms and ADRs with the purpose to personalize patients' therapy, improving the disease outcome. The iPSC model can be a great tool to better understand, and thus prevent, ADRs in particular in comparison to animal models and immortalized cells. Indeed, the predisposition to a specific ADR may be related to the individual genetic patients' background, leading to a wide range of toxicities of different severity[14]. Therefore, the iPSC technology, matching the donor's genetic background, can be extremely helpful for developing patient-specific assays. Indeed, by using iPSCs, it seems reasonable to precisely mimic the patients' susceptibility to an abnormal response to a specific drug, setting up powerful assays useful to identify predictive biomarkers. In the last years, many different models[15] have been developed using the iPSC technology, including the differentiation into pancreatic exocrine cells[16].

PATIENT-SPECIFIC IPSCS AS AN IN VITRO MODEL TO STUDY DRUG-INDUCED PANCREATITIS

Patient-specific iPSCs can be obtained by reprogramming patients' fibroblasts or peripheral blood mononuclear cells using the four Yamanaka's factors OCT4, SOX2, KLF4 and MYC, forcing somatic cells to an embryonic-like state[17,18]. Differentiation of iPSCs allows to generate almost any kind of somatic cells using appropriate protocols. In the literature it is possible to find a wide range of differentiation possibilities including neural-like cells, hepatocytes, enterocytes, pancreatic endocrine cells and many others as recently reviewed by our group[15]. These cells, being patient-specific, have been frequently used to model and study individual susceptibility to develop ADRs. For example, regarding gastrointestinal toxicity, some groups have already tried to model hepatocytes[19-21] and enterocytes[22,23] to study drug-induced liver injury and intestinal toxicity, respectively. However, in comparison to other ADRs, drug-induced pancreatitis has not been deeply studied yet. A limited number of protocols[16,24-26] are available in the literature to generate pancreatic exocrine cells starting from iPSCs in comparison to the endocrine counterpart[15]. To the best of our knowledge, our group recently evaluated for the first time the mechanisms behind TIP predisposition using iPSCs and pancreatic differentiated cells of pediatric patients affected by IBD that developed or not TIP. Differentiation of iPSCs in pancreatic exocrine cells was performed using the protocol developed by Takizawa-Shirasawa *et al*[16]. Briefly, different stimuli were added to the culture medium in 4 different steps (Figure 1). To characterize cells obtained during each differentiation step, genetic expression of specific genetic markers was analyzed and confirmed:
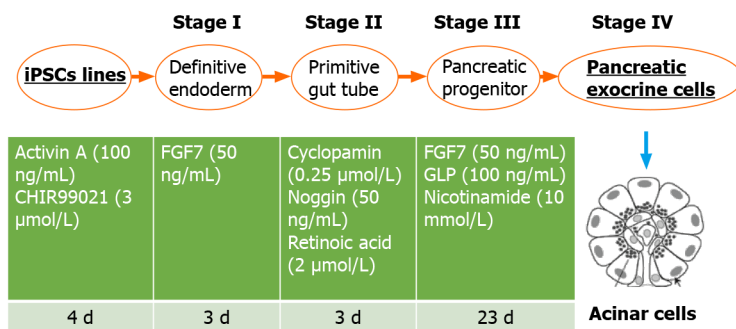
Figure 1 Differentiation of induced pluripotent stem cells into pancreatic cells towards a 4 steps protocol. iPSCs: Induced pluripotent stem cells; d: Days of culture.

OCT4 for undifferentiated cells (iPSCs), *FOXA2* and *SOX17* for definitive endoderm (stage I), *PDX1* for pancreatic progenitors (stage III) and amylase, in particular its pancreatic isoforms *AMY2A* and *AMY2B* for pancreatic exocrine cells (stage IV).

The gold standard of cytotoxicity assay showed an almost double *in vitro* sensitivity of TIP cases cells to thiopurines, more marked in iPSCs rather than in the differentiated counterpart, after mercaptopurine and thioguanine exposure. *TPMT* variants (rs1142345, rs1800460 and rs1800462) were excluded as a possible cause of this different sensitivity because all patients resulted wild-type.

The results obtained are encouraging, however some limitations have to be overcome in the next future. For instance, the differentiation protocol to obtain exocrine pancreatic cells could be further improved in terms of efficiency based on the more recent studies performed by Hohwieler *et al*[24] and Ito *et al*[25] which used 3D culture methods and the distinction between acinar and ductal cell type, by analyzing the expression of different genetic and protein markers such as amylase and chymotrypsin C for acinar cells, and SOX9 and cytokeratin 19 for ductal cells[24,25]. An important point to consider is if the amylase markers are sufficient to reflect terminal differentiation. Beside studies considering the mRNA levels of these markers[24,25], more functional studies, evaluating the amylase protein concentrations and enzyme activity, should be implemented. These comparisons would allow to ensure that terminal differentiation is as representative as possible of the *in vivo* models. Another important point to focus, linked to pancreatic cell generation, is the time necessary that is too long for a clinical application of this *in vitro* model for TIP predisposition screening. Studies are now ongoing to partially resolve this limitation trying to develop more efficient and faster ready-to-use patient-specific pancreatic exocrine differentiated cells. The cost of hospitalization after a pancreatitis event has been recently calculated, resulting in around 8000 € *per* patient[27]. Considering an incidence of pancreatitis of 5%, we can estimate that every 20 patients treated with azathioprine one will be at risk of pancreatitis. Therefore, to be cost-effective, the analysis should amount to 400 €, considering only the cost of the analysis, without evaluating the health benefit[28]. Current costs are still higher but there is a trend toward reduction; indeed, the iPSC technology is still expensive and costs have to be reduced before they can be introduced into clinical practice. In particular, characterization costs are high, but several suggestions to address this limitation have been already proposed such as SNP microarray technology for the routine karyotyping and cost-effective methods such as innovative flow cytometry analyses to assess cell surface expression of pluripotent markers[29].

Beyond technical limitations, it is conceivable that thiopurines do not directly reach the pancreatic tissue unmodified, but rather as metabolites. Therefore, to improve the clinical relevance of the *in vitro* model, patient-specific pancreatic cells would need to be exposed to a representative mixture of thiopurine metabolites or to conditioned media of other thiopurine metabolizing cells such as hepatocytes[30]. Moreover, it is important to keep in mind that TIP predisposition could be influenced by the contribution of the immune system that, in predisposed patients, could be activated for unknown reasons after thiopurine administration attacking the pancreatic tissue. This aspect has to be considered, modeled and studied as well[7,31]. Finally, data obtained have to be confirmed in a larger cohort of patients that now includes 3 cases and 3 controls already analyzed while 2 cases and 2 controls still have to be analyzed.

CLINICAL IMPLICATIONS

Drug-induced pancreatitis represents an important clinical issue for different reasons including therapy interruption, reduction of treatment efficacy, the need for unnecessary diagnostic procedures and treatment for the adverse effect resolution[1] with associated healthcare costs. Moreover, in recent years an increasing number of drugs have been associated with pancreatitis development although its recognition by clinicians is still limited because of the lack of biomarkers useful to prevent this ADR.

CONCLUSION

Drug-induced pancreatitis is a growing problem related to several drugs and TIP recapitulates well all complications related to the development of this ADR. The possibility of studying TIP by an iPSC-based model seems a great opportunity to investigate TIP mechanisms that still remain not clear. The *in vitro* model established in our laboratory has proven to be suitable for studying and investigating TIP predisposition in a personalized way in pediatric IBD patients. Alongside thiopurines, several other drugs such as asparaginase, nilotinib and pazopanib can cause pancreatitis. Therefore, the *in vitro* model developed in this study could be applied also to study the sensitivity of other drugs with the purpose of pancreatitis prevention.

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Screening for nonalcoholic fatty liver disease-when, who and how?

Christoph G Dietrich, Monika Rau, Andreas Geier

ORCID number: Christoph G Dietrich 0000-0001-6927-7970; Monika Rau 0000-0003-1219-4044; Andreas Geier 0000-0002-9626-5083.

Author contributions: Dietrich CG and Geier A developed the concept of this review; all authors contributed to the manuscript.

Christoph G Dietrich, Department of Internal Medicine, Bethlehem Health Center, Stolberg 52222, Germany

Monika Rau, Department of Internal Medicine II, University Hospital Würzburg, Würzburg 97080, Germany

Andreas Geier, Department of Medicine II, University Hospital Würzburg, Würzburg 97080, Germany

Corresponding author: Christoph G Dietrich, MD, PhD, Chief Doctor, Department of Internal Medicine, Bethlehem Health Center, Steinfeldstr. 5, Stolberg 52222, Germany. christoph.g.dietrich@googlemail.com

Abstract

Nonalcoholic fatty liver disease (NAFLD) is becoming a frequent liver disease, especially in patients with metabolic syndrome and especially in Western countries. Complications of NAFLD comprise progressive fibrosis, cirrhosis and hepatocellular carcinoma. NAFLD also represents an independent risk factor for cardiovascular disease, extrahepatic neoplasia and other organ damage, such as renal insufficiency. Given the epidemiological importance of the disease, new developments in specific treatment of the disease and the wide availability of noninvasive techniques in estimating steatosis and fibrosis, NAFLD should be subject to screening programs, at least in countries with a high prevalence of the disease. The review discusses prerequisites for screening, cost-effectiveness, current guideline recommendations, suitability of techniques for screening and propositions for the following questions: Who should be screened? Who should perform screening? How should screening be performed? It is time for a screening program in patients at risk for NAFLD.

Key Words: Screening; Nonalcoholic fatty liver disease; Diabetes; Liver fibrosis; Cirrhosis

Core Tip: Nonalcoholic fatty liver disease (NAFLD) is becoming more important in Western countries and leads to serious complications in patients with progressive disease. The epidemiological, clinical and technical requirements for screening for this disease are fulfilled and are outlaid in this review. It is time to consider a screening program for NAFLD.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is the most common liver disease world-wide, with rising prevalence to an estimate of 25% in Western populations[1]. NAFLD is regarded as one component of metabolic syndrome, including obesity, insulin resistance or type 2 diabetes mellitus (T2DM), hypertension, and dyslipidemia. Recently, the new term metabolic dysfunction-associated fatty liver disease has been proposed to emphasize this association[2]. Over the next decade, the number of patients with advanced fibrosis stages is expected to rise further together with an increasing incidence of complications [nonalcoholic steato hepatitis (NASH)-related end stage liver disease, *e.g.* hepatic decompensation, liver cancer and mortality][3]. In this recent modeling, the number of NAFLD patients in the United States, the EU5 (France, Germany, Italy, Spain, United Kingdom) and China was estimated to be 85.3 million, 72.2 million and 211 million, respectively, whereby in the same countries, more than 17.3 million, 12.6 million and 32.6 million patients were predicted to have NASH[3]. The number of NASH patients with advanced fibrosis is expected to more than double until 2030. Similar but slightly more conservative calculations have been obtained with different modeling methodologies but confirm the extent of the clinical problem[4]. In addition to liver-related morbidity and mortality, it is important to emphasize that NAFLD patients have increased cardiovascular mortality, which to-gether cause an enormous socioeconomic impact in industrialized countries[4]. The fact that NAFLD has become the most frequent disease entity on the liver transplant waiting list in the UNOS network documents the need for early detection and in-tervention in the future[5]. Given the sheer frequency of patients with obesity, me-tabolic syndrome and NAFLD worldwide, it is remarkable that this disease entity has been overlooked by clinicians and the pharmaceutical industry for a considerable period of time, and no widely established algorithms for screening exist. The global burden of disease documents the burning need to establish clinical care structures and diagnostic algorithms to cope with the increasing number of patients at risk.

A multistep diagnostic screening algorithm is recommended in current guidelines in Western countries and combines an initial ultrasound (US) examination with sub-sequent risk prediction tools such as the Fibrosis-4 (FIB-4) or NAFLD fibrosis score (NFS) followed by transient elastography (TE) stratification for liver biopsy[6,7]. Increasing public and professional awareness as well as the implementation of scree-ning algorithms in primary and secondary care will lead to a more frequent diagnosis of NAFLD patients at different stages of the disease (NAFL, noncirrhotic NASH, NASH with cirrhosis) in the near future. For the histological assessment of NAFLD, different systems are used for scoring in clinical practice [*e.g.*, NAFLD activity score (NAS)][8]. The definite histopathological diagnosis of NAFL *vs* NASH is based on the simultaneous presence of steatosis, ballooning and inflammation, which are required for the diagnosis of "NASH" in the European SAF/FLIP algorithm[9].

Of the different histologic features of NASH, fibrosis has been identified as the strongest predictor of adverse clinical outcomes, including decompensation and liver-related death[10-14]. The latest meta-analysis showed that the stage of biopsy-con-firmed liver fibrosis is a strong predictor of future all-cause mortality and morbidity in NAFLD with and without adjustment for key potential confounding variables[15]. It became clear that evaluation of the fibrosis stage is even more fundamental than scoring necroinflammation or diagnosing NASH. Several options for the noninvasive evaluation of liver fibrosis in NASH, such as elastography devices and blood tests, are available[16]. Despite recent progress in noninvasive tests (NITs) for the evaluation of liver fibrosis in NAFLD, the diagnosis of NASH is still often based on liver biopsy, an invasive procedure not suitable for the large proportion of the general population affected by NAFLD. To identify patients with an increased risk, the NFS was in-troduced in 2007 as a simple scoring system to distinguish NAFLD with and without advanced fibrosis (fibrosis stages 3 and 4)[17]. Subsequently, further fibrosis tests, including the FIB-4 index, Fibrotest/Fibrosure, enhanced liver fibrosis (ELF) test, and liver stiffness measurement (LSM) by vibration-controlled TE, have entered clinical practice[18-21]. Of relevance for fibrosis screening, these NITs show excellent

AUROCs for the diagnosis of advanced fibrosis and cirrhosis[22]. Furthermore, repeated testing of FIB-4 within 5 years improved the identification of individuals at an increased risk of severe liver disease in the general population[23]. In light of a multistep screening algorithm, the performance has been further improved by the sequential combination of different NITs for advanced fibrosis, thereby refining the patient referral pathway between primary care or diabetologists and liver specialists [24]. Sequential combinations of FIB-4 (or NFS) and TE with a lower cut-off to rule-out advanced fibrosis and a higher cut-off to rule-in cirrhosis can increase the specificity and thereby reduce the need for liver biopsies from 33% to 19%[25]. The ultimate goal of screening measures is to identify patients at high risk for liver-related events and unfavorable overall outcomes. Longitudinal retrospective studies have demonstrated that NITs calibrated on liver fibrosis are prognostic markers to stratify the risk of liver-related outcomes and mortality in NAFLD patients[26].

Comparative diagnostic accuracy studies for established and novel biomarkers and combinations thereof are ongoing in the European LITMUS and United States NIBLE consortia[27]. It will be interesting to learn whether and which of the novel biomarkers outperforms the established freely available routine scores NFS and FIB-4. At the same time, biomarker screening strategies are currently being tested to establish validated numbers of patients to test to identify NASH patients with advanced fibrosis suitable for specific treatment.

The following review gives an overview of current guideline recommendations and answers the question of when, whom and how to screen in the different clinical settings.

RECOMMENDATIONS FOR NAFLD SCREENING IN RECENT GUIDELINES

Several guidelines worldwide have already taken a position on screening for NAFLD. The consensus is that screening in the general population is not recommended[6,7,28,29]. AASLD also discourages screening in high-risk groups because of the current lack of treatment options, unclear value of screening tests, and unclear cost-effectiveness. However, "a high index of suspicion" for the presence of NAFLD in diabetes mellitus type 2 patients is advised[7]. The Asian guideline takes a similarly noncommittal view, which also does not explicitly recommend screening in risk groups (here T2DM and obesity) but merely describes it as worth considering[29].

In contrast, specific screening recommendations can be found in the Latin American and European guidelines. Here, NAFLD screening is recommended for patients with repeatedly altered liver enzymes, features of metabolic syndrome, or obesity [body mass index (BMI) > 30] according to Latin American guidelines[28]. In the same direction, patients with insulin resistance and metabolic syndrome, especially manifest type 2 diabetes, should also be screened for the presence of NAFLD according to the European recommendation, regardless of the level of liver enzymes[6]. Both guidelines primarily recommend abdominal US as the initial examination to determine the presence of steatosis. Serum fibrosis tests are considered appropriate for further risk stratification[6,28], with the Latin American guideline decidedly recommending determination of FIB-4 and NFS. Elastography, as a more reliable method, is also mentioned[28] but is considered secondary due to its lack of availability in many places.

The guidelines differ in their treatment of patients in whom serum fibrosis scores indicate intermediate fibrosis risk. While the European algorithm recommends both high-risk and intermediate-risk patients for referral to the hepatologist[6], the Latin American guidelines suggest that this should only be the case for patients > 50 years of age with diabetes or obesity[28].

The basis of the differing recommendations is an ultimate lack of data on the efficacy and efficiency of structured screening and on the effectiveness of the therapeutic efforts that begin after NAFLD has been diagnosed in the context of screening. There are also discrepancies between the lack of widespread availability of specific examination procedures and the desire for screening results that are as sensitive and specific as possible and avoid overloading specialists by referring numerous false-positive screened patients.

SCREENING–WHEN? IS IT TIME FOR A NAFLD SCREENING PROGRAM?

Prerequisites for a disease to justify screening

In 1968, Wilson and Jungner formulated basic criteria for the usefulness of screening procedures for a particular disease in a paper by the WHO[30,31]. These criteria include peculiarities of the disease (significant burden of disease in the population and knowledge of etiology and stages of disease) and of reaching a diagnosis (simple test acceptable to patients) as well as organizational requirements (available facilities for diagnosis and therapy). In general, these criteria already apply for NAFLD for some time.

However, the authors also point out that efficient therapy as well as cost-effectiveness of screening must be present[30]. Here, important new developments have occurred in recent years that make screening for NAFLD much more justified than in the past.

The general progress in diagnosing and treating liver disease led an expert group 2016 to the proposal that screening for liver fibrosis (independent from the underlying disease) may now be feasible even for the general population[32].

For a long time, missing therapeutic options were a major argument against NAFLD screening, since lifestyle changes could only be maintained in a minority of patients and NASH-specific drugs were not even developed. In the meantime, several new drugs acting on various pathophysiological processes in NASH have entered clinical development. Current drug classes being investigated for NASH treatment are agonists of nuclear receptors such as FXR agonists (including FGF19), peroxisome proliferator-activated receptors agonists, chemokine receptor inhibitors, thyroid hormone receptor-β agonists and analogs of enterohepatic hormones such as GLP-1 and FGF21 or SGLT2 inhibitors[33]. Despite disappointment by negative interim results from three out of four recent phase 3 trials, the process of approval is ongoing for obeticholic acid as the only drug with a significant benefit in the phase 3 interim analysis. Obeticholic acid is an obvious candidate for the first conditional approval as a NASH therapeutic in the near future. However, even before approval of new drugs, NAFLD patients "at risk" should be offered to participate in ongoing clinical trials, particularly those with drug combinations, since the future will putatively be a more efficient combination therapy of two different drug classes with complementary effects[33].

Cost-effectiveness of NAFLD screening

Decisions on the target population for screening are mostly driven by cost-effectiveness and depend on the prevalence of the disease in the target population and health outcomes measured as quality-adjusted life-years (QALYs). Unfortunately, the cost-effectiveness of noninvasive liver tests in NAFLD is scarcely available in the literature.

However, the cost-effectiveness of noninvasive screening for alcohol-related liver fibrosis has been investigated in more detail[34]. For low prevalence populations, a screening strategy involving a blood-based noninvasive fibrosis test (ELF) in the first-line follow-up with LSM in intermediate- or high-risk individuals in the second-line follow-up was most cost-effective, both short- and long-term, depending on whether diagnostic testing had lasting or temporary effects on abstinence rates. The study documents that the effect of screening measures strongly depends on the therapeutic options and the size of the treatment effect. Moreover, for high-prevalence populations, direct referral to LSM was highly cost-effective.

In contrast to the growing burden of disease, a cross-sectional study of the public health response to NAFLD among experts in 29 European countries in 2018 and 2019 revealed a general lack of national policies, awareness campaigns and civil society involvement and only a few epidemiological registries[35]. Only one-third of the countries reported having national recommendations for NAFLD screening in all patients with diabetes, obesity and/or metabolic syndrome.

Data on cost-effectiveness need to be interpreted in the context of the national health system, economy and availability of treatment. Nevertheless, available data for certain diagnostic measures allow at least some general insight and can be used as part of evidence-informed decision making. As the most basic diagnostic method, ultrasonography screening for NAFLD has been found to be cost-effective in Thailand for patients with metabolic syndrome participating in an intensive weight reduction program when compared with no screening[36]. Differences in the age of the target population have been observed, since screening before 45 years was cost saving, while screening at 45 to 64 years was cost-effective.

The cost-effectiveness of LSM by TE has only been assessed in comparison to liver biopsy as the invasive reference method. In a systematic analysis covering four cost-effectiveness and four cost-utility studies[37], high-quality cost-effectiveness studies suggested that TE is less costly but also less accurate than liver biopsy (which is not surprising since histology is still regarded as the diagnostic gold standard). The incremental cost-effectiveness ratio (ICER) of TE improves with a greater level of diagnostic accuracy and a higher degree of liver fibrosis. Similar data have been obtained in a Canadian systematic review of existing TE cost-effectiveness studies from the perspective of the Ontario Ministry of Health and Long-Term Care[38]. For a primary economic evaluation, decision analytic models were used to compare short-term costs and outcomes of TE compared to liver biopsy. Again, data suggested that TE leads to cost savings but is less effective than liver biopsy in the diagnosis of liver fibrosis. Of note, TE became more economically attractive in a high-risk population with a higher degree of liver fibrosis. No studies have assessed the cost-effectiveness of TE with controlled attenuation parameter (CAP)-based fat quantification for the diagnosis of liver steatosis.

It remains open whether NAFLD screening can become cost-effective in the near future with a further increasing number of at-risk NAFLD patients in Western countries. Investigators from six prospective cohorts in Europe and Asia used patients with mostly alcohol-related liver disease to explore the cost-effectiveness of TE as a screening method to detect liver fibrosis against standard of care in a primary care pathway[39]. In 6295 participants, TE with the proposed cutoffs for the diagnosis of significant fibrosis (≥ F2) of 9.1 kPa in general population settings and 9.5 kPa in at-risk populations outperformed fibrosis scores in terms of accuracy. Screening with TE was cost-effective, with mean ICER ranging from 2570 €/QALY for a population at risk of alcohol-related liver disease (age ≥ 45 years) to 6217 €/QALY in the general population [39]. Overall, there was a 12% chance of TE screening, even though it was cost saving across countries and populations. This study clearly documents that screening for liver fibrosis with TE can be a cost-effective intervention for European and Asian populations, even in primary care, and may even be cost saving.

For various other screening tools, a comparative cost-utility model analysis of different annual noninvasive screening strategies has been conducted in Canada using a third-party payer perspective in a general population compared to screening in a high-risk obese or diabetic population[40]. The investigated screening algorithms involved the NFS, cytokeratin-18, TE and acoustic radiation force impulse (ARFI) imaging for detecting advanced fibrosis (≥ F3). Liver biopsy and magnetic resonance elastography were compared as confirmation methods. Compared with no screening, screening in high-risk obese or diabetic populations was more cost-effective than in the unselected general population. Interestingly, liver biopsy confirmation was not found to be cost-effective. These data suggest that annual NASH screening can be cost-effective in high-risk obese or diabetic populations in a Western country.

Using a different simulation model in the United States, the effectiveness and cost-effectiveness of US screening for NAFLD followed by liver biopsy has been assessed for type 2 diabetic patients[41]. In this more basic NASH screening strategy, all patients received a one-time screening US, individuals with hyperechogenicity on US underwent subsequent liver biopsy, and those found to have NASH received medical therapy to decrease disease progression. Screening for NASH decreased the number of individuals who developed cirrhosis by 12.9% and resulted in an 11.9% reduction in liver-related deaths. However, the screening strategy resulted in only 0.02 fewer QALYs due to the disutility associated with treatment and was dominated by the "no screening" strategy[41]. The impact of treatment efficacy and treatment-related side effects became clear in this study because when the model excluded the treatment-related quality-of-life decrement, screening became cost-effective. This study documents that treatment-associated side effects are relevant for quality of life and impact QALYs and the suitability of screening.

Referral strategies between primary care and secondary care by specialists have also been investigated. Given the high prevalence of NAFLD in Western countries, the optimal evaluation of NAFLD likely involves triage by a primary care physician (PCP) with advanced disease managed by gastroenterologists or hepatologists. Screening in a cohort of 10000 simulated United States-American patients with NAFLD performed in either PCP or referral clinics was simulated[42]. Risk stratification by the PCP using the NFS alone costs approximately 20% more *per* QALY than usual care costs. In the microsimulation, at a willingness-to-pay threshold of $100000, the NFS alone in the PCP setting was the most cost-effective strategy in 94.2% of samples, followed by the combination NFS/vibration-controlled transient elastography in the PCP setting (5.6%) and usual care in 0.2%[42]. This study indicates that risk stratification of pa-

tients with NAFLD in primary care is a cost-effective strategy that should be further explored in clinical practice.

Finally, the outcome of the entire diagnostic chain is relevant for decision making upon screening. This certainly includes the likelihood of referral to the specialist after obtaining a risk surrogate (which is often moderate at best), the availability of effective drugs for the target disease (in case of NASH to be established) and relevant side effects of the treatment impacting quality of life. Taking into account the emerging awareness campaigns among the public and PCPs and ongoing phase 3 treatment studies for NASH patients, it is likely that the impact of screening on the overall outcome could improve over the near future.

WHO TO SCREEN?

NAFLD is an asymptomatic disease in the early phase, often leading to a late diagnosis [43]. In a large population-based, cross-sectional study from Barcelona, the authors found elevated liver stiffness (as defined with TE > 6.8 kPa) in 9% of the participants, and NAFLD was the leading etiology (followed by alcohol risk consumption)[44]. Risk factors for elevated liver stiffness included obesity, type 2 diabetes and the presence of metabolic syndrome (each with a prevalence of elevated liver stiffness in 20%–30%). This study convincingly underlines the importance of NAFLD in the general population but especially in the known risk groups. While the prevalence of NAFLD in the general population is quite high (20%-30%), only approximately 7%-10% of NAFLD patients develop relevant complications of this disease, such as advanced fibrosis, cirrhosis or hepatocellular carcinoma (HCC)[45,46] (Figure 1). Thus, screening the entire population cannot (yet) be justified because too many patients would suffer overdiagnosis and possibly overtherapy. For advanced testing or invasive diagnostic measures such as liver biopsy, which applies to a selected patient population of still 3%-5%, primary testing to rule out low-risk individuals appears mandatory.

These numbers from the general population, however, do not apply to patient groups with increased NAFLD prevalence and increased risk for advanced disease. In the presence of the risk factors diabetes and obesity, the prevalence of NAFLD increases to 75%[47,48]. Diabetes and obesity are clear independent risk factors for the development of NASH-related fibrosis[46,47] and other factors of the metabolic syndrome are closely associated[49]. In addition, patients with these underlying diseases are more likely to develop complications of NAFLD[48]. Consequently, screening in the group of patients with these risk factors for complications is particularly important[50]. Elevated liver enzymes alone are sufficient as a reason for screening but are not sufficient as a sole decision criterion, as relevant NAFLD with fibrosis or cirrhosis may be present even with normal transaminases[51-53].

These facts warrant screening of this risk population[54], especially at higher HbA1c levels[54]. In some cohorts, patients with NAFLD also had an older age > 50 years in addition to the above risk factors[55-57], and an increased prevalence of NAFLD and advanced fibrosis has been shown in men[57]. These risk factors reflect quite well the collective for which screening for NAFLD is repeatedly discussed in the current literature or even concrete recommendations exist[6,7,28,29].

NAFLD is linked to several other diseases and is connected to metabolic disturbances. It is straightforward to consider the presence of NAFLD in patients with such concomitant diseases, one of the most important being coronary heart disease. Additionally, NAFLD should also be considered, depending on the advancement of the respective disease, in diseases such as polycystic ovary syndrome, sleep apnea, hypothyroidism, depression, renal insufficiency or psoriasis[7,58-60]. Making a specific screening recommendation for these patients is probably not warranted at this time; further risk profiles are needed here to justify such screening in selected patient groups with these diseases.

General screening of close relatives is also not reasonable despite some familial clustering and genetic factors (*e.g.*, *PNPLA3*[61]) that may influence the course of NAFLD. The penetrance of these genetic risk factors is too low to justify screening in the presence alone (RR 3.26 for the histological presence of NAFLD *per* effect allele [62]). However, relatives with the presence of the abovementioned risk factors should definitely be screened for the presence of NAFLD[6]. Screening with diabetes type 2 as a central risk factor again has very recently been shown to be cost effective in the United States by avoiding advanced liver-specific disease and endpoints (all calculated screening models based on US and AST, with an ICER between $17000 and $35000/ QALY[63], see also *Cost-effectiveness of NAFLD screening*).
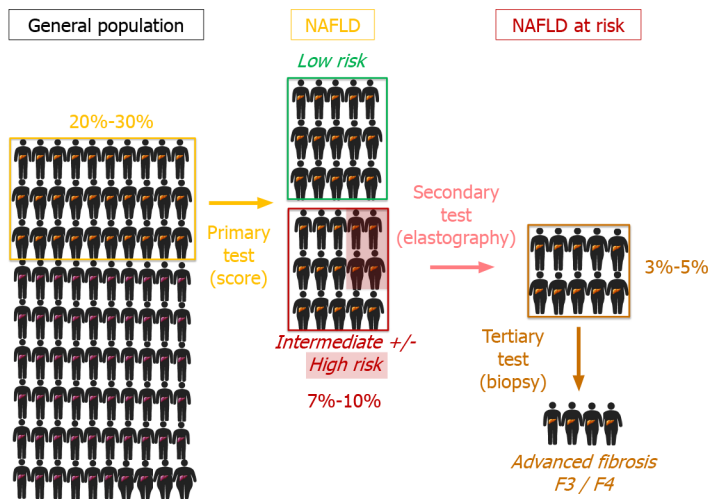
Figure 1 Nonalcoholic fatty liver disease patient proportions according to risk assessment. Stepwise enrichment of nonalcoholic fatty liver disease (NAFLD) patients at risk for advanced fibrosis using a three-step strategy with score-based primary testing in a subgroup of the general population at risk for NAFLD and elastometric secondary testing to identify candidate patients for liver biopsy represents the third and final step in most algorithms. Patients with a diagnosis of NAFLD by either surrogate scores or ultrasound (20%-30% of the general population) are divided into low-risk *vs* intermediate-to-high-risk subgroups (the latter 7%-10% of the general population). After elastometry testing, half of these subjects can be assigned to a high likelihood of advanced fibrosis F3/F4 and should be subjected to liver biopsy. NAFLD: Nonalcoholic fatty liver disease.

WHO SCREENS?

A decision about who carries out screening is determined by the care structures of a particular health care system rather than by the efficacy of particular screening procedures. Even if certain diagnostic procedures proved to be cost-efficient for screening (*e.g.*, LSM as shown above in section 3), the lack of a broad availability of LSM-determining procedures may preclude its application. Consequently, more broadly available blood-based tests are needed, and the design of a screening algorithm must then be aligned with the capabilities of those performing the screening[64-66].

In many countries, almost all patients are primarily cared for by PCPs. A certain proportion of patients defined in the at-risk population (see above) are assigned to specialists (diabetologists/endocrinologists, cardiologists), but numerous patients with diabetes mellitus, obesity, and arterial hypertension are also treated exclusively by PCPs (*e.g.*, in the context of so-called disease management programs). In Europe, screening algorithms are implemented in a total of only 5 countries and are located in the primary health care sector in all of these countries (Belgium, Denmark, Czech Republic, Slovakia, and United Kingdom[35]). However, there are sometimes considerable structural differences in the health care systems of these countries.

Due to access to patients, comprehensive risk population screening in many countries can only be in the hands of PCPs, possibly supported by diabetologists and cardiologists. This group of physicians is particularly suited to broadly identify the major risk diseases for NAFLD and thus to determine the individual NAFLD risk in these patients[67]. This assessment is also in line with existing EASL recommendations [6] and a recently developed algorithm for general practitioners and diabetologists [68]. Direct referral of all patients at risk to hepatologists is not feasible. The need for a screening filter at the primary care level to prevent unnecessary referrals to specialists is shown by data from England ("Camden and Islington NAFLD pathway"[69]) and the United States[70]. In both studies, almost 90% of unnecessary referrals could be avoided by structured screening at the primary care provider level. On the other hand, in an American study, more than 25% of NAFLD patients referred to a hepatologist without screening already had advanced fibrosis (characterized as at least F3 with TE measurement[54]).

Data on awareness of NAFLD at GP level are rare. In the United States, data from the United States Veteran Affairs Database showed that NAFLD is significantly underdiagnosed in primary care patients[71]. Patients with abnormal alanine aminotransferase (ALT)/glutamate pyruvate transaminase (GPT) without other known liver disease (viral hepatitis and alcohol use were largely excluded by data analysis) were detected in only 40% of cases in this study, received a suspected diagnosis of NAFLD in only 21%, received therapeutic counseling in only 15% and were referred to a specialist in only 3% of cases. Initially, there is no reason to assume that the situation

in other countries differs significantly from these results. A study by the professional association of gastroenterologists in private practice in Germany (bng) showed for a cohort of NAFLD patients in secondary care that approximately 10% of these patients already had advanced fibrosis according to FIB-4 screening, but even these patients were not consistently counseled or guided regarding therapy[55]. Only 27% of patients with presumed advanced fibrosis in this study received nutritional counseling. In this respect, education and training activities for PCPs are definitely necessary to increase awareness of the presence and risks of NAFLD and to create acceptance for screening. Diabetologists and cardiologists should also be included by these measures, as they should also be involved in screening due to their spectrum of patients they treat.

Integration of primary care identification of patients at risk for the presence of NAFLD, particularly with advanced fibrosis, into secondary testing facilities at a specialist setting is a crucial issue for the overall efficacy of a screening algorithm (Figure 2). Dedicated elastography platforms have been established at several places, such as in our own center[72]. The likelihood of referral of "intermediate or high risk" individuals to secondary care, the proportion of subjects with "indeterminate" test results (the so-called "gray zone" of respective score-based tests) and the availability of advanced testing platforms for referral are relevant factors at this interface. As pointed out, existing or emerging networks between PCPs and specialists are key to optimizing a bidirectional transition into secondary testing and, in case of "low risk", back to long-term observation and basic treatment in a primary setting.

HOW TO SCREEN?

Value of transabdominal ultrasonography of the liver in NAFLD

US is a widely available, cost-effective, radiation-free method that allows assessment of hepatic fatty degeneration[73]. Hepatic fatty degeneration results in an increase in the echogenicity of the liver parenchyma (*e.g.*, compared with the renal parenchyma). US is thus suitable as a screening method for NAFLD. However, steatosis below 10% of hepatocytes is not detected, and up to 20% is unreliably detected[74] (especially with microvesicular fatty degeneration). In moderate and severe hepatic steatosis, good sensitivity (85%-96%) is achieved with specificity up to 98%[75]. The best results are seen above a liver fat content of 12.5%, where AUROC values under consideration of different echographic parameters reached comparable results to H-magnetic resonance spectroscopy (MRS)[76]. With the above referenced threshold, exclusion of steatosis by US is not completely possible. With regard to possible fibrosis of the liver, US diagnostics do not allow reliable determination and staging[73].

Noninvasive measurement of hepatic steatosis and fibrosis by elastography

US-based shear wave elastography techniques are well suited as a method for measuring liver stiffness to detect or exclude advanced liver fibrosis and cirrhosis in NASH. In addition, FibroScan, for example, now also offers the possibility of quantifying the fat content of the liver *via* the measurement of additional parameters.

The CAP measurement integrated in the FibroScan achieved AUROC values between 0.7[77] and 0.84[78] in studies with more than 400 patients each for (histologically confirmed) steatosis of > 33% and > 66%.

Different elastography techniques are now available on the market, and a differentiated overview cannot be given here but is available elsewhere[79]. While TE using FibroScan requires the purchase of a dedicated device, other techniques, such as ARFI imaging (Siemens), Elast-PQ (Philipps), and supersonic shear-wave elastography (SWE, Aixplorer), offer the advantage of being integrated into routine US equipment [73].

In large cohorts from Europe and Asia, the reliability of TE, its superiority over fibrosis scores, and even its cost-effectiveness have been demonstrated, at least for certain at-risk populations, in determining liver fibrosis of different origins[39]. TE is also well suited for quantifying fibrosis in NAFLD. Here, sensitivity, specificity, and AUROC values improve as fibrosis progresses, reaching values of approximately 92% and 0.89 for cirrhosis (F4), respectively[77,80]. Difficulties in estimating fibrosis in obese patients with the normal (M) probe[81] were countered by the company's introduction of an XL probe for particularly obese patients, which provides reliable values and is automatically chosen if the patient has appropriate physical conditions [82,83].

Apart from slight differences in patients with different body types, the diagnostic value of the different elastography methods in determining liver fibrosis in NAFLD
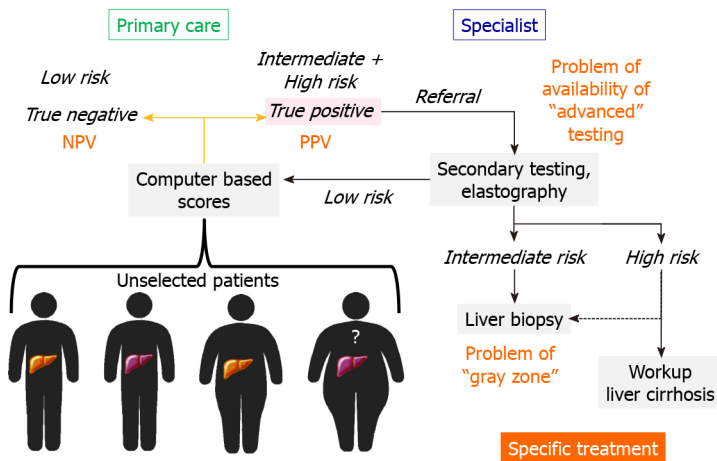
Figure 2 Linking primary care to hepatology. Unselected patients from the general population are most likely in contact with primary care. In primary care, patients at risk for the presence of nonalcoholic fatty liver disease and according to computer-based scores at risk for advanced fibrosis should be transferred into secondary testing facilities at a specialist setting. Critical for the overall efficacy of a screening algorithm are the likelihood of referral of "intermediate or high risk" individuals to secondary care, the proportion of subjects with "indeterminate" test results ("gray zone" of score-based tests) and the availability of advanced testing platforms for referral. NPV: Negative predictive value; PPV: Positive predictive value.

patients appears to be similar. Several studies with different populations and study designs yielded similar AUROC values for TE, SSI, ARFI, and 2D-SWE[84-86]. However, problems with a tendency to overestimate fibrosis occurred in bariatric, extremely obese (median BMI 47 kg/sqm) patients for both TE and ARFI, where the ELF score was actually superior to these two elastography methods[87]. Nevertheless, the procedures should also be well suited for screening most patients. The availability of the methods is very heterogeneous, so broad screening with elastography is currently not possible.

Value of magnetic resonance imaging and computed tomography in the diagnosis and screening of NAFLD

The availability of computed tomography (CT) is bound to institutions with large medical devices but is well reproducible and reliably determines the fat content of the liver by measuring organ density[73]. In a meta-analysis comparing different radiological methods, CT performed rather modestly with a sensitivity of 46%-72%[88]. At least moderate hepatic fatty degeneration can be diagnosed if the density ratio of the liver and spleen on native CT has a cutoff value > 1.1[89]. Dual-energy CT has been able to show promising results for quantifying fat content in the liver in smaller cohorts, even in comparison with magnetic resonance imaging[90]. However, such techniques are poorly validated and not widely available. Overall, CT should not be used as a primary screening method for detecting NAFLD because of its cost, lack of broad availability, and substantial radiation exposure.

Magnetic resonance imaging (MRI), though also a large medical device, is a radiologic imaging modality without any radiation exposure. Certain modalities of MRI can be used to determine both the fat content of the liver and the fibrosis stage quite reliably[73]. MR-based quantification of liver fat content using proton density fat fraction (PDFF) has high linearity and precision with simple postprocessing[91], but it is also not suitable for screening large risk groups because of cost and effort[92]. Compared with histology as a reference standard and in comparison to CAP, PDFF-based determinations have a higher diagnostic accuracy for detecting steatosis (histological grade 1-3) with an AUROC of 0.96 up to 0.99, a sensitivity of 96%, and a specificity of 100%[93,94]. MRS has the highest accuracy for fat assessment in the literature[88,92,95] but is currently limited to research centers due to a lack of standardization of methodology and high costs for hardware and software requirements [73].

MR elastography measures liver stiffness significantly more reliably than US-based elastography techniques[85,96]. In a biopsy-controlled study of 100 patients, an AUROC of 0.98 was achieved at 40 Hz[97]. A joint analysis from 12 studies with over 900 patients still showed summary AUROC values of 0.93-0.95[98]. MR elastography also correlated better to clinical fibrosis parameters and scores than TE[99] but remains restricted to specialized centers[92].

Multiparametric MRI with determination of fat content (by PDFF or spectroscopy) and fibrosis (by MR elastography) was superior to the respective FibroScan-based non-MR methods (CAP for steatosis and TE for fibrosis) in a comprehensive new study [100] and cost-effective for risk stratification of NAFLD in a United Kingdom study [101]. Nevertheless, these methods are not (yet) suitable for broad screening due to lack of availability and high costs.

Laboratory chemistry scores

Because screening must be performed primarily by PCPs, screening tools must be widely available, inexpensive, and noninvasive[58,66,67]. This allows screening to be performed on a day-to-day basis and, more importantly, increases the acceptance of screening by the physicians performing it. The two-step design with the verification of steatosis and fibrosis risk improves the specificity (and in some cases even the sensitivity) of screening[65,102]. Positively screened patients must be transferred to a hepatologist for further evaluation. In this context, the proportion of positively screened patients should not be too large to avoid overloading hepatologists[65,103]. The extent of the diagnostic "gray zone" is of particular importance in this regard and can vary substantially from test to test. In any case, however, patients with prolonged or repeated elevations of GPT/ALT should be referred for further evaluation (as is usually the case), as they are generally at increased risk for liver disease or injury[51, 104,105].

There are significant differences between different countries and health care systems in the availability and cost-effectiveness of different screening tools. However, despite the limited sensitivity of US, this procedure is an attractive screening option for PCPs because of its ease of performance. More technically sophisticated and sensitive procedures such as CAP or elastography are generally not available at this level of care.

Steatosis scores correlate with insulin resistance. Their diagnostic performance for steatosis depends on the degree of fatty degeneration, fibrosis, and inflammation[106]. Assuming at least moderate steatosis is relevant, the performance of the fatty liver index (FLI) and NAFLD liver fat score is best, with the highest AUROC values with a positive predictive value of 99%, but without safe exclusion of steatosis below the cutoff[106-108]. Only the FLI can easily be obtained from routine values in family practice (see Table 1) and should therefore be used when US is not feasible[109].

Fibrosis scores also vary in both availability and quality of information. In this regard, the sensitivity and specificity of each score for significant fibrosis, advanced fibrosis, and cirrhosis are quite different and additionally vary depending on the population screened (population screening *vs* high-risk screening *vs* screening of confirmed NAFLD)[110]. Scores that require the determination of expensive specialty laboratory parameters are not suitable for primary care screening, nor are scores that include, at least in part, unavailable laboratory parameters or instrumental procedures. Although these special scores are superior to routine scores, as expected[111], and would also improve specificity in combination with them[112], the lack of availability and the lack of acceptance of these special scores by general practitioners, based in part on complicated determination, hinder their widespread use. This applies, for example, to the ELF test[113] (hyaluronic acid, TIMP-1, and procollagen peptide), which is of similar prognostic value to liver biopsy[114], and the fibrometer VCTE test (with elastography), which is also superior to purely laboratory chemistry-clinical indices [24].

Scores with readily available routine parameters for fibrosis risk include NFS, FIB-4 score, APRI score, Forns score, and BARD score. The first two (NFS, FIB-4) are superior to the last three (APRI, Forns, BARD) in screening fibrosis in the NAFLD cohort[115,116]. In a recent systematic review, this could be confirmed, especially for the hardest endpoint (mortality)[117]. These two scores (FIB-4 and NFS) are also suitable for screening patients with normal ALT[118] and can be easily determined *via* internet-based calculators.

In population screening, all scores have significant weaknesses and are therefore of limited use for this question[110]. However, the discriminatory performance of all tests is significantly better in high-risk collectives[110]. Although the FIB-4 score was initially developed for the detection of hepatitis C virus fibrosis[119], it has since been validated[120] and compared[121] in NAFLD collectives and may be considered suitable in principle for liver fibrosis of other etiologies. The FIB-4 score has an additional advantage over the NFS in that no albumin value is needed and that the proportion of intermediate tested patients is somewhat smaller[65,116]. However, both scores have lower specificity in patients > 65 years of age[122], which may increase the referral rate to the specialist due to a higher proportion of false-positive screened

Table 1 Scores for diagnosing steatosis and fibrosis with parameters used

Scores for Steatosis	Routine parameters											Special parameters		
	AST	ALT	yGT	Platelets	TG	Bilirubin	BMI	Waist	Age	Sex	Diab.	A2M	HA	Other
FLI			X		X		X	X						
HSI	X	X					X			X	X			
Steato-Test		X	X		X	X			X	X	Gluc	X		Apo-A1, Haptoglobin, Cholesterol
NAFLD-LFS	X	X									X			Insulin
VAI					X		X	X						
TyG					X						Gluc			
Scores for fibrosis														
NFS	X	X		X			X		X		X			Albumin
FIB-4	X	X		X					X					
APRI	X			X										
ELF													X	PIIINP, TIMP-1
Fibrotest		X	X			X						X		Haptoglobin, Apo-A1
Fibrometer (V2G) ((V3G))	X		((X)), for HA	X					X	(X)		X	X	Prothrombin, Urea
NIKEI	X	X				X			X					

New fibrometer versions (V2G, V3G) and their respective parameters labeled with brackets: (V2G) and ((V3G)). AST: Aspartate-aminotransferase; ALT: Alanine-aminotransferase; yGT: gamma-glutamyltransferase; TG: triglycerides; BMI: Body mass index; Diab.: Diabetes; A2M: Alpha-2-microglobulin; HA: Hyaluronic acid; Gluc: Glucose; PIIINP: Procollagen-III-peptide; TIMP-1: Tissue inhibitor of metalloproteinases I; Apo-A1: Apo-A1-lipoprotein; FLI: Fatty liver index; HIS: Hepatic steatosis index; NAFLD-LFS: Nonalcoholic fatty liver-liver fat score; VAI: Visceral adiposity index; TyG: Triglyceride and glucose index; NFS: NAFLD fibrosis score; FIB-4: Fibrosis-4; APRI: AST-platelet-ratio index; ELF: Enhanced liver fibrosis; NIKEI: Noninvasive Koeln-Essen-index.

patients. Data from a screening study of type 2 diabetes patients show that the use of age-adjusted cutoffs on FIB-4 (in delineating negative *vs* intermediate) reduces the number of patients tested intermediate (from 38.3% to 15.4%[65]). Repeated measurements of laboratory scores could also help to identify patients at risk of severe liver disease in the general population, as was recently shown for repeated measurements of FIB-4 within 5 years[23].

The screening strategy proposed in Figure 3 relies on recent proposals and takes into account the aforementioned prerequisites of high-risk screening by PCPs but may not currently be evidence-based in several areas. In particular, this concerns the handling of the intermediate-risk group, the screening interval in low-risk patients, and the cost-effectiveness of the entire algorithm. In addition, the screening recommendation given requires further education and possibly training of PCPs about the prevalence and prognosis of NAFLD.

CONCLUSION

It is time for NAFLD screening. NAFLD is hard to diagnose in the early phase of the disease. The prevalence of this disease is increasing in countries with Western lifestyles, and the complication rate (inflammation, fibrosis, cirrhosis and HCC) is high in patients with metabolic dysfunction. Additionally, there are inexpensive noninvasive tools for the diagnosis of steatosis and fibrosis, leading to a reliable identification of persons at risk who can be referred to hepatologists. Apart from lifestyle modification, there are evolving drug treatments shortly before approval or in the late phases of clinical trials.
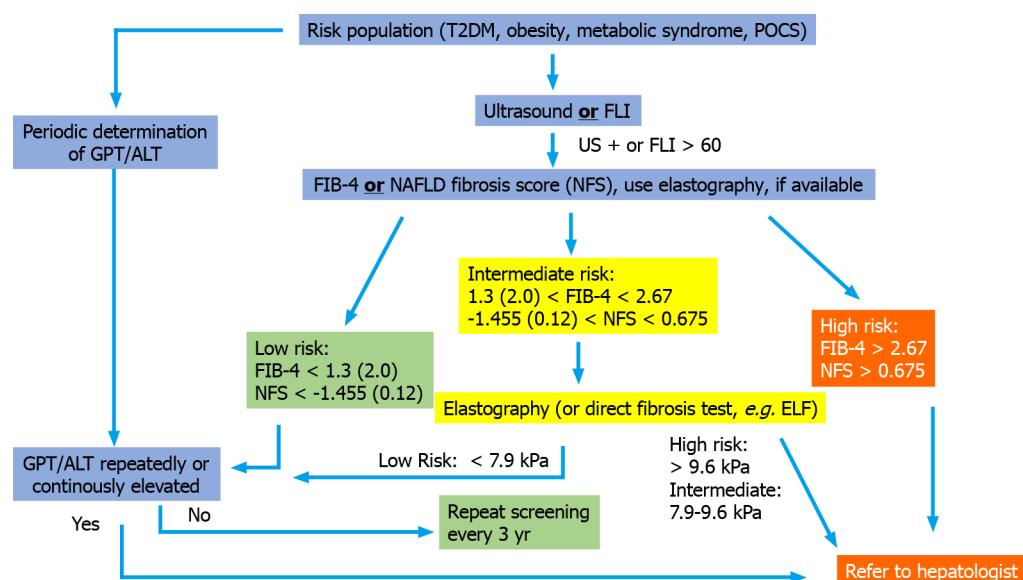
Figure 3 Possible screening algorithm that can be modified according to availability but contains the two main elements (detection of steatosis and fibrosis risk) and can be performed in the primary care physician's office. The algorithm corresponds well to the so-called European algorithm of the EASL-EASD-EASO Clinical Practice Guidelines[6] and to a recently proposed approach for family physicians and diabetologists[68] but is simpler to use. The sequences of fatty liver index and Fibrosis-4 (FIB-4) have been decisively studied for screening in a high-risk population of type 2 diabetes patients[65]. The use of age-adjusted cutoff values (in parentheses) is reasonable to reduce the high proportion of intermediate tested individuals. The sequential use of FIB-4, nonalcoholic fatty liver disease fibrosis score or enhanced liver fibrosis in the intermediate group has not been investigated in studies so far, but there are first studies on the basic sequential use of noninvasive fibrosis scores[123]. FLI: Fatty liver index; FIB-4: Fibrosis-4; T2DM: Type 2 diabetes mellitu; NFS: Nonalcoholic fatty liver disease fibrosis score; GPT: Glutamate pyruvate transaminase; ALT: Alanine aminotransferase.

Studies show that screening for NAFLD, at least for a risk population, is cost effective and will help to prevent serious hepatic consequences of pandemic metabolic dysfunction. However, it will not be easy to implement comprehensive screening programs in all countries since there are large structural differences between national health systems. For example, the extent of availability of elastography will decide in each country, whether this promising technique can be used in broad screening approaches or whether US and lab scores will be necessary for PCPs to conduct screening for NAFLD. Therefore, each screening algorithm (as the one depicted in Figure 3) should be adapted locally depending on the broad availability of methods for detecting steatosis and fibrosis. Additionally, the screening population (*i.e.* the patients with an amount of risk factors high enough for qualifying for the screening program) has to be determined in each country individually depending on the epidemiology of NAFLD in this country.

So what is to be done? We have to increase awareness for NAFLD and its consequences in the population and in primary care. National professional gastroenterology and hepatology societies have to develop guidelines for screening programs depending on the structure of the population and health care system of their respective country. National health systems must implement reimbursement for the tools needed for reliable screening. Hepatologists should prepare for rising numbers of patients referred for risk stratification and specific counseling.

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Environmental perspectives of COVID-19 outbreaks: A review

Palas Samanta, Apurba Ratan Ghosh

ORCID number: Palas Samanta 0000-0001-9369-7502; Apurba Ratan Ghosh 0000-0003-1454-7720.

Palas Samanta, Department of Environmental Science, Sukanta Mahavidyalaya, University of North Bengal, Dhupguri 735210, West Bengal, India

Apurba Ratan Ghosh, Department of Environmental Science, The University of Burdwan, Burdwan 713104, West Bengal, India

Corresponding author: Palas Samanta, PhD, Assistant Professor, Department of Environmental Science, Sukanta Mahavidyalaya, University of North Bengal, Dhupguri, Jalpaiguri, Dhupguri 735210, West Bengal, India. samanta.palas2010@gmail.com

Abstract

The coronavirus disease 2019 (COVID-19) pandemic, caused by the novel virus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), began in December 2019 in China and has led to a global public health emergency. Previously, it was known as 2019-nCoV and caused disease mainly through respiratory pathways. The COVID-19 outbreak is ranked third globally as the most highly pathogenic disease of the twenty-first century, after the outbreak of SARS-CoV and Middle East respiratory syndrome in 2002 and 2012, respectively. Clinical, laboratory, and diagnostic methodology have been demonstrated in some observational studies. No systematic reviews on COVID-19 have been published regarding the integration of COVID-19 outbreaks (monitoring, fate and treatment) with environmental and human health perspectives. Accordingly, this review systematically addresses environmental aspects of COVID-19 outbreak such as the origin of SARS-CoV-2, epidemiological characteristics, diagnostic methodology, treatment options and technological advancement for the prevention of COVID-19 outbreaks. Finally, we integrate COVID-19 outbreaks (monitoring, fate and treatment) with environmental and human health perspectives. We believe that this review will help to understand the SARS-CoV-2 outbreak as a multipurpose document, not only for the scientific community but also for global citizens. Countries should adopt emergency preparedness such as prepare human resources, infrastructure and facilities to treat severe COVID-19 as the virus spreads rapidly globally.

Key Words: COVID-19; SARS-CoV-2 virus; Environmental perspectives; Epidemiological characteristics; Public health; Emergency preparedness

Core Tip: This review is the first attempt to integrate coronavirus disease 2019 (COVID-19) outbreaks (monitoring, fate and treatment) with respect to environmental and human health perspectives. Briefly, the paper systematically addresses the environmental aspects of the COVID-19 outbreak such as the origin of severe acute respiratory syndrome coronavirus 2, epidemiological characteristics, diagnostic methodology, treatment options and technological advancement for the prevention of COVID-19 outbreaks.

INTRODUCTION

A series of patients with unidentified pneumonia, caused by β-coronavirus, was reported in late December 2019 in Wuhan (Hubei Province), China. Coronavirus disease 2019 (COVID-19) outbreaks are clinically very similar to viral pneumonia. A number of experts from the PRC Centers for Disease Control declared that this respiratory disorder (alternatively known as novel coronavirus pneumonia, NCP) was caused by a novel coronavirus[1]. The World Health Organization (WHO) initially named the disease as 2019-nCoV (2019-novel coronavirus) on January 12, 2020. It was officially later named COVID-19 on February 11, 2020 by the WHO. On the same date, the International Committee on Taxonomy of Viruses named the virus as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) after developing the genome sequence from a COVID-19 patient in Wuhan on January 7, 2020. The virus belongs to the β-coronavirus family, which is very prevalent in nature among other families. Similar to other viruses, the SARS-CoV-2 also has many natural hosts including different intermediate and final hosts, which makes it challenging for scientific communities to treat and prevent COVID-19 outbreaks. It has higher transmission and infection potential but causes a lower mortality rate compared with SARS-CoV and Middle East respiratory syndrome (MERS-CoV)[2]. The genomic sequence of SARS-CoV-2 revealed that it has 79.5% and 96% similarity with SARS-CoV and bat coronavirus, respectively[1], which implies that bats might be the source of SARS-CoV-2. Although the COVID-19 outbreak started in China, the virus has spread to over 213 countries with the highest rate of infection in the United States, Italy, France, and Spain among others as per data published by the WHO on December 13, 2020 (Figure 1). There are approximately 202608306 confirmed SARS-CoV-2 cases and 4293591 deaths worldwide. Consequently, COVID-19 has emerged as a global threat to public health and is steadily growing due to human-to-human transmission. Moreover, this transmission also spreads in different environmental sectors such as water, air, soil, sewage and fecal matter[3]. Additionally, this process is accelerated by a number of meteorological factors namely temperature, weather, humidity and air quality parameters including particulate matter, SOx, NOx and carbon, *etc*. Therefore, a better understanding of the global consequences of COVID-19 is required with regard to environmental perspectives. Accordingly, this review will address the origin of SARS-CoV-2, route of transmission, pathogenesis, epidemiological characteristics, diagnostic methodology, treatment options and technological advancement for the prevention of COVID-19 outbreaks with regard to environmental perspectives in order to acquire the latest understanding of this new infectious disease of which certain immediate as well as long-term remedial measures can be explored.

EPIDEMIOLOGY OF THE COVID-19 OUTBREAK

Origin of the COVID-19 outbreak

SARS-CoV-2 is a β-coronavirus and is enveloped with non-segmented Orthocoronavirinae subfamily RNA[4]. Among the four genera, γ- and δ-CoV infect birds while α- and β-CoV infect mammals including humans (Table 1). The α- and β-CoV have six

Table 1 Details of coronavirus (genus, species and receptor)

Genus	Species	Targets	Receptor
α-CoV	Alphacoronavirus 1:	Mammals	
	Feline coronavirus serotype 2		Aminopeptidase N
	Canine coronavirus serotype 2		Aminopeptidase N
	Transmissible gastroenteritis virus		Aminopeptidase N
	Human coronavirus 229E		Aminopeptidase N
	Human coronavirus NL63		ACE2
	Porcine epidemic diarrhea coronavirus		Aminopeptidase N
	Rhinolophus bat coronavirus HKU2		
	Scotophilus bat coronavirus 512/05		
	Miniopterus bat coronavirus 1		
	Miniopterus bat coronavirus HKU8		
β-CoV	Betacoronavirus 1:	Mammals	
	Bovine coronavirus		Neu 5,9 Ac2
	Human coronavirus OC43		Neu 5,9 Ac2
	Equine coronavirus		
	Human enteric coronavirus		
	Porcine haemagglutinating encephalomyelitis virus		
	Canine respiratory coronavirus		
	Murine coronavirus:		
	Mouse hepatitis virus		CEACAM1
	Rat coronavirus		
	Puffinosis virus		
	Hedgehog coronavirus 1		
	Human coronavirus HKU1		
	Middle East respiratory syndrome-related coronavirus		
	Pipistrellus bat coronavirus HKU5		
	Rousettus bat coronavirus HKU9		
	Severe acute respiratory syndrome-related coronavirus		
	SARS-CoV		
	SARS-CoV-2		ACE2
	Rhinolophus bat viruses		
	Tylonycteris bat coronavirus HKU4		
γ-CoV	Avian coronavirus:	Birds	
	IBV (turkey, pheasant, duck, goose and pigeon)		
	Beluga Whale coronavirus SW1		
δ-CoV	Bulbul coronavirus HKU11	Birds	
	Thrush coronavirus HKU12		
	Munia coronavirus HKU13		
	Porcine coronavirus HKU15		

ACE2: Angiotensin-converting enzyme 2; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2.

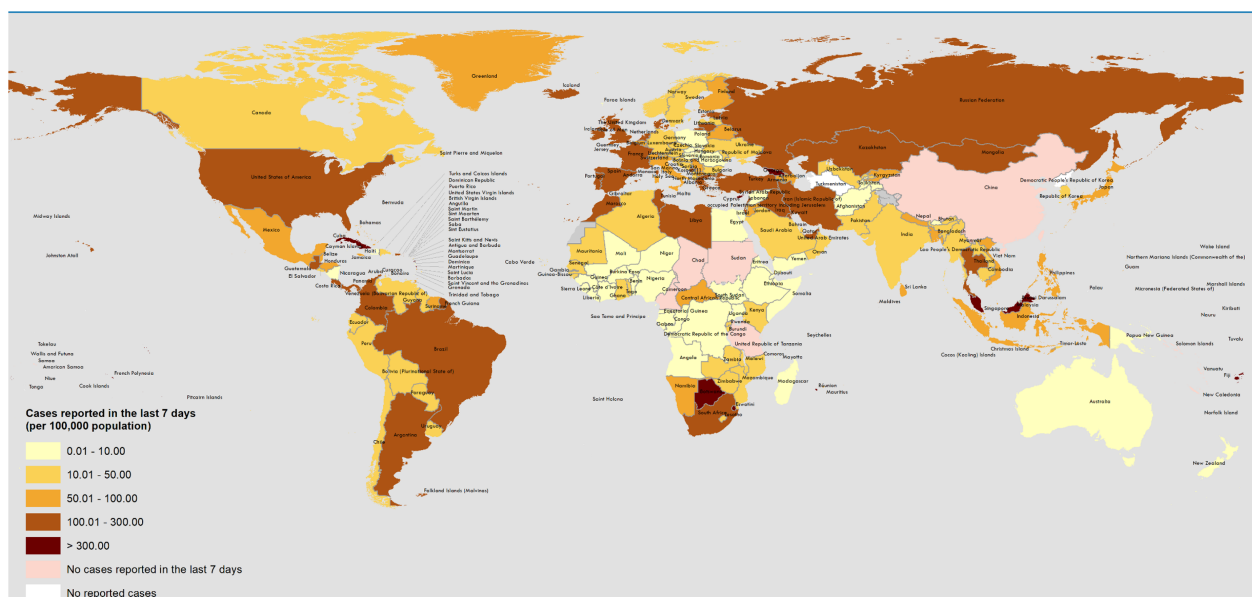variants. Among them α-CoVs variants (HCoV-229E and HCoV-NL63), and β

Figure 1 Geographical distribution of coronavirus disease 2019 outbreaks. Source: https://www.who.int/publications/m/item/weekly-epidemiological-update-on-covid-19---10-august-2021 (data as reported at 4:58 pm CET on August 10, 2021).

-CoVs variants (HCoV-HKU1 and HCoV-OC43) have lower pathogenic capability in humans and cause mild respiratory symptoms similar to the common cold. Only β-CoVs variants (SARS-CoV and MERS-CoV) have severe pathogenic capability in humans. This pandemic started in Wuhan specifically in a seafood wet market, on December 12, 2019. Several studies have demonstrated that bats are natural hosts of SARS-CoV-2 and animals such as snakes, turtles and pangolins are intermediate hosts of SARS-CoV-2.

Previously, snakes were thought to be involved in COVID-19 outbreaks by Ji *et al*[5] but this hypothesis was rejected by Zhang *et al*[6] who did not find any similarity in genome sequence between snakes and COVID-19 patients. In another study, researchers found an approximately 96.2% genome sequence similarity between SARS-CoV-2 and bat coronavirus (CoV RaTG13)[7]. In addition, the genomic sequence of SARS-CoV-2 matched with 79.5% of the genome sequence of SARS-CoV[8]. These findings implied that bats were the suspected source of COVID-19 outbreaks as well as the natural host of this virus. The virus was finally transmitted to humans *via* unknown intermediate hosts from bats. However, few bats are sold in the Wuhan seafood market[9]. Accordingly, scientists are trying to determine the intermediate sources such as snakes, turtles and pangolins. Xu *et al*[10] found approximately 99% genomic similarity between SARS-CoV-2 and pangolins. Furthermore, they revealed that pangolins are the potential intermediate host of SARS-CoV-2. Apart from these studies, to date there is no adequate evidence on the virus origin regarding potential intermediate hosts and the natural host of SARS-CoV-2. Therefore, SARS-CoV-2 could use angiotensin-converting enzyme 2 (ACE2), similar to SARS-CoV receptor for human infection[7]. However, there is controversy regarding the infectious potential of COVID-19 patients to transmit the disease during the incubation period. Recently, the WHO reported that cats may be the carrier of this virus, whereas other domestic animals like ducks, hens and dogs may not be carriers of this deadly virus.

Transmission of COVID-19

The animal-human interface is not a new concept. Zoonotic diseases with a wildlife reservoir have long been recognized as significant public health problems. Indeed, up to three-quarters of infectious diseases that cause human infections are known to be zoonotic[11]. Apart from this, the complexity of animal, human, and environmental factors is thought to play a critical role in its emergence[12]. On the other hand, contact with infected patients and droplets are considered to be major transmission routes of COVID-19. Aerosol transmission is another important route of SARS-CoV-2 infection. By contrast, SARS-CoV and MERS-CoV transmission are mainly reported through nosocomial transmission. However, human-to-human SARS-CoV-2 transmission occurs mainly through close contact between COVID-19 patients or friends or carriers and between family members including relatives. It can be spread rapidly in

healthcare workers (up to 50%) and patients (62–79%) similar to SARS-CoV and MERS-CoV and is considered the most common route of infection[13]. It is also assumed that consumption of wild animals who are the hosts of SARS-CoV-2 and humans in close contact with these animals are suspected to be the route of entry of SARS-CoV-2 and its mode of transmission. However, this route of SARS-CoV-2 transmission remains controversial and requires further study.

To date, 1 million people around the world have tested positive for this virus, but only 4 cases have so far been reported in which pets showed positive for SARS-CoV-2. These involved 2 dogs and 2 cats, the owners had COVID-19 and are believed to be the most likely source of transmission to their pets. The dogs showed clinical signs, but one of the cats did not have signs of illness. In late March 2020, health officials in Belgium reported that a cat from Liège province had also tested positive for SARS-CoV-2. Nevertheless, the US Centers for Disease Control and Prevention (CDC), WHO, and key animal health organizations have all issued statements aiming to calm people's fears about their pets being a source of the novel virus[14-16]. In this regard, the World Organization for Animal Health has emphasized that "there is no justification in taking measures against companion animals which may compromise their welfare". Furthermore, given the speculation that wild live animal species may be linked to this pandemic, this collaborative approach will also require the expertise of wildlife forensic specialists.

SARS-CoV-2 has also been detected in saliva, the gastrointestinal tract, urine and stool. In particular, the gastrointestinal tract or digestive tract has been recognized as another route of SARS-CoV-2 infection based on a bioinformatics study[17]. SARS-CoV-2 has been detected in gastrointestinal mucosal tissue of COVID-19 patients[18]. In addition, it has also been detected in tears and conjunctival secretions of COVID-19 patients[19]. Intrauterine vertical transmission from pregnant women to the newborn is temporarily excluded due to a lack of adequate data on pregnant women infected with SARS-CoV-2[20].

Prevalence of COVID-19

A number of researchers estimated the basic reproduction number (R_0) to calculate the number of people affected by secondary infections. Generally, it represents the number of people with COVID-19 but in a completely susceptible population without intervention[21]. Using the SEIR model, Wu *et al*[22] recorded an R_0 value for SARS-CoV-2 in the range of 2.47-2.86, while Majumder and Kenneth[23] estimated the R_0 value to be 2.0–3.3 based on the IDEA model. By contrast, other β-CoV viruses namely SARS-CoV and MERS-CoV showed an R_0 value in the range of 2.2–3.6 and 2.0–6.7, respectively[24,25], which indicated that SARS-CoV-2 has higher transmissibility than SARS-CoV and MERS-CoV. In China, 87% of cases were in the age group 30 to 79 years and 3% cases were noted to be aged ≥ 80 years, while female cases were only 41.9%[26,27]. Additionally, 81% of cases were classified as mild, 14% cases were severe and 5% cases were very critical. In another study, it was reported that the overall case-fatality rate (CFR) was 2.3%; however, in the age groups 70-79 and ≥80 years, the CFRs were 8.0% and 14.8%, respectively[22]. These findings clearly indicated that elderly males are more susceptible to SARS-CoV-2 compared with other groups. In addition, the virus affected those elderly males with chronic diseases such as diabetes, hypertension, heart disease, *etc*.[20]. In summary, the prevalence of COVID-19 is very high, and it can spread very rapidly within countries and outside countries.

Virus susceptibility and incubation period

Generally, elderly people aged between 55 and 75 years are more susceptible to SARS-CoV-2 infection. Currently, it has been found that the virus is also infecting middle-aged people aged between 25 and 50 years. The average age of patients across 18 studies was 51.97 years (95%CI: 46.06%-57.89%), 55.9% were male (95%CI: 51.6%–60.1%). Additionally, 36.8% cases showed comorbidities (95%CI: 24.7%-48.9%), the most significant being hypertension (18.6%; 95%CI, 8.1–29.0%), cardiovascular disease (14.4%; 95%CI: 5.7%–23.1%), and diabetes (11.9%; 95%CI: 9.1%-14.6%), among others[28]. Children account for 1% to 3% of COVID-19 cases across countries and likely experience an asymptomatic infection (mild or no symptoms on infection) compared with adults. Zhong *et al*[29] demonstrated that the virus has an average median incubation period of about 3 d but it can range between 0 and 24 d, and the average median time from symptomatic onset to death is 14 d. They also found that mortality rises in patients with comorbidities or a surgical history before virus infection. Generally, the average median latency period for SARS-CoV-2 infection was 4 d, the average interval to hospital admission after onset of symptoms was 3.8 d, and the average time to death after admission to hospital was 17.4 d[30]. Another study

reported that the time to appearance of COVID-19 symptoms to death ranged between 6 and 41 d with a median period 14 d[22]. They also showed that this period was age-dependent and related to the patient's immune system status. The prevalence was greater in patients aged over 70 years compared with those less than 70 years. According to the WHO, the incubation period for COVID-19 ranged from 2 to 10 d. By contrast, for MERS-CoV infection the average median latency was 7 d[31]. However, in COVID-19, the maximum latency was observed to be 24 d, which was high compared with SARS and MERS. This indicated that SARS-CoV-2 has a higher risk of transmission. Accordingly, in comparison with SARS and MERS, SARS-CoV-2 has a shorter median incubation period. Recent data showed that elderly people (aged above 75 years) have a shorter median interval, *i.e.,* 11.5 d from symptom onset to death in comparison to COVID-19 patients (20 d). This finding indicated that disease progression is more rapid in elderly people compared to younger people[1].

GENOMIC STRUCTURE AND PATHOPHYSIOLOGY

Genomic structure

SARS-CoV-2, a β-coronavirus, is a single-stranded RNA virus with a diameter ranging between 80 nm and 120 nm. Currently, four types of coronavirus are present in nature: α-, β-, δ- and γ- coronavirus. The γ- and δ-CoV infect birds, while α- and β-CoV infect mammals. Details of these coronaviruses are presented in Table 1. There are six coronaviruses causing human infection including SARS-CoV and MERS-CoV. The complete genome sequence of SARS-CoV-2 is closest to SARS-like bat CoV (MG772933). There is approximately 79% homology in genome sequence between SARS-CoV-2 and SARS[9]. In addition, the complete genomic sequence of SARS-CoV-2 is approximately 29.9 kb, while SARS-CoV and MERS-CoV have a genome length of 27.9 kb and 30.1 kb, respectively[8,32]. The SARS-CoV-2 genome contains a variable number of open reading frames (ORFs) ranging between 6 and 11[33]. Two-thirds are located mainly in the first ORF (ORF1a/b) which encodes 16 non-structural proteins (NSP) and translates polyproteins (pp1a and pp1ab), while the remaining ORFs encode accessory and structural proteins. The remainder of the RNA virus encodes four essential structural proteins, including the spike (S) glycoprotein, small envelope (E) protein, matrix (M) protein, and nucleocapsid (N) protein, and several accessory proteins, that interfere with the host innate immune response[34]. Frameshift mutation between ORF1a and ORF1b is mainly responsible for the production of pp1a and pp1ab polypeptides that are regulated by chymotrypsin-like protease (3CLpro) or main protease (Mpro), and this process produces 16 non-structural proteins (NSPs) with the help of papain-like proteases[35]. Therefore, SARS-CoV-2 pathophysiology and virulence are thought to be linked with NSPs and structural protein functions.

Pathophysiology

The pathophysiology of COVID-19 produces pneumonia which seems to be very complex. The pathological mechanism is presented in Figure 2. A group of researchers claimed that viral infection is caused by an immune reaction through the "cytokine storm"[36,37]. The main protagonist of this "cytokine storm" is interleukin 6 (IL-6). Generally, activated leukocytes are primarily responsible for IL-6 production and IL-6 acts on a number of cells and tissues. It stimulates acute phase protein production and regulates thermoregulation, bone structure and central nervous system functions[36, 37]. However, its main role is pro-inflammatory actions. COVID-19 enhances IL-6 level, which is implicated in the pathogenesis of the cytokine release syndrome (CRS), which is an acute systemic inflammatory syndrome characterized by fever and multiple organ dysfunction[36,37].

Another group of researchers demonstrated that SARS-CoV-2 uses angiotensin converting enzyme 2 (ACE2) receptor for both cross-species and human-to-human transmission[1,38]. The virion S-glycoprotein present on the virus surface interacts with ACE2 receptors on human cells to spread the infection[39]. S-glycoprotein contains two subunits, S1 and S2. The S1 determines the virus-host range and cellular tropism in the key function domain – RBD (receptor-binding domain), while S2 is responsible for cell membrane-virus fusion by two tandem domains, heptad repeats 1 (HR1) and HR2[40,41]. Following membrane fusion, viral RNA is released into the cytoplasm, and the uncoated RNA is induced to produce pp1a and pp1ab polypeptides with the help of either chymotrypsin-like protease (3CLpro) or main protease (Mpro), which encode 16 non-structural proteins (NSPs) in the presence of papain-like proteases, and finally form a replication-transcription complex (RTC) in double-
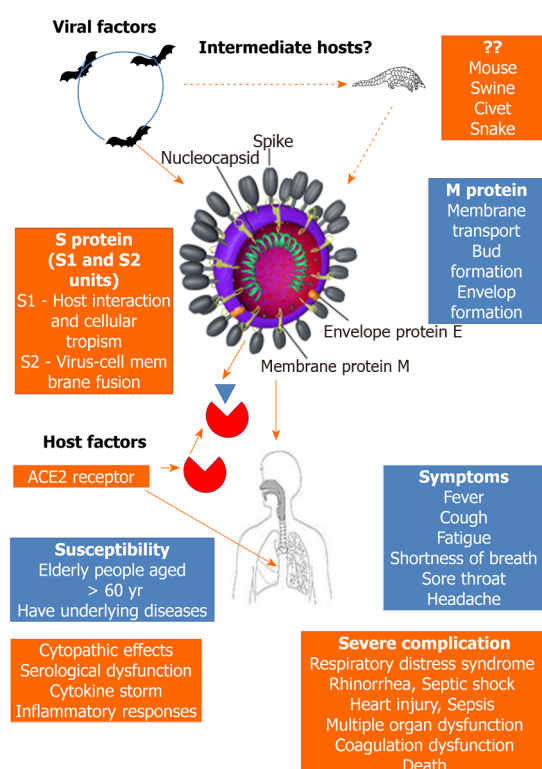
Figure 2 Pathogenesis of severe acute respiratory syndrome coronavirus 2 (viral and host factors). ACE2: Angiotensin-converting enzyme 2.

membrane vesicles[8]. Subsequently, the RTC replicates continuously and synthesizes sub-genomic RNAs[42] to encode accessory proteins and structural proteins. This newly formed genomic RNA, envelopes glycoproteins and nucleocapsid proteins mediated through the endoplasmic reticulum (ER) and Golgi[43] are assembled together to form viral buds. Finally, these newly formed virion-containing vesicles are fused with plasma membrane to release the virus and cause infection through mucous membranes, especially nasal and larynx mucosa, and then enter the lungs through the respiratory tract.

These ACE2 receptors are very important in the spread of COVID-19. They are mainly found in the lower respiratory tract of humans. After entry through mucous membranes, especially nasal and larynx mucosa, the virus enters directly into the lungs through the respiratory tract. In the next step, the virus attacks other target organs which contain ACE2 receptors, such as the lungs, heart, renal system and gastrointestinal tract[36,37]. Accordingly, the binding affinity of this virus-receptor has been intensively studied using different approaches. Systematic detection analysis showed that SARS-CoV-2 S-glycoprotein binding capacity with ACE2 was 10-fold higher than SARS-CoV as shown under cryo-electron microscopy of the SARS-CoV-2 S protein in pre-fusion conformation[39]. Recently, Wu *et al*[9] demonstrated moderate genomic and phylogenetic similarity with SARS-CoV but higher similarity with bat CoV genome sequence, particularly in the S-glycoprotein and RBD. They also found that there were no amino acid substitutions occurring in the NSP7, NSP13, envelope, matrix, or accessory proteins p6 and 8b at the protein level, except in NSP2, NSP3, spike protein, underpinning the subdomain, *i.e.*, RBD. Another recent study demonstrated that mutation of NSP2 and NSP3 plays an important role in infection and SARS-CoV-2 differentiation. However, this mechanism of SARS-CoV-2 infection in humans *via* S-protein binding with ACE2 is unclear, as is the interaction strength for risk transmission. Accordingly, the WHO was also unable to clarify the mechanism of COVID-19. This has led to further investigations regarding potential human-to-human transmission and the pathophysiological mechanisms of COVID-19 outbreaks.

CLINICAL CHARACTERISTICS OF COVID-19 INFECTION

Being an acute respiratory infection, COVID-19 is initiated in the respiratory tract, primarily by droplets, respiratory secretions, and direct contact. After entry, the virus

affects a number of organs or systems (Figure 3). The clinical symptoms of COVID-19 vary from asymptomatic or paucisymptomatic forms to clinical conditions. In particular, all patients are divided into general, severe, and critical patient groups. The most common clinical symptoms of COVID-19 are fever (87.9%), cough (67.7%), fatigue (38.1%), sputum production (33.4%), shortness of breath (18.6%), sore throat (13.9%), and headache (13.6%)[27,44]. The development of these symptoms may occur within 3 d of viral infection. On the other hand, other symptoms may occur 9 d after virus infection. Of these, fever and cough are the dominant COVID-19 symptoms. The incidence of diarrhea (3.7%) and vomiting (5.0%) is very rare[27,44]. However, it is very difficult to accurately distinguish COVID-19 from other viral respiratory infections. The CDC included loss of taste or smell, pink eye, muscle pain, intense chills, headache and sore throat as COVID symptoms. In severe cases, symptoms such as acute respiratory distress syndrome, rhinorrhea, dyspnea, gastrointestinal disorders, septic shock, mental stress, acute heart injury, sepsis, multiple organ dysfunction syndrome (MODS), secondary infection and even death may occur[8,34]. Critical COVID-19 patients with severe respiratory failure require an intensive care unit (ICU) or ventilation support. However, the occurrence of upper respiratory symptoms and gastrointestinal symptoms are very rare compared with other symptoms. In addition to this, the elderly and those who have underlying diseases (*i.e.,* chronic obstructive pulmonary disease, hypertension, diabetes, cardiovascular disease) are very prone to COVID-19 and develop symptoms such as metabolic acidosis, acute respiratory distress syndrome, coagulation dysfunction and even death [8,45]. Sometimes, COVID-19 patients experience acute heart injury, arrhythmia, impaired renal function and abnormal liver function such as the formation of microvesicular steatosis (50.7%) at the time of admission[1,45,46].

Hematological assays revealed that most patients had decreased white blood cell counts, and lymphocytopenia[27]. In the case of critical patients, neutrophil count, D-dimer, blood urea, creatinine and lymphocyte levels decreased markedly. In another study, a reduction in albumin level (75.8%; 95%CI, 30.5%-100.0%), higher C-reactive protein (58.3%; 95%CI: 21.8%-94.7%) and lactate dehydrogenase (LDH) levels (57.0%; 95%CI: 38.0%-76.0%), higher lymphopenia level (43.1%; 95%CI, 18.9%-67.3%), and higher erythrocyte sedimentation rate (ESR) (41.8%; 95%CI: 0.0-92.8%) and other clinical manifestations were recorded[28]. Additionally, inflammatory factors, which indicated the immune status of patients, namely IL-6, IL-10, and tumor necrosis factor-α (TNF-α) are also markedly increased. In critical patients (admitted to the ICU), higher IL-2, IL-7, IL-10, granulocyte colony-stimulating factor (GCSF), 10 kD interferon gamma-induced protein (IP-10), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein 1-α (MIP-1α), and TNF-α levels in plasma were observed[8,45]. In patients with severe COVID-19 [admitted to the ICU; 20.3% cases (95%CI, 10.0–30.6%)], 32.8% of patients experienced ARDS (95%CI: 13.7%–51.8%), 13.0% patients had acute cardiac injury (95%CI: 4.1%-21.9%), 7.9% patients experienced acute kidney injury (95%CI: 1.8-14.0%), 6.2% cases (95%CI: 3.1%-9.3%) developed shock and 13.9% cases (95%CI 6.2%-21.5%) experienced fatal outcomes[28]. Furthermore, 96.8% of all patients (95%CI: 94.9%-98.7%) had RNAemia in blood and nasopharyngeal aspirates (NPA)[28].

IMMUNOPATHOLOGICAL RESPONSES

Immunological symptoms are generally caused due to binding of virus S proteins with ACE2 at the receptor, usually in the endosome Toll-like receptor (TLR) 3, TLR7, TLR8, and TLR9[8,47]. Retinoic-acid inducible gene I (RIG-I) of the virus, melanoma differentiation-associated gene 5 (MDA5) of the cytosol and nucleotidyltransferase cyclic GMP-AMP synthase (cGAS) are generally responsible for the spread of COVID-19[8, 48,49]. Viral infection activates nuclear factor-κB (NF-κB) and interferon regulatory factor 3 (IRF3) to produce type I interferons (IFN-α/β) and pro-inflammatory cytokines as immune mediators (*i.e.,* innate immunity) to prevent infection[8,50]. As a result, the plasma levels of some cytokines and chemokines are elevated in COVID-19 patients such as IL-1, IL-2, IL-4, IL-7, IL-10, IL-12, IL-13, IL-17, GCSF, macrophage colony-stimulating factor (MCSF), IP-10, MCP-1, MIP-1α, hepatocyte growth factor (HGF), IFN-γ and TNF-α[20,45,51]. Generally, these inflammatory responses were noted in the lower airway and lung[52]. Consequently, these trigger immune signaling and produce the "cytokine storm' within the body leading to a very critical condition in COVID-19 patients.
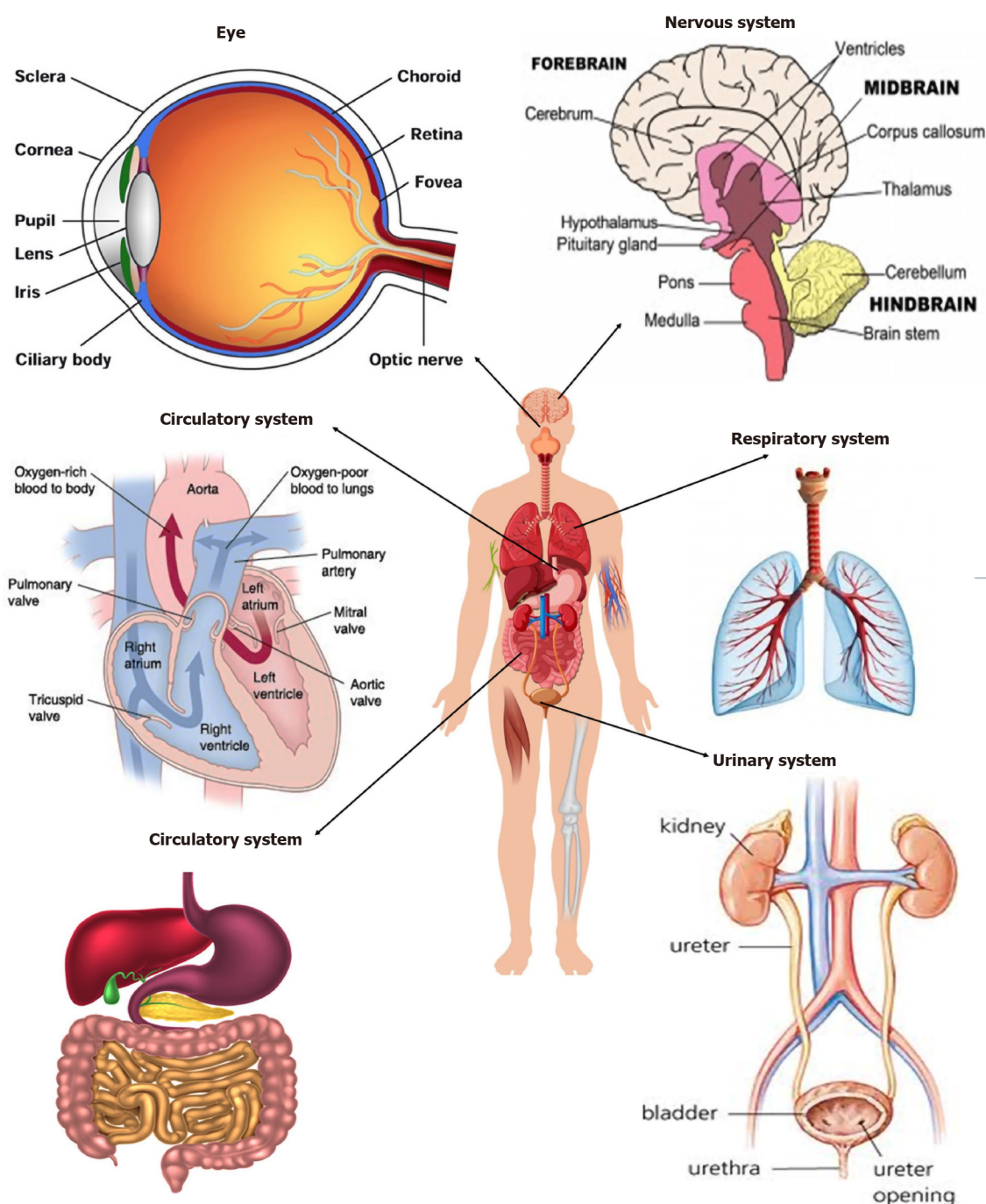
Figure 3 Coronavirus disease 2019 in organs or systems (Images were taken www.google.com).

DIAGNOSIS OF COVID-19

Since the outbreak of COVID-19, a number of diagnostic tools have been used to detect the infection. The classical Koch's postulates method was used to detect the infection in Wuhan[22]. This method is very expensive and time-consuming as it uses electron microscopy. In some countries, radiography was used to detect the viral infection such as a chest computed tomography (CT) scan. CT scan is an important tool in diagnosing COVID-19 pneumonia. Typical COVID-19 pneumonia features were observed by CT. and CT imaging showed ground-glass opacities (56.4%-65%), an air bronchogram (47%), bilateral patchy shadowing (51.8%), consolidations (50%), smooth or irregular interlobular septal thickening (35%), thickening of adjacent pleura (32%), sometimes rounded morphology, peripheral and lower lobe involvement and a peripheral lung distribution in COVID patients[27,53,54]. A very recent study recorded bilateral chest CT findings in 90% patients, and proved its sensitivity (97%) in detecting COVID-19 [55]. However, in another study clinical scientists found that some patients with

confirmed COVID-19 had normal CT scans[53]. Therefore, the diagnosis of COVID-19 is very confusing. Moreover, this technique mainly determines pneumonia. Accordingly, scientists are looking for an alternative method which is more reliable and confirmative. The detection of viral nucleic acid from nasal and throat swab samples, cough, sputum or other respiratory tract samples is the golden diagnostic method for COVID-19 detection. This method uses RT-PCR technology to detect viral infection. Although, this method has high specificity, false-negative results may occur due to low sensitivity and the testing time is too long. In the case of false-positive tests, the WHO recommends resampling and further testing. In this regard, serologic testing is an important diagnostic tool to detect patients who have either current or previous infection but have a negative PCR test[56,57]. In this technique, basic parameters are tested to detect the COVID-19, namely white blood cell count, neutrophil and lymphocyte count, D-dimer, blood urea, and creatinine estimation to identify the appearance of leukopenia, leukocytosis, and lymphopenia as COVID-19 symptoms[58, 59]. In another study, it was demonstrated that 82.1% of COVID patients are lymphopenic, 33.7% patients are leukopenic and 36.2% patients are thrombocytopenic [1]. In addition, another group of researchers recommended elevated plasma levels of C-reactive protein, lactate dehydrogenase, creatinine kinase, transaminase, abnormal myocardial enzyme spectrum or creatinine as COVID-19 indicators[27,45]. They also showed that cytokine release syndrome is an important vital indicator of disease progression. On the other hand, Wan *et al*[60] demonstrated higher IL-6 and IL-10 levels, and lower CD4+T and CD8+T levels as indicators of COVID-19.

Currently, a number of technological inventions are ongoing to detect COVID-19 in a simplistic pathway. Different technological inventions such as the more organized sequencing library (SHERRY) in China, SHERLOCK technology in China, FELUDA in India *etc.*, have been developed as testing tools for rapid detection of COVID-19[6,61]. However, clinical verification of these technological inventions has not been undertaken to date, and once approved, they will be a major breakthrough in techno-logy to diagnose COVID-19 rapidly and economically.

GLOBAL SCENARIOS OF COVID-19 OUTBREAKS

Since its outbreak in Wuhan, China in late December 2019, SARS-CoV-2 infection is spreading very rapidly across the globe. COVID-19 has affected 202608306 people and caused around 4293591 deaths (Table 2). The inter-continental spread is described in Table 2. Figure 4 shows COVID-19 outbreaks in different countries. In the beginning, the Asian countries namely China and South Korea were the epicenter of COVID-19 until the first week of February. Up to August 10, 2021, there have been 93826 confirmed cases and 4636 deaths in China (WHO). In Korea the first COVID case was recorded on January 20, 2020. Since then, about 212448 cases have been confirmed and 2125 deaths recorded in Korea. The epicenter then moved from Asian countries to European countries mainly Italy and Spain. COVID-19 was recorded in Italy on January 30, 2020, and was found in France and Spain on January 24, 2020 and January 31, 2020, respectively. In particular, in Italy, the United Kingdom, France, Germany and Spain it affected people more seriously; approximately 4400617, 6094243, 6310933, 3800048, and 4627770 confirmed cases and 128242, 130357, 112288, 92291, and 82125 deaths were recorded in these countries, respectively, up to August 10, 2021. Among the European countries, mortality rate was highest in Italy due to its travel connection with China. In the middle of March, the virus epicenter moved to the United States and other American countries. The United States and Canada were the most affected countries during this phase. Although the first COVID-19 patient was recorded in late January, 2020 the first death was confirmed in February. In the USA, the first COVID-19 patient died in the middle of March. On August 10, 2021, the USA had recorded the greatest number of confirmed cases and deaths worldwide. The death rate is 206 per million people, which is the tenth highest rate globally. The first COVID-19 patient in Canada was reported on January 27, 2020. On August 10, 2021 there have been 36780480 and 1442087 confirmed cases in the USA and Canada, respectively, and 633799 and 26678 deaths, respectively. In the middle of April, the virus epicenter moved to Russia and India. As of August 10, 2021, there have been 6469910 and 31997017 confirmed cases in Russia and India, respectively, and the number of deaths is 165650 and 428715, respectively. However, the first confirmed COVID-19 case was recorded on January 30, 2020 in Kerala state and January 31, 2020 in Russia. The virus infection in these countries took a very long time to spread due to the implementation of different control measures. The details of COVID-19 cases in India are presented in

Table 2 Coronavirus disease 2019 outbreaks based on the World Health Organization (data as reported at 7.07 PM CEST on August 10, 2021)		
Items	Confirmed cases	Deaths
Globally	202608306	4293591
Africa	5156790	122537
Americas	78718104	2032256
Eastern Mediterranean	13169171	243217
Europe	61333662	1231439
South-East Asia	39271048	593565
Western Pacific	4958767	70564
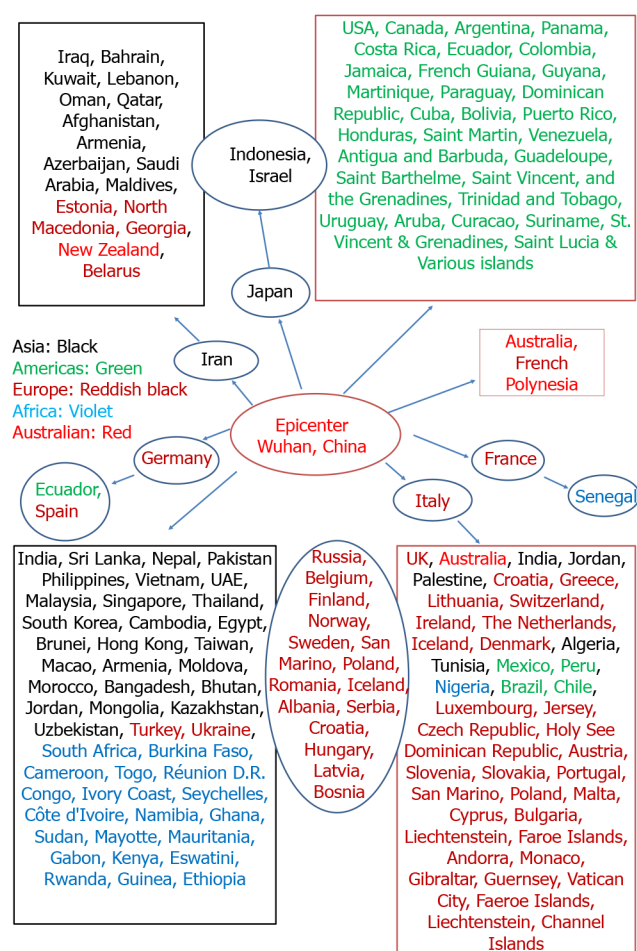
Figure 4 Coronavirus disease 2019 routes of transmission across countries. Figure modified after Ali and Alharbi (2020)[68], an Elsevier journal.

Table 3. However, according to fatality rate data, Belgium (15% fatality) is highest, followed by the United Kingdom (15%), France (14.7%), Italy (13.6%) and the Netherlands (12.3%) (John Hopkins Bulletin).

TREATMENT OF COVID-19

Antiviral drug treatment

Presently, COVID-19 treatment is based on symptomatic findings. To date, there is no precise treatment method, but currently the WHO, CDC and Food and Drug Administration have recommended certain drugs for COVID-19 treatment. The effectiveness

Table 3 Coronavirus disease 2019 state-wise status in India (as on August 10, 2021; Ministry of Home Affairs, GoI)

No.	Name of State / UT	Total confirmed cases*	Cured/discharged/migrated	Deaths**
1	Andaman and Nicobar Islands	7546	7412	129
2	Andhra Pradesh		1950623	13549
3	Arunachal Pradesh	50372	47520	246
4	Assam		558720	5404
5	Bihar		715303	9646
6	Chandigarh	61984	61146	811
7	Chhattisgarh		988004	13540
8	Dadar Nagar Haveli	10656	10612	4
9	Delhi		1411235	25067
10	Goa		167884	3164
11	Gujarat		814778	10077
12	Haryana		759769	9650
13	Himachal Pradesh		202569	3519
14	Jammu and Kashmir		316957	4390
15	Jharkhand		342074	5130
16	Karnataka		2859552	36817
17	Kerala		3377691	17852
18	Ladakh	20393	20117	207
19	Madhya Pradesh		781307	10514
20	Maharashtra		6151956	134064
21	Manipur		96128	1657
22	Meghalaya	69358	63450	1174
23	Mizoram	44520	32854	168
24	Odisha		971391	6554
25	Puducherry		119031	1800
26	Punjab		582753	16320
27	Rajasthan		944670	8954
28	Tamil Nadu		2522470	34340
29	Telengana		637789	3828
30	Tripura	80208	77230	767
31	Uttarakhand		328569	7368
32	Uttar Pradesh		1685449	22774
33	West Bengal		1505808	18240
34	Nagaland	28709	25906	585
35	Sikkim	27908	24544	355
36	Lakshadweep	10257	10112	51

and limitations of each drug are summarized in Table 4[62]. The existing drugs for treating COVID-19 patients are remdesivir, chloroquine, hydroxychloroquine, tocilizumab, lopinavir-ritonavir, azithromycin, baloxavir, favipiravir, *etc.*[63]. Remdesivir, is most prominent for treating COVID-19 patients[64]. The efficacy of remdesivir in treating patients has been reported globally[63-65]. Recently, the ChAdOx1 vaccine developed by the University of Oxford's Jenner Institute and the Oxford Vaccine Group has proved effective in combatting COVID-19. More recently,

Table 4 Recommended drugs for coronavirus disease 2019 treatments (Food and Drug Administration and World Health Organization)		
Common drugs	**Dose**	**Mechanism**
Chloroquine; Antimalarial	50% for GFR < 10 mL/min	*In vitro* activity and has immunomodulating properties
		Inhibits viral enzymes or processes such as viral DNA and RNA polymerase, viral protein glycosylation, virus assembly, new virus particle transport, and virus release
		ACE2 inhibition due to acidification at cell membrane surface, inhibits fusion of virus, and cytokine release
Hydroxychloroquine; Antimalarial	800 mg orally on day one, followed by 400 mg/d orally for four to seven days	Same as chloroquine
Chloroquine phosphate; Antimalarial	1 g orally on day one, followed by 500 mg/d orally for four to seven days	Same as chloroquine
Remdesivir; Nucleoside Analogue	200 mg IV on day 1 followed by 100 mg IV daily on days two to five or 200 mg IV on day 1 followed by 100 mg IV daily on days two to ten	*In vitro* activity; Inhibitor of RNA-dependent RNA polymerases (RdRps)
		Remdesivir-TP competes with adenosine-triphosphate for incorporation into nascent viral RNA chains
		Once incorporated into the viral RNA at position i, RDV-TP terminates RNA synthesis at position i+3
		Because RDV-TP does not cause immediate chain termination (*i.e.*, 3 additional nucleotides are incorporated after RDV-TP), the drug appears to evade proofreading by viral exoribonuclease (an enzyme thought to excise nucleotide analogue inhibitors)
Azithromycin; Macrolide Antibacterial	500 mg on day one, followed by 250 mg daily for four days	Prevents bacterial superinfection, has immunomodulatory action on pulmonary inflammatory disorders
		Downregulates inflammatory responses and reduces excessive cytokine production associated with respiratory viral infections; however, its direct effects on viral clearance are uncertain
		Immunomodulatory mechanisms include reducing chemotaxis of neutrophils (PMNs) to lungs by inhibiting cytokines (*i.e.*, IL-8), inhibition of mucus hypersecretion, decreased production of ROS, accelerating neutrophil apoptosis, blocking activation of nuclear transcription factors
Lopinavir; Ritonavir; HIV protease inhibitor	400 mg/ritonavir 100 mg orally twice daily for up to 21 d	*In vitro* animal model studies show potential activity for other coronaviruses (SARS-CoV and MERS-CoV)
		Lopinavir and ritonavir may bind to Mpro, a key enzyme for virus replication and suppress virus activity
Tocilizumab; Interleukin-6 (IL-6) Receptor-InhibitingMonoclonal Antibody	4-8 mg/kg infused over more than 60 min (additional dose after 12 h)	Cytokine release syndrome; Inhibits IL-6-mediated signaling by competitively binding to both soluble and membrane-bound IL-6 receptors. IL-6 involved in T-cell activation, immunoglobulin secretion induction, hepatic acute-phase protein synthesis initiation, and hematopoietic precursor cell proliferation and differentiation stimulation
Baloxavir; Antiviral	80 mg orally on day 1 and on day 4, and another dose of 80 mg on day 7 (as needed); not to exceed 3 total doses	Active against influenza viruses; *In vitro* antiviral activity against SARS-CoV-2 demonstrated in one trial
Favipiravir; Antiviral	1600 mg twice daily on day 1, then 600 mg twice daily for 7-10 d; Severe: 1600 mg every 12 h on day 1, then 600 mg every 12 h days 2-10	*In vitro* activity against Vero E6 cells

SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2.

Russia has reportedly developed a coronavirus vaccine named Sputnik V.

Chinese medicine treatment

A number of Chinese medicines have been used to treat COVID-19 patients. According to the Academy of Sciences, Shuanghuanglian oral liquid is most prominent and inhibits SARS-CoV-2. Several studies reported that baicalin, chlorogenic acid and forsythin present in Shuanghuanglian oral liquid have certain inhibitory effects on various viruses and bacteria including SARS-CoV-2[66]; however, the detailed mechanism is not yet known. Lianhuaqingwen capsules have also been used to treat

SARS-CoV-2 infected people as well as other diseases such as influenza viruses, including H7N9 by reducing inflammatory factors[1,17].

Unani medicine treatment

These are plant-based treatments, called Ayurvedic treatments, and these treatments are nontoxic and have no side effects. Different plant parts are used to treat anti-viral activities[67]. The most important plants are *Glycyrrhiza glabra, Allium cepa, Allium sativum, Ocimum sanctum, Ocimum tenuiflorum, Piper nigrum, Cinnamomum verum, Daucus maritimus, Curcuma longa, etc.* Administration of the aqueous extracts of these plants along with lemon juice and honey is very effective for flu and the common cold [68]. According to Fiore *et al*[69] *Glycyrrhiza glabra* plant extract is effective in treating viruses such as SARS related coronavirus, HIV-1, respiratory syncytial virus, varicella zoster, hepatitis A, B, C, and cytomegalovirus herpes. Similarly, Wang *et al*[70] indicated that *Glycyrrhiza glabra* also has antiviral and antimicrobial activities. Therefore, *Glycyrrhiza glabra* plant extract along with other plants may be useful in controlling COVID-19. Accordingly, the Government of India has recommended Ayurveda treatment methods to improve immunity (Table 5).

Homeopathic treatment

Arsenic album-30 is considered beneficial for viral infections. Recently, the Directorate of AYUSH, New Delhi, India has issued an order on January 30, 2020 to take prophylactic medicine to avoid coronavirus infection. Dr Rajan Sankaran has recommended Camphor 1M as a potential medicine for COVID-19 (https://www.boomlive.in/coronavirus-outbreak/homoeopathy-can-be-used-as-adjuvant-to-covid-19-treatment-dr-anil-khurana-7997). They recommended 4 pills of Arsenic album-30 medicine once daily on an empty stomach for 3 d. It is highly diluted arsenic trioxide and works as a homeopathic prophylaxis. Accordingly, the Homeopathy Department of Kerala Government is administering *Arsenicum Album* 30C as a preventive medicine to boost immunity in COVID-19 patients and it was approved by the Department of AYUSH, GoI (https://gulfnews.com/world/asia/india/covid-19-kerala-government-distributes-homeopathy-medicine-to-boost-immunity-1.1588091249686). However, to date, there is no clinical evidence that Arsenic album-30 is an effective medicine. As a result, the use of these medicines to manage COVID-19 has been criticized globally. Mathie *et al*[71] reported that *Arsenicum album* medicine is effective in reducing fever, runny nose, headache, and sore throat in patients with swine flu. Therefore, the use of homeopathy in COVID-19 management is debatable and requires further scientific study.

Immuno-booster treatment

Boosting the body's immunity is a potential individual protocol as COVID-19 pathogenesis is caused by a disproportionate immune response. Therefore, it is important to take supplements to boost both innate and adaptive immune response. Interferon is reported to inhibit viral infection and in particular, recombinant interferon α is effective for SARS-like viruses. Additionally, interferon was reported to be an effective inhibitor of MERS-CoV replication[72]. These findings indicated that interferon could be used to treat COVID-19 infection. Intravenous immunoglobulin might be the safest immune modulator for all age groups, and could help to inhibit pro-inflammatory cytokine production and to increase anti-inflammatory mediators[1, 73]. Moreover, thymosin alpha-1 (Ta1) is used as an immune booster for SARS patients to effective control the disease[74]. Accordingly, intravenous immunoglobulin and Ta1 may also be used for the treatment of COVID-19. Recently, different immune-booster drugs have been used to treat COVID-19 such as neuraminidase inhibitors (*e.g.,* oseltamivir used to treat influenza). Apart from these, citrus fruits, dry fruits (almonds, walnuts, and dates) are very effective in improving the immune system. Vitamin A, C, D and E, and zinc supplements are effective in older patients. Additionally, adequate sleep, regular exercise and stress avoidance is essential to boost the immune system[68].

Plasma therapy

Due to lack of appropriate vaccines and specific drugs, plasma therapy could be an effective way to treat COVID-19. Previously, convalescent plasma therapy was proved to be an effective treatment option for SARS patients and those with H1N1 influenza [75,76]. From an immunological perspective, it was observed that recovered COVID-19 patients produced specific antibodies against SARS-CoV-2, and therefore their serum could be used to prevent re-infection. Additionally, these antibodies can limit the

Table 5 Unani drugs for coronavirus disease 2019 treatment (Source: Department of AYUSH, Government of India)	
Unani drugs	Doses
Symptomatic treatments	
SharbatUnnab	10-20 mL twice a day
TiryaqArba	3-5 g twice a day
TiryaqNazla	5 g twice a day
KhamiraMarwareed	3-5 g once a day
ArqAjeeb	4-8 drops in fresh water and four times a day
Habb e IkseerBukhar (fever)	2 pills with lukewarm water twice daily
SharbatNazla	10 mL mixed in 100 mL of lukewarm water twice daily
Qurs e Suaal	2 tablets to be chewed twice daily
Decoction	
Behidana	3 g
Unnab	7 nos
Sapistan	7 nos
Darchini	3 g
Banafsha	5 g
Berg-e-Gaozabaan	7 g
Sore throat	
Khashkhash; Bazrulbanj; Post Khashkhash; Barg e Moard (Habbulaas); Tukhm e kahuMukashar; GuleSurkh	Any of them @12 g (each)

production of virus in the acute phase and help to clear the virus if injected during the first week of the viremia peak. Therefore, plasma globulin specific to SARS-CoV-2 has to be prepared from recovered COVID-19 patients. Recently, the Delhi Government successfully applied plasma therapy to treat COVID-19 patients.

In summary, in addition to the abovementioned treatments for COVID-19, auxiliary blood purification treatment (mainly used for severe NCP patients) could be used as an alternative therapy. According to Zarbock *et al*[77] the ACE2 receptor, the key receptor of SARS-CoV-2, is highly expressed in human kidney (100 times higher than in the lung). Kidney is one of the target organs for SARS-CoV-2; therefore, continuous blood purification could reduce renal recovery during COVID-19. Additionally, the kidney suffers from cytokine storms under severe COVID-19 infection. Therefore, blood purification technology could be an alternative method for removing inflammatory factors, eliminating cytokine storms, correcting electrolyte imbalances and maintaining acid–base status[1]. In addition, randomized double-blind clinical trials should be used as standard methodology for large sample sizes to determine antiviral drug efficacy in clinical practice. Currently, in India the discharge policy for COVID-19 recovered patients is based on 3 tier COVID-19 facilities and the categorization of patients is based on clinical severity. The revised discharge policy is indicated in Figure 5.

PREVENTION OF COVID-19 OUTBREAKS

COVID-19 has affected all sectors of society. Therefore, prevention is the best practice to reduce the impact of COVID-19 considering the lack of effective treatments. This can be achieved through a variety of means as follows:

Individual measures

Individual measures are essential in reducing the spread of COVID-19 at the community level. Community level spread is mainly caused when an infected person is in close contact with other healthy individuals. According to the WHO, the following individual measures should be taken to reduce the contamination level such
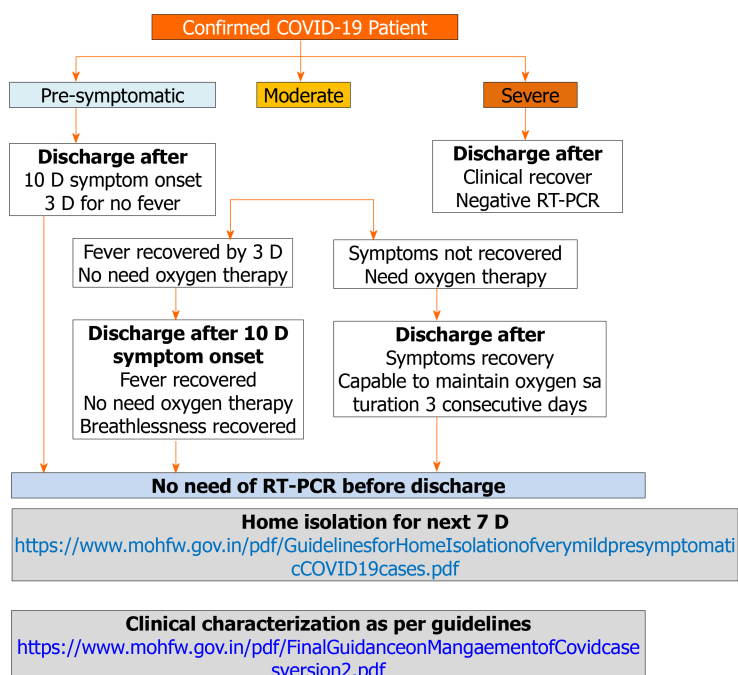
Figure 5 Discharge policies adopted by the Indian government. COVID-19: Coronavirus disease 2019.

as the use of face masks; respiratory hygiene by covering the mouth and nose with a bent elbow or tissue during coughing or sneezing; washing hands regularly with soap or disinfectant (containing at least 60% alcohol); avoiding contact with infected people, maintaining an appropriate distance (at least 2 m) from coughing or sneezing people; refraining from touching eyes, nose, and mouth with unwashed hands and finally, following advice from the healthcare provider.

Community level measures, social lockdown
Social lockdown is the restriction of inter-individual physical contact. Generally, it is a community level measure. The prime objective of social lockdown is to avoid two people from different families or nearby inhabitants coming in close contact with each other[78]. However, minimal and emergency movement of the general public is allowed under this condition. The emergency services (medical care, food security, general security and medicine supply) vary in different countries. However, in severe situations, emergency services such as the food and medical supply chain can also be closed as external or internal body fluid discharges such as coughs, sneezes, saliva *etc.* from COVID-19 patients infect healthy persons due to its easy transmissibility. Another objective of social lockdown is to allow the community to develop mild or full resistance to a mutated virus[78]. Moreover, it provides researchers more time to work on medicine or vaccines production. Considering the advantages of social lockdown, many nations across the globe have started different degrees of social lockdown to prevent SARS-CoV-2 infection.

International social lockdown progress
Some of the international social lockdown campaigns have been addressed here to understand COVID-19 preventive measures. Since the outbreak of COVID-19, China was the first country to implement social lockdown, which occurred in the last week of January 2020 in Wuhan city, the epicenter of the COVID-19 outbreak. During lockdown, buses and cars were allowed to run but domestic flights and trains were cancelled in various cities, and around 760 million people were under lockdown[29]. Accordingly, the WHO praised China as they had taken "perhaps the most ambitious, agile and aggressive disease containment effort in history"[79-81]. After China, Italy was the second country to adopt social lockdown. In Italy, social lockdown was declared on February 21, 2020 in northern Italy covering only 50000 people. Considering the disease incidence, the Federal government of Italy declared whole country lockdown on March 9, 2020. Only public transport was partially allowed, and a public pass system was initiated to ride buses or board flights on an emergency basis [82].

COVID-19 in the USA was spreading very rapidly with a high death rate since its first official COVID-19 case. Higher infection was mainly due to either higher migrant movement or a higher rate of clinical diagnosis[83]. Hence, following the high death and infection rate in the USA, the Trump government implemented the first lockdown on March 19, 2020 but to achieve total control of COVID-19, the American government extended the lockdown period to April 30, 2020 on March 30, 2020. The Trump government explained the second lockdown as follows "The better you do, the faster this whole nightmare will end. Therefore, we will be extending our guidelines to April 30th to slow the spread." Accordingly, the Director of NIH recommended the people of the USA to adapt to the lockdown voluntarily and stringently[84]. Most of the African countries had started to implement social distancing in the middle of March and ended it between May 10 and May 20, 2020. The same window was also used by most European countries. Social distancing in Bangladesh was implemented by Prime Minister Sheikh Hasina very late on March 25, 2020 and ended on May 16, 2020. Other countries such as Pakistan and Sri Lanka started to implement social distancing on March 24, 2020 which ended on May 9, 2020. Additionally, Sri Lanka declared a curfew to maintain strict social distancing.

Social lockdown status in India

Being a populous country, a large portion of the population lives in places of high density and their unhygienic lifestyle results in frequent infectious and epidemic diseases[85]. Therefore, as World Bank data have indicated India is still struggling to improve its health care system and is unable to provide sufficient hospital beds for its citizens. India can only afford 0.7 hospital beds per 1000 people, the doctor: population ratio is 1:1800 (standard is 1:1000), and the total number of ventilators available is 48000[86]. Considering this, the Government of India under Prime Minister Narendra Modi declared a Janata Curfew for 14-h (from 7 a.m. to 9 p.m.) on March 22, 2020 prior to total lockdown. Except for 'essential services' (police, medical services, media and home delivery) everyone took part in the curfew. According to Swiss firm IQAir, at least 75 Indian districts took part and helped to control the spread of SARS-CoV-2, which had an immediate positive effect, especially in Delhi, which is known as one of the world's most polluted capital cities. This resulted in a massive change in New Delhi's Air Quality Index (AQI). This was mainly due to a huge reduction in vehicular traffic; during lockdown there was a 70% reduction in the demand for petroleum oil. India is the third largest user of oil, after the USA and China. After that a nationwide lockdown for 21 days (except emergency services) was declared on March 24, 2020. The government implemented the following restrictions: (1) ban on people from stepping out of their homes; (2) closed all services and shops except pharmacies, hospitals, banks, grocery shops and other essential services; (3) closed all commercial and private establishments (only work-from-home allowed); (4) suspended all educational, training, and research institutions; (5) closed all places of worship; (6) suspended all non-essential public and private transport; (7) prohibited all social, political, sports, entertainment, academic, cultural, and religious activities; and (8) suspended entry of all international commercial flights from March 22. During the first phase of lockdown, the infection rate was not as high as that in the USA, Spain and Italy. It was previously reported that temperature may adversely affect virus infection [87]. Considering the influence of the upcoming Indian hot and humid summer, the health experts urged the Government to extend the lockdown. Many international news agencies described this strict lockdown by the Indian government as harsh, intensive and mismanaged[88,89]. However, the WHO declared that "the measures taken by India to break the community spread of COVID-19 by the lockdown was a very early, scientific and timely decision"[90]. In the words of Dr. David Nabarro, special envoy on the disease, WHO "*The lockdown in India was quite early on, when there was relatively a small number of cases detected. This was really a far-sighted decision because it gave the whole country the opportunity to come to terms with the reality of this enemy. People understood that there is a virus in our midst. It gave time to develop capacities at the local level for interrupting transmission and sorting out hospitals. Of course, there is a lot of debate and criticism, and inevitably with a lot of frustration and anger that life is being disturbed in this way. It is very, very upsetting. I think it is courageous of the government, honestly, to take this step and provoke this enormous public debate and let the frustration come out, to accept that there will be hundreds of millions of people whose lives are being disrupted. For poor people on daily wages, this is a massive sacrifice they are making. And to do it now at an early stage as opposed to waiting three or four weeks later when the virus is much more widespread was very courageous*[91]."

In the second phase, PM Modi extended the nationwide lockdown on April 14, 2002 until May 3, with a conditional relaxation after April 20. On April 16, lockdown areas

were classified as "red, orange and green zones", indicating the presence of infection hotspots, some infection, no infections, respectively. On April 20, the government announced relaxations in different sectors such as agriculture including dairy, aquaculture and plantations, selling of farming products, cargo transportation including trucks, trains and planes following social distancing norms[92]. On April 25, the government allowed the opening of small retail shops with half-staff following social distancing norms. On April 29, the Ministry of Home Affairs allowed inter-state movement of migrant people following the guidelines laid down by the government. An additional extension (May 4 – May 17) was granted by Government of India on May 1, 2020 with additional relaxation to curb the infection.

In this phase, the whole country was categorized into three zones namely red zones (130 districts), orange zones (284 districts) and green zones (319 districts). Red zones were areas with high infection and a high doubling rate, orange zones had comparatively fewer cases and green zones had no cases in the past 21 days. Normal movement was allowed in green zones with buses (50% capacity). In orange zones, only private and hired vehicles but no public transportation was allowed, while red zones were under complete lockdown. The government then implemented a fourth phase of lockdown to prevent COVID-19 between May 18 and May 31, 2020. On May 30, the government extended the ongoing lockdown until June 30 for only containment zones with services resumed in a phased-manner from 8 June. This was termed "Unlock 1.0". The second phase of unlock, called Unlock 2.0, was announced for the period of 1 to 31 July, followed by the easing of restrictions. Currently, Unlock 3.0 has been announced for August.

ENVIRONMENTAL PERSPECTIVES: INFLUENCE AND IMPACTS

The lockdown period has greatly helped the environment to rejuvenate, simply due to a reduction in pollution level to a large extent.

Longevity of SARS-CoV-2 in the environment

SARS-CoV-2 can remain suspended for approximately 30 min as an aerosol (< 5 μm). SARS-CoV-2 remained viable in aerosols for up to 3 h, with a reduction in infectious titer from $10^{3.5}$ to $10^{2.7}$ $TCID_{50}$ per L of air. SARS-CoV-2 is more stable on plastic and stainless steel than on copper and cardboard[78]. The virus has the longest life on plastic and steel, surviving up to 72 h but the total number of virus particles decreases sharply over this time ($10^{3.7}$ to $10^{0.6}$ $TCID_{50}$ per mL of medium after 72 h on plastic and $10^{3.7}$ to $10^{0.6}$ $TCID_{50}$ per mL after 48 h on stainless steel). On copper, it survives up to 4 h [78]. On cardboard, it survives up to 24 h, which suggests packages that arrived in the mail should have only low levels of the virus. On copper and cardboard, the virus is undetectable by 8 and 48 h, respectively[78]. The half-life of SARS-CoV-2 is similar to SARS-CoV-1 in aerosols, with a median of approximately 1.1 to 1.2 h and 95% credible intervals of 0.64 to 2.64 for SARS-CoV-2 and 0.78 to 2.43 for SARS-CoV-1[78]. The half-life of these two viruses is also similar on copper. On cardboard, the half-life of SARS-CoV-2 is longer than SARS-CoV-1. The longest viability was detected on stainless steel and plastic; the estimated median half-life of SARS-CoV-2 is 5.6 h on stainless steel and 6.8 h on plastic[78].

Meteorological influence

The COVID-19 pandemic is spreading globally irrespective of meteorological influence. Meteorological factors such as temperature, weather conditions and humidity are thought to play a vital role in COVID-19 transmission. At the beginning of the outbreak, it was speculated that COVID-19 may decrease with increasing air temperature as the outbreak occurred in the winter months[93]. Additionally, air temperature was relatively low in those months in comparison with Spring and/or Summer months. Accordingly, Zhou and Xie[94] demonstrated there is no concrete evidence of a decrease in COVID-19 when ambient temperature increases. Recently, Ma *et al*[95] indicated the positive influence of temperature and humidity on COVID-19 *i.e.*, increase in temperature and humidity decreases the number of COVID-19 deaths. This study was also conducted in same time period (January-February) as the study by Zhou and Xie[94]. A similar positive influence of meteorological factors on COVID-19 in various countries[96,97] was demonstrated. In addition to meteorological factors, Ramadhan[96] highlighted very high mobility and high density of people resulted in fast transmission of COVID-19 in Jakarta.

Influence on air quality

COVID-19 transmission has a direct impact on air quality namely particulate matter, SOx, NOx and carbon, *etc*. Standard air quality is essential in maintaining human health. However, almost 91% of the world's population lives in very poor air quality that exceeds the permissible limits[98], resulting in approximately 8% of deaths globally mainly in Asia, Africa and parts of Europe[98]. Coccia[99] demonstrated that cities (North Italy) with poor air quality (PM_{10} or ozone) increased the probability of COVID-19, mainly due to air pollution-to-human rather than human-to-human transmission. Another study from the same city indicated that prolonged exposure to poor air quality (PM_{10}, $PM_{2.5}$, O_3, SOx and NO_2) boosts COVID-19 incidence and even death in elderly people who have severe respiratory and cardiovascular disorders[97].

On the other hand, COVID-19 has significantly improved the air quality globally, particularly during lockdown periods due to the cessation of social activity, industrial activity, institutional activity, *etc*. Columbia University reported that the amount of carbon monoxide and carbon dioxide in New York City was reduced by 5% and 10%, respectively. During February 2020, carbon emission was decreased by 25% in China, which was last recorded during the economic crisis of 2008-2009. NASA's OMI instrument measured a 36% reduction in NO_2 concentration in China as well as in Italy, Spain, and France during February 2020 (these countries declared lockdown before other European nations). The level of particulate matter ($PM_{2.5}$) in London, Cardiff, and Bristol was less following the implementation of lockdown. PM induces inflammation in lung cells and exposure to PM increases the susceptibility and severity of COVID-19 symptoms.

In China, there was a profound decline in air pollution (greenhouse gases) during January and February as recorded by NASA using satellite images due to the decrease in industrial, business and transportation activity. Accordingly, the China's Ministry of Ecology and Environment declared that it is 'good quality, air days'.

An approximately 43%, 31%, 10%, and 18% decrease in $PM_{2.5}$, PM_{10}, CO, and NO_2 levels, respectively, were observed in India during COVID-19 lockdown compared to previous years[100]. The AQI was reduced by 44%, 33%, 29%, 15% and 32% in north, south, east, central and western India, respectively. In New Delhi, the AQI was reduced to as low as 93, and in Mumbai it decreased to 90 from 161 and 153, respectively.

Due to quarantine, NO_2 level was reduced by 22.8 $\mu g/m^3$ and 12.9 $\mu g/m^3$ in Wuhan and China, respectively. $PM_{2.5}$ level dropped by 1.4 $\mu g/m^3$ in Wuhan but in another 367 cities it was decreased by 18.9 $\mu g/m^3$[103]. After two weeks of lockdown in Spain, the black carbon and NO_2 level decreased markedly (-45 to -51%)[102]. However, O_3 level increased (+33 to +57%, 8 h daily), probably due to lower titration of O_3 by NO due to lower NOx level[102]. Additionally, the Copernicus Atmosphere Monitoring Service (CAMS) of the European Union observed a drop in $PM_{2.5}$ level during February 2020 in comparison with the previous three years. In China, according to CAMS[103], an approximately 20%–30% decrease in $PM_{2.5}$ was recorded in different parts of China during February 2020 compared with monthly averages in February 2017, 2018 and 2019. It is likely that the improvement in air quality around the globe was recorded due to COVID-19 control measures mainly by lockdown and quarantine[104-108]. During this period the demand for petroleum oil was reduced by 20% worldwide.

Furthermore, different national and international media on 10th February reported increased SO_2 concentration of approximately 1,350 $\mu g/m^3$ in Wuhan and Chongqing cities due to mass cremation of COVID-19 victims based on a screenshot image from *windy.com*. These were the results of the GEOS-5 Model. On the other hand, The Sun showed that this was not certain but mainly due to the cremation of virus-infected victims. Accordingly, The Sun (https://archive.is/ShAfz), *WION* (https://archive.is/Cdz4d) and IndiaTimes (https://timesofindia.indiatimes.com/times-fact-check/news/fact-check-satellite-images-showing-high-levels-of-sulphur-dioxide-indicate-mass-cremations-in-china/articleshow/74130633.cms) demonstrated that the mass cremations in Wuhan and Chongqing cities could be the prime reason for increased SO_2 concentration. Dr Arlindo M da Silva, from the Global Modeling and Assimilation Office, stated that GEOS-5 sulfur dioxide models do not "assimilate real satellite data" to confirm the image of *windy.com.* The China National Environmental Monitoring Center and the Center for Satellite Application on Environment and Ecology and the Chinese Academy of Sciences explained that the SO_2 data fluctuated between 4 and 8 $\mu g/m^3$, which was over 200 times less than the data shown on the website.

Influence on noise level and water quality

Environmental noise produced mainly by industrial or commercial operations, transit

vehicles, and many other sources cause serious health problems in the population [109]. The implementation of quarantine and lockdown due to COVID-19 preventive measures by most governments around the globe has compelled people to stay at home. The use of private and public transportation including trains and planes decreased significantly. Additionally, all commercial activities, shopping complexes and industrial operations stopped almost entirely. Accordingly, it is thought that noise level should have reduced; however, there are currently no studies on this issue. Most studies are confined to air quality assessment. Therefore, more attention should be focused on this environmental aspect.

Water quality in freshwater and marine ecosystems is also expected to improve globally. The lack of tourists, as a result of social distancing, has caused a significant change in beaches around the world. Coastal areas are important natural assets, which provide recreation and tourism, and fishing activities. These services are crucial for the nutrition and survival of coastal animals and human communities, and impart intrinsic values[110]. The lack of tourists has resulted in less pollution, especially plastics and wastes as well as reduced drainage volume into water bodies. A lower pollution level in aquatic ecosystems improves the health of the ecosystem by improving the health of aquatic organisms. In undisturbed habitats, olive ridley turtles were able to lay their eggs in Odisha's Gahirmatha beach and Rushikulya roockery. A number of dolphins were observed jumping in the water at the Marine Drive of Mumbai in the Arabian Sea, and the Canals of Venice are now full of fish and dolphins, as the water has sufficient time for sediments to settle to the bottom. According to Sunita Narain, the environmental activist, also the Director General of the Centre for Science and Environment (CSE), explained that, *"Right after this health crisis subsides, it is imperative to get the economy back in shape. People need to get back to work and continue leading their lives. This is just a phase. People can learn from it. However, we require long-term solutions like that of the utilization of clean energy, conservation of forests, and efficient waste management systems in order to see real impact."* According to R. Ramamurthy, COVID-19 is an eye-opener. For example, beaches such as those of Acapulco (Mexico), Barcelona (Spain), or Salinas (Ecuador) are now cleaner with crystal clear waters[101]. This aspect also needs further study to understand the impact of COVID-19.

Influence on waste generation and waste recycling

A number of environmental issues such as air and water pollution, soil erosion, and deforestation are responsible for direct or indirect generation of organic and inorganic waste[111]. Home quarantine measures, established across most countries as COVID-19 measures, have expanded online shopping dramatically. Accordingly, online procurement systems enhanced the generation of inorganic waste due to packaging, in addition to enhanced organic waste generation by households. Furthermore, medical waste generation is also high. In Wuhan, around 240 metric tons of medical waste is generated per day since the COVID-19 outbreak, which is too high compared with previous years (average 50 tons)[45]. Calma[112] reported that in countries like the USA garbage generation due to personal protective equipment such as masks and gloves have increased significantly compared with previous years.

Waste recycling is a common and effective way to prevent pollution, save energy, and conserve natural resources; simultaneously, it is a major environmental problem across the globe[113,114]. Although wastes are generated in high volume globally, at present it is impossible for all countries to recycle these wastes due to the further spread of SARS-CoV-2 infection. Accordingly, the USA has closed waste recycling totally due to COVID-19. Affected European countries have also restricted waste management during this outbreak[101]. For example, Italy totally prohibited infected residents from sorting their waste. Industry also seized the use of reusable bags, as single-use plastic can harbor viruses[115]. China has implemented the use of additional disinfectant in wastewater treatment plants to strengthen their disinfection process to prevent the new coronavirus spreading *via* wastewater. However, to date, there is no evidence of the survival of SARS-CoV-2 in drinking water or wastewater [116].

Other indirect influences on the environment

Wildlife is also affected by SARS-CoV-2. In a USA sanctuary, one tiger was reported to be coronavirus positive. In a Chinese sanctuary, two pangolins died due to the virus infection. It also affected the movement of migratory birds. Different migratory birds are now visiting places where they never visited before due to high pollution levels. It has also forced the UN organization to postpone the Annual Climate Change Conference, *i.e.,* COP-26, which was scheduled to be held at Glasgow in the UK in

November 2020.

SOCIAL IMPACTS

COVID-19 outbreaks have adversely affected different sectors of society with big losses globally in terms of both monetary and personal loss, which cannot be accurately estimated. However, some aspects can be addressed here. Globalization is a chain process; therefore, it will collapse if a single chain stops working. In particular, the economy of countries is adversely affected. Functions, especially business meetings, sports events, scientific conferences, running educational institutes, fashion shows, and wedding parties are to be avoided, which has a big social impact on society. In the educational sector, many countries banned the running of schools, colleges and universities as well as students attending classes, which has deprived the students of a good quality education. This loss poses a large problem not only in monetary matters but also a big disadvantage to the students and their families mainly due to psychological stress. Apart from this, the tourism sector and industrial sectors are facing a major problem due to lack of labor. Prices of commodities are increasing, which has had a negative impact on poor people worldwide. Implementation of lockdown has had an enormous negative impact on poor people especially their daily wage as they are unable to earn. According to the ILO, half of permanent employees will be deprived of work, particularly in the Asia and Pacific regions. In India, 90% of workers from unorganized sectors were highly affected. In addition, production in eight major sectors was reduced by 6.5%, which obviously affected the industrial production index. According to an estimate by the IATA there was a loss of about $113 billion during the lockdown period so far. However, the positive effect of social lockdown is spending more time with family members as well as friends but without physical meetings. It has positive effects on health and accordingly improves immunity.

This pandemic has had a serious impact on major festivals around the world, which may lead to secondary epidemic burnout and stress-related absenteeism. The Public Health Department of England has mentioned 14 ways to protect mental health during the pandemic. The WHO has recommended two most effective protocols, the R-TEP (Recent Traumatic Episode Protocol) and G-TEP (Group Traumatic Episode Protocol) to treat the invisible and psychological wounds of trauma in these situations. *'The Lancet'* documented the psychological impact of quarantine in people which included low mood, insomnia, stress, anxiety, anger, irritability, emotional exhaustion, depression and post-traumatic stress symptoms. Some people have a higher risk because of long-term absenteeism from work due to illness and burnout, which has led to a loss of productivity of approximately 35% in these workers (America's State of Mind Report). In the case of patients who are in quarantine with their children they are facing major mental disorders such as trauma-related health disorder.

It is obvious that this pandemic has both long- and short-term implications on public mental health. Poor mental health may be the result of social isolation and loneliness. It is reported that 47% cases showed negative mental health effects due to worry or stress related to coronavirus, in particular, the situation is very pronounced among older adults and households with adolescents. Research has shown that older adults are at higher risk of poor mental health due to loneliness and bereavement. It also showed that job loss enhances depression, anxiety, distress, and low self-esteem and a higher rate of disorders. In the USA, 30 million students and subsequently their families face physical, social, and mental health impairment. During this pandemic, mental health illness among adolescents has been exacerbated, and over 12% of adolescents aged between 12 and 17 years have depression and/or anxiety. Closures of non-essential businesses and disruption to livelihood have a negative impact on mental health. It has been observed that people with low incomes (about 26%) experience major negative mental health impacts (worry, 17% and stress, 14%) compared with high income groups. Presently, the Coronavirus Aid, Relief, and Economic Security Act (CARES Act) endorsed the need for emergency services to improve the mental health conditions of remote people. According to the CDC, people who suffer from chronic illness such as chronic lung disease, asthma, chronic cardiovascular disease, and diabetes are at high risk of severe illness due to COVID-19.
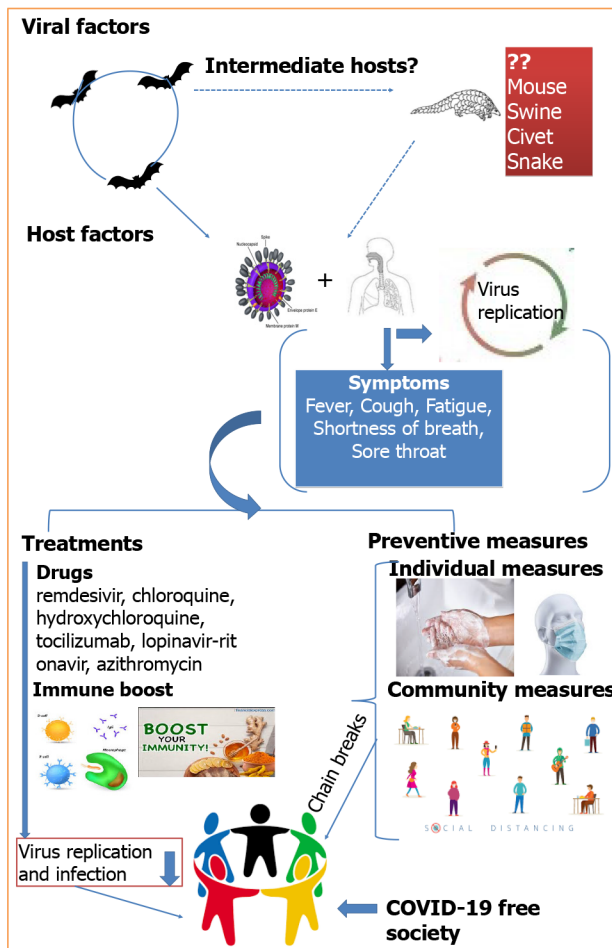
Figure 6 Schematic presentation of the management of coronavirus disease 2019 outbreaks. COVID-19: Coronavirus disease 2019.

RECURRENCE OF COVID19

Although a large number of individuals recover from COVID-19, the incidence of SARS-CoV-2 RNA recurrence has been recorded in various countries. To date, the incidence of recurrent SARS-CoV-2 in recovered individuals ranges from 7.35 to 21.4% [117]. Bonifácio *et al*[118] reported the recurrence of COVID-19 in a female nurse from Brazil. Following her recovery, two family members developed flu-like symptoms and tested positive for COVID-19 by RT-PCR. The next day, the nurse experienced malaise, myalgia, severe headache, fatigue, weakness, feverish sensation, sore throat, anosmia and dysgeusia. Hoang[119] estimated that 15% (95%CI, 12% to 19%) of patients (among 3,644 patients, recovering from COVID-19) tested positive for SARS-CoV-2. In addition, Hoang[119] documented that the proportion was 14% (95%CI, 11% to 17%) in China and 31% (95%CI: 26%-37%) in Korea. Furthermore, he demonstrated that among recurrent cases, 39% (95%CI: 31%-48%) experienced at least one comorbidity. The estimates for times from disease onset to admission, from admission to discharge, and from discharge to RNA positive conversion were 4.8, 16.4, and 10.4 d, respectively [119]. Loconsole *et al*[120] reported the recurrence of COVID-19 in a 48-year-old man from Italy who developed dyspnea and chest pain. The recurrence of COVID-19 has been reported around the world, and raises questions about the durability and quality of immune protection from SARS-CoV-2 as well as the quality of treatment options.

FUTURE PERSPECTIVES

COVID-19 has been an unprecedented disaster around the globe in every aspect, especially environmental health, social and economic aspects. This pandemic originated from bats. People worldwide are consuming different animals including bats, cats, snakes, mice, rats, pigs, dogs, *etc.,* as food stuff. Accordingly, our future generation must be provided with substantial knowledge before consuming these

animals as food. Furthermore, people should be informed about the negative impact of these foods as they may harbor dangerous microbes. Emphasis should be given to providing adequate health care facilities to all people across countries including a greater number of health care systems, health insurance *etc*. This pandemic has highlighted the lack of health care facilities across the globe. Therefore, investment is needed in science and technology to establish specialized research centers to fight against such disasters in the future. In addition, more scientific studies are needed especially on viral diseases, mosquito-and insect-based diseases, bacterial infections, cancer, *etc.,* to combat any future pandemics. Currently, no medicine or vaccines have been identified to treat or eradicate COVID-19. Therefore, efforts should be focused on developing effective medicine or vaccines to treat COVID-19 through technological advancements.

CONCLUSION

This review provides an insight into the current status of COVID-19 (to date) from an environmental perspective. COVID-19 is a zoonotic disease, which originated from bats in Wuhan, China and was declared a pandemic by the WHO. The main symptoms are high fever, cough, shortness of breath and fatigue, which are similar to those of SARS. COVID-19 is highly infectious and transmissible through either aerosol droplets or close contact. The virus has spread to 213 countries/territories with approximately 202608306 confirmed cases and 4293591 deaths up to August 10, 2021. SARS-CoV-2 binds to human ACE2 and infects humans. Elderly people are more prone to SARS-CoV-2 compared to other age groups. To date, there is no specific medicine or vaccines for COVID-19. Currently, drugs such as remdesivir, chloroquine, hydroxychloroquine, tocilizumab, lopinavir-ritonavir, azithromycin, *etc.,* are used to treat SARS-CoV-2 infection. However, no drug is able to induce full recovery in COVID-19 patients. Remdesivir is effective in treating the virus. Recently, the ChAdOx1 vaccine was developed by the University of Oxford's Jenner Institute and the Oxford Vaccine Group. More recently, Russia has developed a coronavirus vaccine, named Sputnik V but these are still in the testing phase. Therefore, boosting the immune response could be an effective way to improve viral resistance. Accordingly, prevention and management are currently the best solution to control COVID-19. Therefore, it is essential that we follow the preventive measures, management and quarantine strictly laid down by the concerned government (Figure 6). Source reduction as an individual protective measure is the best way to control the infection. Lockdown as a social strategy is considered an indirect, but effective alternative tool to control spread of the virus. Additionally, the pandemic has had a direct impact on the environment, society and economy. Therefore, we should promote science and technology to develop vaccines or specific drugs to combat COVID-19.

ACKNOWLEDGEMENTS

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Pancreatic cancer in 2021: What you need to know to win

Valeria Tonini, Manuel Zanni

Valeria Tonini, Department of Medical Sciences and Surgery, University of Bologna- Emergency Surgery Unit, IRCCS Sant'Orsola Hospital, Bologna 40121, Italy

Manuel Zanni, University of Bologna, Emergency Surgery Unit, IRCCS Sant'Orsola Hospital, Bologna 40121, Italy

Corresponding author: Valeria Tonini, MD, PhD, Professor, Surgeon, Surgical Oncologist, Department of Medical Sciences and Surgery, University of Bologna- Emergency Surgery Unit, IRCCS Sant'Orsola Hospital, Via Massarenti 9, Bologna 40121, Italy. valeria.tonini@unibo.it

Abstract

Pancreatic cancer is one of the solid tumors with the worst prognosis. Five-year survival rate is less than 10%. Surgical resection is the only potentially curative treatment, but the tumor is often diagnosed at an advanced stage of the disease and surgery could be performed in a very limited number of patients. Moreover, surgery is still associated with high post-operative morbidity, while other therapies still offer very disappointing results. This article reviews every aspect of pancreatic cancer, focusing on the elements that can improve prognosis. It was written with the aim of describing everything you need to know in 2021 in order to face this difficult challenge.

Key Words: Pancreatic cancer treatment; Advanced pancreatic cancer; Metastatic pancreatic cancer; Pancreatic cancer surgery; Pancreatic cancer chemotherapy; Pancreatic cancer screening

Core Tip: Pancreatic cancer is a very dangerous enemy and the results are still very unsatisfactory. But we have not given up. Research is running fast on many paths, without losing its enthusiasm.The number of articles published on this subject in the last two years is impressive. I have tried to summarize all the most significant data from the different lines of research, ranging from screening and early diagnosis to new developments in surgery and associated therapies. I hope I have succeeded in the task of describing as comprehensively as possible the most promising fields of research available to us today, in order to achieve the improved results we desire.

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INTRODUCTION

Pancreatic cancer is currently the seventh leading cause of cancer death worldwide and the fourth following lung, colorectal and breast cancers in the United States and Europe. It will become the third by 2030. It is an age-related neoplasm and this trend is similar between males and females. In particular the number of both deaths and incident cases peaked at the ages of 65-69 years in males, whereas the peak in females was observed at the ages of 75-79 years[1-4]. The commonly used term "pancreatic cancer" usually refers to ductal adenocarcinoma (PDAC), which represents 85% of all pancreatic tumor[4]. Complete surgical resection significantly prolongs survival, but the tumor is often diagnosed at an advanced stage and only a small percentage of patients are therefore candidates for surgery. Moreover, surgery is still associated with high post-operative morbidity. Despite ongoing developments, PDAC remains one of the most difficult tumors to treat, and the five-year survival rate is less than 10%[5]. There are four fundamental challenges that underlie the high mortality. First, the retroperitoneal location of the pancreas, deep in the abdomen, protects growing tumors from detection. The symptoms are late and therefore the diagnosis is made when the tumor is already in an advanced stage. Second, PDAC has an aggressive biology characterized by early metastasis and 50% of patients has metastatic disease at presentation. In addition, a large number of patients undergoing surgery develop metastases within 4 years. This suggests the presence of micrometastasis in apparently localized cases[6]. Third, pancreatic cancer dramatically weakens patients, limiting their ability to withstand aggressive treatments. Finally, it shows resistance to many antineoplastic therapies[7,8]. Advances in prevention, screening, early detection, and therapy, particularly on new frontiers, are essential to improve outcomes. This article has been written with the aim of describing everything you need to know in 2021 in order to face this difficult challenge.

NON-FAMILIAL RISK FACTORS AND PREVENTION

Identification of risk factors, high-risk populations and early detection markers is the first and crucial step to change the pancreatic cancer horizon[9]. PDAC incidence rates are nearly four times higher in high-income countries such as the United States and Western European countries than in middle- and low-income countries[3]. The different incidence seems to be related with different lifestyles.

Obesity, smoking, alcohol consumption and type 2 diabetes are considered non-familial risk factors for pancreatic cancer. Chronic pancreatitis, cystic fibrosis and intraductal papillary mucinous neoplasm (IPMN) should also be considered. An increased risk of pancreatic cancer has been observed following gastrectomy[10-17].

One-third of all cancers could have been prevented through lifestyle correction[18]. A 2020 European prospective study (EPIC) evaluated the association between the healthy lifestyle index score and PDAC[19-22]. Healthy lifestyle habits were inversely related to the risk of PDAC. Adherence to healthy behaviors, corresponding to a three-point increase in the score, was associated with a 16%-23% lower risk. The result summarizes many previous studies[23-29] and support the adoption of healthy lifestyles in PDAC prevention.

A recent nutrigenomic study has highlighted nutrients capable of preventing cancer through epigenetic modifications. An optimal diet should include omega 3 fatty acids, polyphenols, folic acid, selenium and zinc. Particularly important for PDAC prevention could be the epigallocatechin, a polyphenol from tea and green tea[30,31].

Data linking type 2 diabetes with pancreatic cancer suggest that the new onset of diabetes in a lean older adult should prompt consideration of PDAC. This is even more valid if new-onset diabetes is associated with unintentional weight loss[32-34]. A Mayo Clinic study evaluated the use of computed tomography (CT) at the time of diabetes diagnosis in otherwise asymptomatic patients. A higher likelihood of showing potentially resectable tumors was observed compared with scans performed six months later[32]. However, CT screening of all elderly subjects with new-onset

diabetes is not feasible[33]. With the identification of these characteristics that differentiate pancreatic cancer-associated diabetes from other cases of new-onset diabetes, perhaps the guidelines will updacate[35].

HEREDITARY RISKS FACTORS

PDAC can be hereditary. There are two categories of inherited risk for PDAC: Genetic syndromes (20% of cases) and familial pancreatic cancer (80%). Familial pancreatic cancer is defined as a predisposition that is based on familial clustering in families in which there is at least one pair of first-degree relative (FDR) relatives with PDAC in the absence of a known genetic syndrome. Genetic syndromes that predispose to pancreatic cancer are listed in Table 1. Table 1 also shows in parentheses the frequencies of mutated genes in PDAC patients[36-42].

Knowledge of inherited risk factors is important because it allows us an effective stratification and management of patients. According to American Society of Clinical Oncology and National Comprehensive Cancer Network guidelines, all patients diagnosed with PDAC should be evaluated to understand if there is a risk of familial predisposition to cancer. All patients should undergo risk assessment for syndromes associated with an increased risk of PDAC. Germline genetic testing is recommended for patients with PDAC and an unremarkable family history[43,44].

SCREENING

Screening aims to detect preinvasive lesions (IPMNs and pancreatic intraepithelial neoplasias) with high-grade neoplastic changes and early invasive tumors that are more amenable to potentially curative resection[45-49].

Candidates for screening

(1) Patients with Peutz-Jeghers syndrome or CDKN2A mutation, regardless of family history; (2) BRCA2 mutation with at least one affected FDR or at least two affected relatives of any degree; (3) BRCA1, partner and localizer of BRCA2 (PALB2), ataxia-telangiectasia mutated (ATM), and Lynch syndrome mutation carriers with one or more affected FDRs; (4) Hereditary pancreatitis with a PRSS1 mutation; and (5) Regardless of gene mutation status: (a) At least three affected relatives on the same side of the family, of whom at least one is an FDR of the individual being considered for surveillance; (b) At least two affected relatives who are FDRs of each other, of whom at least one is an FDR of the individual being considered for surveillance; and (c) At least two affected relatives on the same side of the family, of whom at least one is an FDR of the individual being considered for surveillance.

General population-based screening for average-risk patients is not recommended [33] because the average lifetime risk for developing PDAC is too low[49].

Screening modality

The current recommendation provides for the execution of endoscopic ultrasonography (EUS) or magnetic resonance imaging (MRI)/magnetic resonance cholangiopancreatography (MRCP). It has been demonstrated that they detect more lesion as compared with CT scan[50]. Screening is recommended at age 50 years or 10 years younger than the youngest relative with PDAC in familial pancreatic cancer relatives. In other cases, screening is carried out between 35 and 45 years. For patients with a normal pancreas on imaging, repeat the procedure every year alternating EUS and MRCP. The age for stopping screening should be individualized based on each patient's medical status, life expectancy, and preferences.

SURGICAL RESECTION FOR IPMNS AND OTHER CYSTIC LESIONS

Surgical resection is indicated in patients with any of the following[45]: (1) Solid pancreatic lesion ≥ 5 mm of indeterminate pathology or if additional evaluation does not yield a definitive preoperative diagnosis; (2) Any positive fine-needle aspiration (FNA) result, except for a pancreatic neuroendocrine tumor; (3) Main-duct IPMNs with any one of the following: (a) Main pancreatic duct dilation of ≥ 10 mm; (b) Main pancreatic duct stricture; or (c) Mural nodules; (4) Branch duct IPMNs (BD-IPMNs)

Table 1 Genetic syndromes predisposing to pancreatic cancer (the frequency of mutated genes among patients with pancreatic ductal adenocarcinoma is indicated in brackets)

Genetic syndrome	Mutated genes
Hereditary breast/ovarian cancer syndrome[36,37]	*BRCA1* (0.7%), *BRCA2* (1.4%), *PALB2* (1%)
Familial atypical multiple mole melanoma syndrome[38]	*CDKN2A* (0.7%)
Peutz-Jeghers syndrome[39]	*STK11*
Familial adenomatous polyposis	*APC* (0.4%)
Lynch syndrome[40]	*MLH1, MSH2* (0.4%), *PMS2* (0.3%)
Hereditary pancreatitis[41]	*PRSS1, SPINK1*
Ataxia telangectasia[42]	*ATM* (1.4%)
Li-Fraumeni syndrome[42]	*P53* (0.4%)

with any one of the following: (a) Rapid growth (> 5 mm over six months); (b) Mural nodules or an enhancing solid component; (c) Abrupt main pancreatic duct caliber change with distal atrophy (even if no mass is visible); (d) Main pancreatic duct dilation of ≥ 10 mm; (e) Positive cytology; or (f) Associated symptoms of pancreatitis, jaundice, or pancreatic-type pain; or (5) Asymptomatic main pancreatic duct stricture with an associated suspicious mass.

For patients who do not meet these criteria for surgery, repeat imaging in three months if worrisome features are present[47,51]. Worrisome features include the following: (1) Solid lesion with main pancreatic duct size of 5 mm to 9 mm in diameter; (2) Main pancreatic duct stricture and/or dilation ≥ 6 mm of unknown etiology without an associated mass; and (3) Solid lesion < 5 mm of uncertain significance.

Repeat imaging in six months is recommended for patients who have the following imaging abnormalities: (1) Cystic lesion (presumed BD-IPMN) ≥ 3 cm in size; (2) Cystic lesion with associated main pancreatic duct 5 mm to 9 mm; (3) Cystic lesion associated with lymphadenopathy; (4) Cyst growth rate of ≥ 5 mm in two years; and (5) Increased serum carbohydrate antigen 19-9 (CA 19-9).

Individuals without worrisome features of malignancy should undergo repeat imaging in 12 mo[47,51].

Screening/surveillance should be continued until the patient is no longer a surgical candidate.

A 2020 paper analyzed the benefits of screening. Nine out of 10 screen-detected PDAC were resectable, with a three-year survival of 85%, compared with 25% in PDAC detected outside surveillance. With continued follow-up of patients with resectable PDAC, the five-year overall survival (OS) rate was 60%[49].

BIOMARKERS AND EARLY DETECTION

Different biomarkers are being evaluated to improve early diagnosis of tumor not detectable by imaging and to differentiate cancer and high-grade dysplasia from benign disease[52].

Blood tests

The most useful serum tumor marker for PDAC is CA 19-9. It is recommended adding this test when there are worrisome features on abdominal imaging. The sensitivity and the specificity of elevated CA 19-9 to detect PDAC are 79% and 82%, respectively[53-55]. It becomes more precise when used in combination with CA 125[56,57]. Other carbohydrate markers, such as CA 50, CA 72.4 and CA 242, were extensively analyzed in PDAC patients. Although they exhibited less sensitivity than CA 19-9 for the diagnosis, they improved specificity[58-61]. Satake and Takeuchi[62] also studied SPan-1 and DUPAN-2. SPan-1 has a high sensitivity for PDAC (81.4%), but the specificity (67.5%) and diagnostic accuracy (71%) are lower than those of CA19-9. SPan-1 may be considered as an additional useful serum marker, but it does not significantly improve the diagnostic accuracy obtained with CA 19-9. In contrast, DUPAN-2 has a high specificity (85.3%) and low sensitivity (47.7%). Furthermore, it seems that serum levels of DUPAN-2 are influenced by liver function. SPan-1 and DUPAN-2 unfortunately have not yet shown the sensitivity and specificity needed to

be used for early detection[62,63].

A huge step forward in the early detection of pancreatic cancer could come from studying cell-free DNA (cfDNA), which consists of circulating double-stranded DNA molecules that can be found in plasma or blood serum. From the analysis of these molecules, it is possible to understand if we are in the presence of a tumor DNA and to go back to the tissue of origin. By analyzing the methylation status of two genes in cfDNA, ADAMTS1 and BNC1, early stage cancer can be identified with a sensitivity of 94.8% and a specificity of 91.6%[64].

Innovative discoveries have also been made in the field of RNA. Abnormal microRNA expressions are potential diagnostic markers for several cancers, including PDAC. Multiple microRNA tests performed in combination with CA 19-9 can improve diagnostic accuracy, particularly miR-216[65-69]. Permuth *et al*[70] demonstrated that a combination of eight lncRNAs helps in the differential diagnosis between malignant and non-malignant IPMNs. Furthermore, three lncRNAs (HAND2-AS1, CTD-2033D15.2, and lncRNA-TGF) could be exploited as early diagnostic biomarkers of IPMN[71,72].

Pancreatic juice and pancreatic cyst fluid

Pancreatic juice collected at the time of ERCP and cyst fluid obtained by EUS-guided FNA can be analyzed for molecular markers. These procedures also have broad potential in terms of early diagnosis of PDAC. Next-generation sequencing can be performed at low cost to detect low-frequency mutations. Potential markers include mutant GNAS (specific for IPMNs) and mutant KRAS. TP53, SMAD4, PIK3CA, PTEN, and AKT1 mutants are also useful as they correlate with IPMN-associated tumors[73-75]. According to Suenaga *et al*[76], a pancreatic juice collection, to ensure optimal yield of mutations for pancreatic screening assays, should be performed 10 min after secretin administration. The authors detected 40 patients with KRAS mutations in pancreatic juice out of 45 undergoing surveillance with EUS, reconfirming the usefulness of these analysis[76].

There are many other biomarkers that are currently being validated for clinical use, such as mucins (MUC). Normal pancreatic ductal epithelium expresses low levels of MUC, while an upregulation of MUC occurs in BD-IPMNs and more pronounced changes in expression in PDAC. Normal pancreatic ductal epithelium expresses low levels of MUC, while upregulation of MUC occurs in BD-IPMN and PDAC[77-83]. The analysis of mucin changes in the fluid of pancreatic cysts allows us to differentiate mucinous from non-mucinous pancreatic cysts with high sensitivity and specificity and to diagnose PDACs associated with IPMN at an early stage[84]. MUC4 and MUC16 have been reported to be 100% specific for PDAC, while associated with sensitivities of 63% and 67%, respectively[85].

Interesting data were reported about interleukins (IL). Higher concentrations of IL-1b, IL-5, and IL-8 have been identified in cystic lesions with high grade dysplasia or malignancy[86]. IL-1b is a potentially useful factor in differentiating high-risk from low-risk pancreatic cysts.

The Das-1 monoclonal antibody is also capable of detecting pancreatic cysts at risk of malignancy with high levels of sensitivity (88%) and specificity (98%)[87,88]. Das-1, IL and MUC could be used in conjunction with clinical guidelines to identify patients at risk for malignancy.

Saliva

Saliva is a suitable substance for screening because it is obtained in a simple and noninvasive manner. In addition, salivary mRNA is relatively stable and informative for disease diagnosis, including cancer. Zhang *et al*[89] identified 7 up-regulated genes (MBD3L2, KRAS, STIM2, DMXL2, ACRV1, DMD, and CABLES1) and 5 down-regulated genes (TK2, GLTSCR2, CDKL3, TPT1, and DPM1) in subjects with PDAC compared with healthy controls or those with chronic pancreatitis. A combination of 4 mRNAs (MBD3L2, KRAS, ACRV1, and DPM1) can discriminate diseased patients from healthy ones with sensitivity and specificity over 90%[89]. Xie *et al*[90] worked on miR-3679-5p and miR-940. The former is down-regulated, while the latter is up-regulated in PDAC patients compared to controls. The combination of the two miRNAs identifies diseased subjects with sensitivity and specificity of 70%. The same group evaluated the expression of salivary long non-coding RNAs (lincRNAs). They identified HOTAIR and PV1T as significantly up-regulated lincRNAs in the PDAC group compared with controls and benign pancreatic tumors. The combination of salivary HOTAIR and PVT1 differentiated PDAC from healthy controls with a sensitivity of 78.2% and specificity of 90.9% and PDAC from benign tumors with a sensitivity of 81.8% and specificity of 95%[90,91]. Another important mRNA studied in

serum, urine, and saliva is MIR1246. Salivary expression of miR-1246 is related to serum CA19-9 levels[92]. Significantly higher expression of MIR1246 in serum and urine was observed in patients with cancer compared with healthy controls. Ishige *et al* [93] observed an AUC for MIR1246 in serum of 0.87 (sensitivity, 92.3%; specificity, 73.3%), for MIR1246 in urine of 0.90 (sensitivity, 90.2%; specificity, 83.3%). Combining the expression of MIR1246 in serum and urine resulted in a sensitivity of 85%. These results indicate that MIR246 may be a useful diagnostic biomarker for pancreatic cancer. The accuracy further increases if we consider miR-1246 and miR-4644 simultaneously[92].

Urine

Several biomarkers have also been evaluated in urine. Radon *et al*[94] used three protein biomarkers (REG1A, TFF1 and LYVE1) to form a powerful urinary panel that can detect patients with stages I-II PDAC, with over 90% accuracy. Brezgyte *et al*[95] found four miRNAs (miR-143, miR-204 and miR-223) in significantly higher amounts and one miRNA (miR-30e) in lower amounts in the urine of PDAC Stage I patients compared to the healthy population. These miRNAs (except for miR-204) also showed a decreased expression in Stage II-IV compared to Stage I[95]. However, more studies are needed to validate the clinical utility of these biomarkers.

CLINICAL FEATURES

The presenting symptoms in patients with PDAC varies according to location. Tumors in the body and tail present with pain and weight loss, while tumor of the head cause jaundice and steatorrhea[96]. Pain associated with PDAC is usually insidious, visceral, generally epigastric, radiating to the sides or straight through to the back. It is worse by eating or lying supine at night. Rarely, it develops acutely on account of acute pancreatitis due to tumoral occlusion of the main pancreatic duct[97]. Pancreatic cancer may result in an onset of diabetes mellitus[98,99]. The hypercoagulable state that accompanies PDAC can result in Trousseau syndrome, which consists of superficial, sometimes migratory thrombophlebitis[100]. Thromboembolic complications occur more commonly in patients with tumors arising in the tail or body of the pancreas[101]. Skin manifestations could occur as paraneoplastic phenomena[102]. Rarely, erythematous subcutaneous areas of nodular fat necrosis (pancreatic panniculitis), typically located on the legs, may be evident. It is more frequent in patients with the acinar cell variant of PDAC. It is not pathognomonic for an PDAC, because it has also been described in associated with pancreatic neuroendocrine tumors, IPMNs and chronic pancreatitis[103].

When assessing symptoms, it should be borne in mind that PDAC tends to infiltrate nearby organs and structures and to give distant metastases very early. Local extension typically involves adjacent structures, such as the duodenum, the portal vein (PV), or the superior mesenteric vessels. PDAC also show a striking tendency toward perineural invasion, both within and beyond the pancreas. The difficulty in achieving a wide resection margin due to the proximity to the vessels accounts for the fact that the retroperitoneal tissue behind the head of the pancreas represents the most common site of disease recurrence. Sometimes the tumor extends to the spleen, adrenal glands, vertebral column, transverse colon, and/or stomach. In these cases, tumors are not resectable. Tumor may metastasize to regional peripancreatic lymph nodes or less often to distant lymph node, peri-gastric, mesenteric, omental or porta-hepatic nodes. Distant metastasis may affect the liver, peritoneum, lungs, and less frequently, bone. Signs of advanced, incurable disease include an abdominal mass, ascites, Virchow's node, Sister Mary Joseph's node or a palpable rectal shelf. Pancreatic cancer is the origin of a cutaneous metastasis to the umbilicus in 7% to 9% of cases[104].

DIAGNOSIS

CT

CT is considered the gold standard for pancreatic cancer's diagnosis. Protocol pancreatic CT is performed for evaluation of suspected PDAC or if a routine CT scan was not sufficient for initial staging[105,106]. This protocol consists of evaluating the patient at different stages of contrast injection. The arterial phase provides excellent opacification of the celiac axis, superior mesenteric artery (SMA), and peripancreatic

arteries. An attenuation difference between tumor and normal pancreas is best achieved after peak enhancement of the aorta in the arterial phase but before the one of the liver, in the portal venous phase. This is sometimes termed the "pancreatic phase". The portal venous phase provides better enhancement of the superior mesenteric vein (SMV), splenic and PVs. In addition, peak hepatic enhancement, which optimizes the detection of hepatic metastases, also occurs in the portal venous phase[107,108].

The typical CT appearance of a PDAC is an ill-defined hypoattenuating mass within the pancreas. Smaller lesions may be iso-attenuating, making difficult their identification[109]. Secondary signs of PDAC include a dilatation of the pancreatic duct or common bile duct, parenchymal atrophy, and contour abnormalities. Dilation of both the pancreatic duct and the common bile duct, commonly referred to as the "double duct sign" is not diagnostic for a pancreatic head malignancy[110]. Routine preoperative CT helps to identify hepatic vascular anatomy and prepares the surgeon for any potential vascular anomalies. It can detect hemodynamically significant arterial stenosis[111]. The contrast-enhanced CT scan is the best technique for PDAC staging [112] and it is essential to detect vascular invasion. CT criteria for vascular invasion include arterial embedment in the tumor mass or venous obliteration, tumor involvement exceeding one-half the circumference of the vessel, vessel wall irregularity, vessel caliber stenosis, or a "teardrop" sign of the SMV[113]. Classic CT criteria for vascular involvement are not reliable in patients who have undergone neoadjuvant therapy with a highly active chemotherapy combination such as mFOLFIRINOX (mFFX). In such cases, surgical exploration may be the only method to assess resectability[114].

MRI

Contrast-enhanced MRI of the pancreas may be useful in staging patients at initial presentation. MRI is the best technique for detection of small liver metastases[115]. The importance of MRI also lies in the ability to diagnose pancreatic cancer by identifying changes in the body that indicate systemic effects of PDAC. It has been well recognized that anorexia, sarcopenia, and weight loss are hallmarks of PDAC. Consequently, it can be used to measure adipose and muscle mass in high-risk populations to identify early disease[116-118].

EUS

EUS is considered the most sensitive method to detect early neoplasia in the pancreas. PDAC on EUS appears as a hypoechoic mass, typically with dilation of the proximal pancreatic duct and the border of the lesion may have an irregular contour. This is the best accurate technique for local T and N staging, and for predicting vascular invasion. However, EUS is inferior to CT for evaluation of distant metastases. In addition, the specificity of EUS for excluding vascular invasion in small tumors is limited, particularly when inflammatory changes are present[119].

EUS is mainly used as part of the workup to obtain fine needle aspiration or biopsy material in patients suspected of having a PDAC[120]. EUS is not readily accessible and as a result is considered a complementary modality to the pancreatic protocol CT. Emerging area for endoscopic ultrasound includes the incorporation of elastography. Elastography shows significantly lower elasticity values for PDAC than for normal pancreatic tissue[121]. Incorporation of elastography in the evaluation of solid pancreatic lesions improves diagnostic accuracy[122,123].

Endoscopic retrograde cholangiopancreatography

A meta-analysis demonstrated a 92% sensitivity and 96% specificity of endoscopic retrograde cholangiopancreatography (ERCP) in the diagnosis of PDAC[124]. Findings suggestive of a malignant tumor of the pancreatic head include stenosis or obstruction of the common and pancreatic bile ducts (the "double duct" sign), a pancreatic duct stenosis greater than 1 cm in length, and pancreatic duct obstruction. In addition, ERCP provides an opportunity to collect tissue samples for cytohistologic analysis [124].

Some early-stage pancreatic tumors are not detected by CT, MRI, or EUS. Especially for carcinoma in situ, localized stenosis of the main pancreatic duct is often the only imaging finding. Pancreatic duct imaging evaluation by ERCP and subsequent pancreatic juice cytology are critical for diagnosis.

On the other hand, ERCP is an invasive procedure that can cause acute pancreatitis, bleeding, and cholangitis. Consequently, it has purely therapeutic value for patients with cholestasis due to tumor obstruction of the biliary system and require placement of a biliary stent[125].

Positron emission tomography

The role of positron emission tomography (PET) is limited for PDAC due to the high number of false positives and false negatives[126]. However, the degree of fluorodeoxyglucose (FDG) uptake correlates with histopathology, aggressiveness, and metastatic potential[127,128]. According to a meta-analysis, PET/CT is more accurate than CT in detecting distant metastases. Preoperatively, it may therefore be useful in avoiding unnecessary resection if unexpected metastases are found[129,130]. After treatment, FDG-PET is instead used to detect residual or recurrent cancer. It can also be applied to assess and monitor response to therapy in unresectable or metastatic disease[127,131].

Other molecular imaging agents including overexpressed proteins, signaling pathways, and tumor stroma may also be used[132]. Among these, promising results appear to involve 68Ga-cicratide, an integrin αvβ6-specific radiotracer, which has favorable pharmacokinetics and is capable of detecting pancreatic cancer lesions and monitoring response to therapy[133]. Another molecular imaging method that is of interest for early detection is hyperpolarized MRI. It can identify metabolic aberrations in the pancreas that indicate preneoplasia[134].

Staging laparoscopy

Sub-centimeter metastases of the liver or peritoneum that are rarely visible by CT, MRI or PET may be visualized laparoscopically. Up to one-third of patients thought to be resectable by imaging will be found to be unresectable based upon laparoscopic findings[135,136].

Some experts suggest a selective approach to staging laparoscopy, limiting the procedure to those with the highest likelihood of occult metastatic disease[137,138]. First, this includes tumors of the body or tail of the pancreas that appear potentially resectable by CT scan. Second, it includes large (> 3 cm) primary tumors and patients with a high initial CA 19-9 level (> 100 units/mL)[139].

Biopsy

Biopsy of a pancreatic mass can be performed either percutaneously or *via* EUS. EUS-guided FNA is the best modality for obtaining a tissue diagnosis. EUS-FNA is a safe method with a 0.98% morbidity and a 0.02% mortality. Although the most common adverse events of EUS-FNA include pancreatitis and postprocedural pain, there is also some concern regarding tumor cell seeding[140]. According to a study by Yane *et al* [141] the cumulative needle tract seeding rate at five years was 3.8%. However the preoperative EUS-FNA has no negative effect on recurrence-free survival and OS.

In many cases, the diagnosis will not yet be histologically confirmed. Once PDAC is suspected on imaging studies, the next step is generally a staging evaluation rather than biopsy. Patients who are fit for major surgery and who appear to have potentially resectable PDAC, they do not necessarily need a biopsy before surgery. Biopsy could be indicated if there is evidence of systemic spread or local evidence of unresectability on staging studies. It is also indicated if the patient is unfit for major surgery or if other diagnoses need to be excluded[142,143].

Pancreatic incidentaloma

A 2014 systematic review[144] evaluated 5 studies enrolling patients with incidentalomas and concluded that most solid lesions are malignant. Histologic definition of a solid lesion of the pancreas should be the first option, as opposed to radiologic monitoring alone. It is important to avoid operating on benign solid lesions such as chronic focal pancreatitis or autoimmune pancreatitis.

In case of cystic lesion, surgery is the first option for cystadenomamucinous and IPMN with high-risk stigmata. A recent review defined high-risk stigmata as the presence of obstructive jaundice, vascularized mural nodules ≥ 5 mm, main duct diameter ≥ 10 mm[145].

STAGING

The goal of the staging workup is to delineate the extent of disease spread and to identify patients who are eligible for resection with curative intent. Patients with PDAC can be staged according to the eighth edition of TNM system of American Joint Committee on Cancer (AJCC). However, most clinicians use a four-tiered staging system including resectable, borderline resectable, locally advanced (LA), and

metastatic cancer[146,147] (Table 2). In 2017, a classification was published, by the International Association of Pancreatology, which redefines the concept of resectability in relation to biological risk and patient conditions[148]. Table 3 summarizes the different resectability criteria assumed by the different scientific societies.

SURGERY

Surgical resection is the only potentially curative treatment. Unfortunately, PDAC is often diagnosed at an advanced stage and radical surgery could be performed in a very limited number of patients. The surgical interventions that can be performed are different depending on the tumour location and extension. In all cases the operation involves the removal of the tumour with free margins and at least twelve lymph nodes, which are necessary for staging. Tumors of the head require more complex operations, which still have a high operative morbidity. In high-frequency surgical centres mortality after pancreatoduodenectomy (PD) is now less than 2%, but post-operative morbidity remains high, 30%-50%. Anastomotic dehiscences, are the most serious post-operative complication. They are difficult to manage and are unfortunately associated with a still high mortality rate. Tumors of the tail and body require easier operations than head tumors, with a low operative morbidity and mortality. Unfortunately, because of their late symptomatology, they are more frequently unresectable.

Pancreaticoduodenectomy

PD is the classic operation performed for pancreatic tumors of the head or uncinate process. Conventional pancreaticoduodenectomy involves removal of the pancreatic head, duodenum, first 15 cm of the jejunum, common bile duct, gallbladder, and a partial gastrectomy. It is a complex procedure and patients may experience several complications. These complications could be intra-operative or post-operative[149, 150].

The most important intraoperative complication of PD is bleeding. Most patients undergoing PD for PDAC have an obstructive jaundice with associated coagulopathy. Bleeding can occur from multiple sites during the various phases of mobilization and resection, so hemostasis must be monitored and assured before reconstruction begins.

Postoperative complications can be further divided into short-term and long-term complications. The short-term ones are pancreatic fistula, delayed gastric emptying, and postoperative bleeding. The long-term ones are biliary stenosis and cholangitis, pancreatitis, peptic ulcer disease, small bowel obstruction, and incisional hernia[149, 150].

Modifications of the conventional PD procedure have been developed in an attempt to improve outcomes or minimize the morbidity associated with this operation. The pylorus-preserving pancreaticoduodenectomy preserves the gastric antrum, pylorus, and proximal 3 cm to 6 cm of the duodenum. It can decrease the incidence of post-operative dumping, marginal ulceration, and bile reflux gastritis, without negative effect on the morbidity, mortality and long-term survival[151]. Instead, the subtotal stomach-preserving pancreaticoduodenectomy is performed with the aims to preserve as much stomach as possible, minimizing the delayed gastric emptying that are associated with preserving the pyloric ring in the face of vagal denervation. In this procedure, the duodenum, pylorus, and 1 cm to 2 cm of stomach are resected with the pancreatic specimen. Although described, this modification has yet to be validated, and it is uncommonly performed[152].

The "Artery-first" approach is a surgical technique or set of techniques that have in common the dissection of the main arterial vasculature involved in pancreatic cancer, prior to performing any irreversible surgical step (transection of the pancreatic neck or bile duct division). The "Artery-first" approach has the potential to reduce blood loss and increase R0 resection rates and OS, as demonstrated in a recent meta-analysis[153].

Modified child reconstruction aims to reduce the incidence of cholangitis due to digestive reflux through hepatic-digiunal anastomosis. In case of pancreatic-digiunal anastomosis, the hepatic-digiunal anastomosis is made downstream of the previous one. In case of pancreatico-gastric anastomosis, the hepatico-digiunal anastomosis is made near the previously closed loop. Whatever the type of pancreatico-digestive anastomosis, the digestive anastomosis (gastro-digiunal or duodeno-digiunal) is made 60 cm downstream of the hepatico-digiunal anastomosis, to reduce digestive reflux into the biliary tract.

Table 2 Resectability criteria

Resectability status		Resectable	Borderline resectable	Locally advanced
Arterial involvement	Celiac artery	None	≤ 180°; > 180°, without involvement of aorta o GDA (body/tail)	>180° (head/uncinate); Solid tumor contact with CA and aorta
	SMA common hepatic artery	None	≤ 180°; Solit tumor contact without extension into CA or hepatic artery biforcation	> 180°
Venous involvement (portal vein/smv)		None; ≤ 180° contact without contour irregularity	> 180°; ≤ 180° with contour irregularity or thrombosis, with reconstructible PV/SMV; Solid tumor contact with IVC	Unreconstractible PV/SMV due to tumor involvement or occlusion

CA: Celiac artery; GDA: Gastroduodenal artery; IVC: Inferior vena cava; PV: Portal vein; SMA: Superior mesenteric artery; SMV: Superior mesenteric vein.

Table 3 Resectability criteria and societies

Vessel involvement	NCCN 2019	MDACC	ACTO	AHPBA/SSAT/SSO
CA abutment (≤ 180°)	Borderline	Borderline	Borderline	Unresectable
CA encasement (> 180°)	Borderline (body/tail); locally advanced (head/uncinate)	Unresectable	Unresectable	Unresectable
SMA abutment (< 180°); SMA encasement (> 180°); CHA abutment or encasement	Borderline; Locally advanced; Borderline	Borderline; Unresectable; Borderline	Borderline; Unresectable; Borderline	Borderline; Unresectable; Borderline
PV/SMV encasement (> 180°) or abutment (≤ 180°) with contour abnormality	Borderline	Borderline	Borderline	Borderline

ACTO: Alliance for Clinical Trials in Oncology; AHPBA: American Hepato-Pancreato-Biliary Association; CA: Celiac artery; CHA: Common hepatic artery; MDACC: The University of Texas MD Anderson Cancer Center; NCCN: National Comprehensive Cancer Network; PV: Portal vein; SMA: Superior mesenteric artery; SMV: Superior mesenteric vein; SSAT: Society for Surgery of the Alimentary Tract; SSO: Society for Surgical Oncology.

Post-operative pancreatic fistula (POPF) is the main and most frequent complication after pancreatic resection surgery. It is caused by leakage of pancreatic juice into the abdominal cavity, which is collected and conveyed to the outside by the drains normally placed at the end of surgery or during postoperative care if necessary. The diagnosis is made on the basis of the quality of the drainage fluid (varying from transparent to coffee-colored to brown) and the value of amylase in the fluid itself, greater than three times the normal limit of serum amylase[149,150].

POPFs are classified into three grades based on clinical impact. Grade A fistulas do not involve any special intervention and do not significantly modify the postoperative hospital stay. Grade B fistulas require a longer postoperative stay, the retention of surgical drains, the possible placement of additional drains under radiological guidance, antibiotic therapy and the use of artificial nutrition (enteral or parenteral). In grade C fistulas, reoperation is required to resolve the complication.

Several methods have been used to reduce the risk of pancreatic fistula, including the use of octreotide, pancreatic duct occlusion, pancreatic duct stenting, pancreaticoje-junostomy, anastomosis modification, and pancreaticogastrostomy. The efficacy of octreotide in preventing POPF is still a hotly debated topic. According to a 2020 meta-analysis[154], somatostatin analogs did not affect POPF after PD, but rather appeared to be associated with a lower rate of POPF after distal pancreatectomy. Therefore, reconstruction technique is the most important factor in reducing the risk of this complication. Recently, interesting results concern the blumgart anastomosis (BA), which combines the duct-mucosal principle with the transpancreatic U-suture technique. Unlike other duct-mucosal anastomoses such as Cattell-Warren anastomosis and Kakita anastomosis, U-shaped sutures and horizontal mattress suture technique are used in BA. The difference is that Blumgart's technique involves the placement of 3 to 6 transpancreatic and digestive seromuscular U-sutures to bring the pancreatic stump and jejunum closer together. A meta-analysis conducted by Ricci *et al* [155] demonstrated the ability of BA to reduce the risk of pancreatic fistula compared with non-blumgart duct-to-mucosal anastomoses (non-BA DtoM). The reduction seems clinically significant, with a number needed to treat of 9 which means that one pancreatic fistula can be avoided every ten patients treated with BA instead of non-BA

DtoM[155,156].

Indications for the preoperative treatment of jaundice in patients who are candidates for surgery are still under debate. It increases post-operative complications and should be reserved to patients with cholangitis or with bilirubin levels greater than 15 mg/dL[157].

Distal pancreasectomy

Distal pancreasectomy with splenectomy is the conventional operation for PDAC located in the body or tail of the pancreas. It can provide a margin-negative resection and ensure a sampling of at least 12 regional lymph nodes. A systematic review, that included 29 observational studies, found less blood loss and reduced length of hospital stay in patients operated with laparoscopic approach. However, the laparoscopic technique has some disadvantages that may lead to inadequate resection margins: Technical difficulties, inability to palpate the gland, difficulty in closing the pancreatic stump. Generally, surgeons advocate an open approach when the concern for malignancy is high, reserving laparoscopic resection for benign or premalignant indications[158-160].

Petrucciani *et al*[161] evaluated the prognosis of patients with positive surgical margin (R1). A better OS was observed in patients with R0 margin *vs* R1. However, an extension of the surgical resection following R1 pancreasectomy did not improve long term survival.

Total pancreasectomy

Sometimes, because of the extent or location of the tumor, a total pancreasectomy is required to achieve microscopically negative resection margins[162,163]. However, the metabolic consequences of this procedure, which include permanent exocrine insufficiency and brittle diabetes, have a detrimental impact on the quality of life and long-term survival[164]. A recent study showed a moderately reduced summary score of 76%, compared with a general population score of 86% using the EORTC QLQ-C30 questionnaire to evaluate the overall quality of life. Diarrhea is the most important symptom[165].

Lymphadenectomy

Tomlinson *et al*[166] evaluated the minimum number of lymph nodes removed during pancreasectomy that are essential for proper staging. They consider a number of 15 Lymph nodes as the optimal cut-off. Therefore, the cut-off of 12 lymph nodes reported by Schwarz, represents a more easily threshold value, but sufficient for correct staging.

Standard lymphadenectomy should strive to resect lymph node stations 5, 6, 8a, 12b1, 12b2, 12c, 13a, 13b, 14a, 14b, 17a, and 17b[167].

In some centres, mainly in Japan, surgeons routinely perform extensive lymph node dissection, including all 8, 9, all 12, all 14, 16a2, and 16b1 lymph nodes. A systematic review comparing standard *vs* extended lymphadenectomy demonstrated that there are no differences in OS between the two groups at one, three, or five years. However, the risk of complications was significantly increased after extended lymphadenectomy [168].

Vascular resection

If the pancreatic tumor involves the PV or SMV, pancreatic resection with PV or SMV resection may be considered: (1) When the vascular resection allows for adequate vascular flow; (2) When the tumor does not involve the SMA or hepatic artery; and (3) When an R0 resection can be accomplished. Nevertheless, many surgeons prefer to treat patients with PV or SMV involvement with neoadjuvant systemic chemotherapy before surgery.

A systematic review of 12 single-center reports concluded that pancreasectomy with PV/SMV resection is a safe and feasible procedure. It increases the number of patients who can undergo curative surgery and improves long term prognosis in a selected group of patients[169]. However, post-operative morbidity and mortality increase markedly when arterial resections are performed and few data are available to support these procedures[170-172].

Open vs minimally invasive approach

A systematic review identified 27 retrospective studies, including close to 7000 patients who underwent pancreasectomy (1306 minimally invasive, 5603 open)[173]. The laparoscopic approach was associated with longer operative times [mean difference (MD) 71 min], but lower intraoperative blood loss (MD -300 mL). The rate of

lymph node retrieval was significantly higher in the minimally invasive group (MD 1.34 nodes), and the likelihood of an R0 resection was also higher (odds ratio 1.45). Hospital stay, postoperative hemorrhage and wound infection were significantly lower in the laparoscopic group, while the rate of overall mortality, reoperations, vascular resection, pancreatic fistula, delayed gastric emptying and bile leak were similar between the two groups[174-176].

In some high-volume surgical centres, robotic-assisted pancreatic resection has been adopted. Experienced surgeon reported the same morbidity and mortality of open surgery. Decreased blood loss, higher number of adequate lymphadenectomy and improved gastric emptying are reported in some studies. These results may improve OS, but, because robotic-assisted pancreasectomy is still in its infancy, available long-term oncologic outcomes are limited[177-181].

CHEMOTHERAPY FOR RESECTABLE AND BORDERLINE RESECTABLE PANCREATIC CANCER

The only treatment with curative potential for pancreatic cancer is surgery. Five-year survival ranges from 10% to 25%.

For patients with PDAC resectable or borderline resectable, surgical resection is followed by adjuvant chemotherapy. Some high-volume centers also use neoadjuvant therapy in these categories of patients[182,183].

Adjuvant chemotherapy

Several adjuvant chemotherapy regimens have been evaluated in randomized controlled trials[184-190]. Currently, mFFX is the recommended therapy for patients with a good performance status. Gemcitabine (+/- capecitabine) remains a treatment option for patients not sufficiently fit or with contraindications to mFFX[182,191]. Because mFFX has a high toxicity, Brown University Oncology Research Group suggests FOLFOX + nab-paclitaxel (FOLFOX-A) as an alternative[192].

According to a meta-analysis[193], S1 was ranked best for overall and disease-free survival followed by mFFX. Whilst there was no significant difference between S1 and mFFX for OS, S1 had significantly longer disease-free survival (MD 2.8 mo) and was ranked best for lowest overall and haematological grade 3/4 toxicities[194]. However, the results should be interpreted with care, as S-1 has shown good results in the Asian population, but its performance in Caucasians remains unclear due to the different expression of cytochrome P-450.

Adjuvant chemotherapy should be administered between 28 and 59 d after surgery. This timing appears to provide better survival than administering before 28 or after 59 d[182,194].

A 2020 study compared the efficacy between adjuvant chemotherapy and chemora-diation therapy in relation to AJCC stage. Monochemotherapy and combination chemotherapy + chemoradiotherapy (CRT) showed better OS and disease free survival than CRT alone in patients with AJCC stage III, whereas there was no significant difference in OS in patients with AJCC stage I/II[195].

Neoadjuvant chemotherapy

The main purpose of neoadjuvant chemotherapy (NACT) differs according to the stage. For patients with BR-PDAC the objective of the therapy is to decrease tumor size and to control the micro metastases. For patients with primary resectable PDAC the purpose is to increase the proportion of patients receiving chemotherapy, because half of patients undergoing surgery, do not receive adjuvant chemotherapy due to postoperative morbidity or poor general condition[196].

In 2020, important advances were made in this field. For patients with BR-PDAC several studies confirmed the benefits on R0 resection rates and survival of NACT with mFFX[197-200] or multi-agent gemcitabine[201]. Moreover, in the PREOPANC-1 trial, patients receiving neoadjuvant CRT with gemcitabine obtained the same benefits of mFFX[202]. A study of the University of Texas showed that patients who received neoadjuvant CRT had significantly improved R0 resection rates, lymph node resection rates, and locoregional recurrence rates, compared with those who received NACT [203]. Although early data suggest the importance of integrating both NACT and CRT into the treatment, large prospective trial data are lacking[204]. New evidence for a standard regimen for BR-PDAC will be established by the result of the ESPAC-5F trial (ISRCTN89500674)[205].

For primary resectable cancer, the potential benefit of NACT has been validated, particularly when initiated within 6 wk of diagnosis[206]. The SWOG S1505 study observed that patients who received gemcitabine and nab-paclitaxel had a greater pathologic response and median survival comparable to those who received mFFX [207]. Several chemotherapeutic agents for resectable pancreatic cancer are currently being studied in several RCTs[208]. The NorPACT-1 study[209] and the Panache-01 study[210] are evaluating the effect of NACT with mFFX, and the NEONAX study [211] of NACT with 2 cycles of nab-paclitaxel/ gemcitabine.

In the Asian population, treatment regimens differ. The Prep-02/JSAP-05 study study demonstrated, in patients with resectable PDAC, that NACT with gemcitabine plus S-1 (GS therapy) improves median OS compared with initial surgery (37 mo vs 27 mo). The resection rate and morbidity of surgery remain the same[212].

Based on these results, the latest Japanese guidelines recommend GS therapy as standard neoadjuvant therapy for patients with resectable PDAC. In this regimen, patients receive intravenous gemcitabine at a dose of 1000 mg/m2 on days 1 and 8, plus oral S-1, twice daily, at a dose based on body surface area (80, 100, 120 mg/d) on days 1-14 every 3 wk for 2 cycles. For patients with BR-PDAC, they recommend NACT, but have refrained from recommending any specific regimens[212-214]. Among several ongoing RCTs on treatments for borderline resectable pancreatic cancer, a Japanese trial is comparing neoadjuvant therapy with gemcitabine plus nab-paclitaxel and CRT therapy with S-1[215].

A subset of patients does not respond to NACT. There is therefore a need to find markers that can predict response to NACT. At the moment the best ones seem to be GRP78, CADM1, PGES2 and RUXF[216] (Table 4).

CHEMOTHERAPY FOR LA PANCREATIC CANCER

Thirty to forty percent of patients with PDAC are initially diagnosed LA PDAC[182, 215]. LA PDAC is still nonmetastatic, but due to the local growth, curative resection is not possible at the time of diagnosis. Treatment involves chemotherapy with regimens that are also used in the metastatic setting, such as mFFX or gemcitabine plus nab-paclitaxel[217-219]. A small percentage of patients, with excellent response to chemotherapy, may become eligible for surgical resection. The majority have incurable disease. A systematic review of studies investigating mFFX in LA-PDAC revealed a median OS ranging from 10.0 mo to 32.7 mo[220], while in the LAPACT study, about the Nab-Paclitaxel + Gemcitabine regimen, OS 18.8 mo[221]. Recently, Kunzmann *et al* [222] compared two different NACT regimens, mFFX and gemcitabine plus nab-paclitaxel. The mFFX was superior in both the conversion rate to surgery (45.0% *vs* 30.6%) and the rate of R0 resections achieved (74% *vs* 68%). A subsequent study confirmed that mFFX patients had greater tumor size reduction, fewer positive lymph nodes, longer OS and distant metastasis-free survival compared to the nab-P/G patients[223].

The role of CRT for LA disease is still unclear. According to the LAP07 study, CRT improves the rate of local control but does not prolong survival in patients with LA PDAC after treatment with chemotherapy (gemcitabine with or without erlotinib) [224]. It is unclear whether these conclusions still hold true in the setting of newer combination chemotherapy regimens and improved radiation therapy techniques, such as stereotactic radiation therapy and proton therapy. The PAULA-1 study compared two cohorts of LAPDAC patients treated with stereotactic body radiotherapy (SBRT) ± chemotherapy *vs* CRT ± chemotherapy in terms of local control, distant metastases-free survival (DMFS), progression-free survival (PFS), OS, and toxicity. Patients treated with SBRT showed higher local control rate and similar OS, DMFS, PFS and toxicity compared to CRT[225].

CHEMOTHERAPY FOR METASTATIC PDAC

Half of patients have metastatic disease at the time of diagnosis. The primary treatment is systemic chemotherapy, with the goal of increasing survival and palliating cancer-related symptoms. Both mFFX and gemcitabine plus nab-paclitaxel improve median OS compared to gemcitabine monotherapy[226,227]. In clinical practice, for patients who are fitter, mFFX is generally preferred, reserving gemcitabine plus nab-paclitaxel as a second-line option if they have adequate performance status[228,229]. For patients who have received first-line gemcitabine and

Table 4 Phase of trial and level of evidence of trial about chemotherapy for resectable and borderline resectable pancreatic ductal adenocarcinoma

Ref.	Phase of trial	Level of evidence
Neoptolemos *et al*[185]	III	II
Oettle *et al*[186]	III	I
Neoptolemos *et al*[187]	III	I
Neoptolemos *et al*[188]	III	I
Conroy *et al*[189]	III	I
You *et al*[195]	III	II
van Roessel *et al*[198]	IV	II
Versteijne *et al*[202]	III	II
Ghaneh *et al*[205]	II	II
Sohal *et al*[207]	IV	II
Labori *et al*[209]	III	II
Schwarz *et al*[210]	II	I
Ettrich *et al*[211]	II	II
Motoi *et al*[212]	III	II
UMIN-CTR Clinical Trial[215] (UMIN000026858)	III	II

have progressed, a good option might be the combination of fluorouracil plus leucovorin with nanoliposomal irinotecan[230]. Golan *et al*[231] evaluated patients with metastatic PDAC and BRCA1-2 germline mutation. In these patients, disease progression had not occurred during at least 4 mo of first-line platinum derivative-based chemotherapy. Patients were randomized to receive olaparib or placebo. Olaparib showed a benefit in terms of PFS and a relatively safe toxicity profile. Although AIFA has not yet approved the indication, this study suggests a role for olaparib as maintenance therapy[231].

Finally, we look forward to the results of the AVENGER 500 trial (NCT03504423) to evaluate the efficacy of mFFX with or without CPI-613. CPI613 (devimistat) is an inhibitor of pyruvate dehydrogenase and a-ketoglutarate, key enzymes of the Krebs cycle. It has already shown good results in a phase I study[232].

STROMA-TARGETING THERAPY

Although chemotherapy is the recommended treatment for patients with advanced PDAC, its efficacy is not satisfactory. The major hurdle is considered the dense dysplastic stroma. The stroma components occupy more than 70% of the total tumor volume. The dense desmoplastic stroma of PDAC leads to vascular compression and a hypoxic microenvironment, which in turn influences drug pharmacokinetics/pharmacodynamics. It also prevents proper action of immune system cells, which are unable to reach the target site. The result is a chemoresistant and immunoresistant tumor[233,234].

One of the major components of the PDAC stroma is hyaluronic acid (HA). HA promotes the survival, proliferation, and migration of tumor cells[235]. HA is a potential therapeutic target using pegylated hyaluronidase (PEGPH20). The HALO-109-202 study demonstrated that PEGPH20, combined with Abraxane (nab-paclitaxel) and gemcitabine, improves progression-free and OS in patients with high HA levels [236]. However, poor results were obtained from the subsequent HALO-109-301 study (NCT02715804). Another element to be acted upon is the Hedgehog signaling pathway, which is generally overactivated in pancreatic cancer. Vismodegib, in combination with gemcitabine or erlotinib, was studied for this purpose. It did not significantly affect survival compared with these two drugs administered as monotherapy[237,238].

In tumors, Angiotensin II activates transforming growth factor-β through the AT1R and stimulates proliferation, so several angiotensin system inhibitors have been used to target PDAC stroma[233]. One study evaluated the efficacy of mFFX combined with losartan in a neoadjuvant regimen in patients with LA PDAC. The therapy was associated with an increased R0 resection rate[239].

A clinical trial evaluated the efficacy of focused ultrasound combined with gemcitabine microbubble delivery in PDAC patients. Patients treated with the combination tolerated multiple chemotherapy cycles of gemcitabine. A prolongation of median survival by almost 9 mo and, in 50% of cases, a reduction in tumor size were observed[240].

Poor results were obtained from stroma depletion in clinical settings. They are due to the fact that, although stroma-targeting therapy enhances the delivery of chemotherapeutic agents, it might also promote tumor chemoresistance and metastasis (a double-edged sword)[241]. According to several experts, future research should focus on the tumor ECM biology, biomarkers correlated with treatment benefit (as ADAM12)[242] and pharmacological agents able to alter the tumor microenvironment (TME). One of the most interesting discoveries in this regard involves clodronate liposomes. They prevent metastasis formation by inhibiting the activity of PDAC-associated macrophages and altering the microenvironment of key organs that are sites of metastatic invasion. They are therefore valuable candidates to be evaluated in combination with target therapy against stroma[243].

IMMUNOTHERAPY

Immune checkpoint inhibitors

Checkpoint inhibitors activates the function "kill the tumor" of the immune system, targeting immune checkpoint molecules (PD-1, PD-L1, CTLA-4) that negatively regulate T-cell function. Although they resulted in remarkable successes in other cancers, ipilimumab, BMS-936559 and tremelimumab showed little efficacy in PDAC [244-247]. The reasons of failure of immune checkpoint inhibitors are the low baseline PD-1+ T-cell infiltration into the tumor and a paucity of neoepitopes[248,249]. Indeed, in a very small subset of PDAC patients with a high burden of microsatellite instability (MSI-high) PD-1 inhibitor is effective and was recently FDA approved[250,251].

Currently, the development of immune checkpoint inhibitors for PDAC is focused on combination therapy with chemotherapeutic agents[252-255].

Therapeutic cancer vaccines

Therapeutic cancer vaccines present of immunogenic tumor antigens to the immune system, resulting in activation of the anti-cancer response. GVAX is an allogeneic vaccine irradiated with tumor cells engineered to express GM-CSF. It was studied alone and in combination with CRS-207 and cyclophosphamide, however it didn't correlate with improved survival[256,257].

More promising results were instead obtained with KIF20A-66[258-260].

K-RAS vaccines have been tested in the past, but data remain unclear and with no prominent advantages in metastatic patients[261-264].

We are currently awaiting the results of some studies: (1) TLP0-001, a phase III study of a dendritic cell (DC) vaccine loaded with WT1 peptides in patients with advanced PDAC refractory to standard chemotherapy[265,266]; (2) A clinical trial using GV1001 with GM-CSF in patients with LA-PDAC in combination with gemcitabine chemotherapy, tadalafil and radiation therapy (NCT01342224); and (3) NCT01836432, NCT02405585 and NCT01072981 evaluating algenpantucel-L in combination with chemotherapy and CRT therapy. They involve patients with borderline resectable and LA unresectable PDAC.

CAR-T cell

CAR-T cell therapy is a type of adoptive cell therapy. CAR-T cells are T lymphocytes that are extracted from a patient's blood sample or from a donor by apheresis, genetically modified to express the receptor for chimeric antigen (CAR), and cultured in the laboratory. They are then re-infused into the patient. The resulting T cells are able to recognize tumor cells and activate the immune system response against the disease[267]. The target antigens of CAR-T cells include mesothelin, prostate stem cell antigen (PSCA), CEA, HER2, MUC-1, and CD133[268,269]. In a study of metastatic PDAC, autologous mesothelin-specific T lymphocytes improved PFS in two patients of the six examined. An additional patient had complete remission of all liver metastases

[270].

Combination of immunotherapy drugs was experienced and showed good results over time. Le *et al*[271] compared the efficacy of Ipilimumab as monotherapy (arm 1) and Ipilimumab in combination with GVAX (arm 2) in patients with already treated PDAC. Combination therapy showed an increase in median OS (5.7 mo *vs* 3.6 mo) and 1-year OS (27% *vs* 7%). Chung *et al*[272] evaluated the combination of Pembrolizumab with modified p53-expressing Ankara vaccinia virus (p53MVA). Three of eleven patients experienced disease stabilization by 30, 32, and 49 wk. Good OS and PFS results were also obtained using DC and cytokine-induced killer cell immunotherapy in combination with S-1 chemotherapy, compared with chemotherapy or supportive care alone[256].

Several trials of immunotherapy-based treatment combinations with targeted agents are ongoing for patients with pancreatic cancer[273-275].

Oncolytic viruses

Oncolytic viruses are modified therapeutic drugs that selectively infect and self-replicate in tumor cells with tumor-dissolving effect. They also activate the anti-tumor immunity and change the TME from an immunosuppressed state to an immune-activated state. Futhermore, oncolytic viruses have the advantages of specificity, low toxicity, and low drug resistance[276]. Adenovirus, Herpes Simplex Virus, Protoparvovirus, Reovirus and Vaccinia Virus have been tested. However most of the studies have shown unsatisfactory results. The only positive results derive from ParvOryx02 (NCT02653313). A single-arm study published in 2020 showed an encouraging efficacy of pembrolizumab in combination with Pelareorep and chemotherapy in patients progressed after first-line treatment[277-281] (Table 5).

GENETIC MUTATION AND TARGET THERAPY

Some genetic alterations produce cellular changes in neoplastic cells that are potentially therapeutically targetable. BRAF mutations occur in 1%-3% of PDAC. They showed to be targetable in metastatic colon cancer where the combination of Encorafenib and Cetuximab has recently been approved[282,283]. Encorafenib and Cetuximab should also be evaluated in PDAC. Furthermore, pancreatic tumors with NTRK gene fusions can be treated with tropomyosin receptor kinase inhibitors[284, 285]. Similarly, some wild-type Kras pancreatic tumors hosting somatic NRG1 gene fusions respond to treatment with a kinase inhibitor of the HER family[286,287].

However, the results of the targeted therapies have been unsatisfactory, mainly due to the low life expectancy. There is no time to sequence the tumors and develop a treatment based on mutations[288].

The exceptions were the germline alterations. Patients with mutations of BRCA1, BRCA2 or PALB1 are remarkably sensitive to treatment with DNA cross-linking agents, such as platinum-based drugs, and poly(ADP-ribose) polymerase (PARP) inhibitors[289-291]. Patients with Lynch syndrome (MSI-high) respond well to treatment with immune checkpoint inhibitors[292-294] and those with ATM mutations could respond to the drugs, targeting the ATR-checkpoint kinase 1 (Chk1) pathway [295,296].

The elephant in the targeted therapy room remains Kras[297]. It has been considered "undrinkable"[297-299] because the protein lacks an efficient small-molecule binding pocket and has a high affinity for cellular guanosine triphosphate (GTP), which is highly concentrated in the cytoplasm. Furthermore, other than the GTP/GDP binding pocket, KRAS has no other pockets for small-molecule inhibitor binding. A druggable variant of Kras appears to be G12C. Enormous progress has been made in this regard and several drugs (AMG 510, MRTX849, JNJ-74699157 and LY3499446) are currently in clinical trials[299]. The importance of these can be deduced from the fact that 95% of pancreatic cancers harbor mutations in the Kras gene (the four Kras mountains, TP53, CDKN2A and SMAD4 present in > 50% of tumors)[300,301]. Although Kras G12C mutations are only a small fraction of Kras mutations in PDAC, these drugs represent a chance to take down a previously thought invincible adversary.

PANCREATIC CANCER AND GUT MICROBIOTA

Recent studies have shown the gut microbiota (GM) may play a role in the

Table 5 Phase and level of evidence of trials about immunotherapy for pancreatic ductal adenocarcinoma

Ref.	Phase of trial	Level of evidence
Royal *et al*[245]	II	II
Brahmer *et al*[246]	I	I
O'Reilly *et al*[247]	II	II
Tumeh *et al*[248]	II	III
Le *et al*[250]	II	II
Le *et al*[251]	II	II
Wainberg *et al*[252]	I	II
Weiss *et al*[253]	Ib/II	II
National Institute of Public Health[254] (JapicCTI-184230,ONO-4538)	II	II
Wang-Gillam *et al*[255]	II	II
Le *et al*[257]	IIb	I
Asahara *et al*[258]	I/II	II
Suzuki *et al*[259]	II	III
Miyazawa *et al*[260]	II	II
Wedén *et al*[261]	IV	III
Toubaji *et al*[262]	I	III
Abou-Alfa *et al*[263]	I/II	III
Cohn *et al*[264]	I	III
Katsuda *et al*[265]	III	I
Katsuda *et al*[266]	I/II	II
Beatty *et al*[270]	I	III
Le *et al*[271]	Ib	II
Chung *et al*[272]	I	III
Wang-Gillam *et al*[273]	I	III
Reiss *et al*[274]	II	III
Desai *et al*[275]	Ib/II	Ongoing trial
Chang *et al*[278]	I	III
Noonan *et al*[279]	II	II
Mahalingam *et al*[280]	Ib	III

development of PDAC and its response to therapy. GM alterations result in reduced mucus thickness, leading to decreased antimicrobial defenses and increased exposure to bacterial components such as LPS, flagellin, single or doubled DNA and CpG DNA. These agents activate Toll-like-receptors and trigger chronic inflammation that are related to carcinogenesis. Moreover, inflammation and dysbiosis lead to mutation of Kras, that accelerates carcinogenesis, activating nuclear factor-κB pathway[302-304].

Several bacterial products are considered potential carcinogens. Cyclomodulins promote tumorigenesis through active interference with host cell cycles. Colibactin and Bacteroides fragilis toxin act synergistically with Escherichia coli to create double-stranded DNA damage[305]. E. coli cytotoxic necrotizing factor and CagA lead to uncontrolled cell proliferation, while cytolytic distending toxin and cycle inhibitory factor participate in genetic alterations and induce hyperploidy even in the absence of cell division[306]. The presence of an *Helicobacter pylori* infection and high concentrations of Fusobacterium spp and Porphyromonas gingivalis (bacteria generally present in the oral cavity) are associated with an increased risk of pancreatic cancer [307-310].

Moreover, other studies correlated a large number of microbes with immune suppression, downregulation of tumor suppressive pathways and the upregulation of oncogenic pathways[311].

Dysbiosis is also related to obesity, chronic pancreatitis and diabetes, well-established risk factors of PDAC[312,313].

Because it participates in drug metabolism and biotransformation and immune regulation, the GM is implicated in the efficacy of chemotherapeutic agents[314]. The innate immune response activated by the GM potentiates the action of oxaliplatin [315]. Gentamicin activity may be reduced by the enzymes pyrimidine nucleoside phosphorylase and cytidine deaminase, which are produced by Gamma-proteo-bacteria and mycoplasmas within PDAC. Thus, these data suggest the possibility of modulating GM to counteract the chemoresistance characteristic of pancreatic cancer [316].

Intratumoral microorganisms can play a key role in anticancer therapy[317]. Indeed, they can stimulate host immune responses with positive or negative impacts on therapy. *Gammaproteobacteria*, *Escherichia Coli* and *Fusobacteria* are most commonly present in PDAC. Gamma proteobacteria contain the enzyme CDD which could be responsible for the ineffectiveness of gemcitabine[318]. Escheria Coli is capable of inducing chemical changes in the structure of gemcitabine, fludarabine, cladribine, and CB1954[319]. The desmoplastic response induced by tumor cells is dependent on MyD88. It is activated by Fusobacterium species.

The intratumoral microbiota thus emerges as a major proponent of the chemo-immunoresistant phenotype of pancreatic cancer and is related to long-term survival in PDAC patients.

PROGNOSIS

The most important prognostic factor is tumor stage. The median survival time after resection for patients with stage IA, IB, IIA, IIB, and III was 38, 24, 18, 17, and 14 mo, respectively[320]. Other factors may influence the prognosis of PDAC after surgery: Surgical margin status, tumor grading, presence of lymphatic invasion, preoperative and postoperative serum levels of CA 19-9, and cigarette smoking[321-329]. Squamous subtypes have a poor prognosis. They are enriched with TP53 and KDM6A mutations, upregulation of TP63ΔN transcriptional network, hypermethylation of pancreatic endoderm cell fate determining genes[330].

Several studies have investigated novel factors influencing prognosis: (1) Increased expression of CDK1 and CCNA2 is associated with poor prognosis, although they may be potential therapeutic targets[331]; (2) The autophagy regulatory genes MET and RIPK2 play a prognostic role in PDAC[332]; (3) High expression of GPDAC2, GPDAC3 and GPDAC5 has been significantly associated with favorable survival[333]; (4) High expression of Hic-5 is negatively correlated with postoperative survival time, as Hic-5 stimulates tumor proliferation, migration, and invasion[334]; (5) PRMT1 promotes pancreatic cancer growth by increasing cellular β-catenin levels and predicts poor prognosis[335]; (6) Patients with first recurrence in the lung have a better prognosis than patients with first recurrence in the liver[336]; (7) Increased levels of ZIP4 correlate with poorer survival. ZIP4 inhibits the expression of the gemcitabine transporter ENT1, so that cells take up smaller amounts of the drug. Activation of this pathway participates in the chemoresistance of pancreatic cancers[337]; (8) The highly upregulated in liver cancer (HULC) lncRNA distinguishes patients with pancreatic cancer, patients with benign pancreatic disease, and healthy subjects and correlates with TNM stage. Subjects with low HULC expression have significantly higher 3- and 5-year OS than those with high expression. Therefore, HULC lncRNA could be considered an effective marker for the diagnosis and prognosis of PDAC[338]; (9) Upregulation of TYMS leads to unfavorable OS and RFS[339]; and (10) The GINS complex has four subunits, encoded by the GINS1, GINS2, GINS3, and GINS4 genes, all of which are overexpressed in PDAC. The expression of each member is associated with the histological grade of PDAC and is a negative prognostic marker[340].

CONCLUSION

Pancreatic cancer is a very treacherous, dangerous enemy and the results are still very unsatisfactory. But we have not given up. Research is running fast on many paths, without losing its enthusiasm. It is proof that we are encircling it, and at the end, we

will win. The success of a fight is linked to the ability to move from one failure to another without losing one's enthusiasm.

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Gastrinoma and Zollinger Ellison syndrome: A roadmap for the management between new and old therapies

Roberta Elisa Rossi, Alessandra Elvevi, Davide Citterio, Jorgelina Coppa, Pietro Invernizzi, Vincenzo Mazzaferro, Sara Massironi

ORCID number: Roberta Elisa Rossi 0000-0003-4208-4372; Alessandra Elvevi 0000-0001-9841-2051; Davide Citterio 0000-0002-0708-8733; Jorgelina Coppa 0000-0003-2466-0524; Pietro Invernizzi 0000-0003-3262-1998; Vincenzo Mazzaferro 0000-0002-4013-8085; Sara Massironi 0000-0003-3214-8192.

Roberta Elisa Rossi, Davide Citterio, Jorgelina Coppa, Vincenzo Mazzaferro, HPB Surgery, Hepatology and Liver Transplantation, ENETS Center of Excellence, Fondazione IRCCS Istituto Nazionale Tumori (INT, National Cancer Institute), Milan 20133, Italy

Roberta Elisa Rossi, Department of Pathophysiology and Transplantation, University of Milan, Milan 20122, Italy

Alessandra Elvevi, Pietro Invernizzi, Sara Massironi, Division of Gastroenterology and Center for Autoimmune Liver Diseases, Department of Medicine and Surgery, University of Milano-Bicocca, Monza 20900, Italy

Alessandra Elvevi, Pietro Invernizzi, Sara Massironi, European Reference Network on Hepatological Diseases (ERN RARE-LIVER), San Gerardo Hospital, Monza 20033, Italy

Vincenzo Mazzaferro, Department of Oncology and Hemato-Oncology, University of Milan, Milan 20122, Italy

Corresponding author: Roberta Elisa Rossi, MD, PhD, HPB Surgery, Hepatology and Liver Transplantation, ENETS Center of Excellence, Fondazione IRCCS Istituto Nazionale Tumori (INT, National Cancer Institute), *via* Giacomo Venezian, Milan 20133, Italy. robertaelisa.rossi@gmail.com

Abstract

Zollinger-Ellison syndrome (ZES) associated with pancreatic or duodenal gastrinoma is characterized by gastric acid hypersecretion, which typically leads to gastroesophageal reflux disease, recurrent peptic ulcers, and chronic diarrhea. As symptoms of ZES are nonspecific and overlap with other gastrointestinal disorders, the diagnosis is often delayed with an average time between the onset of symptoms and final diagnosis longer than 5 years. The critical step for the diagnosis of ZES is represented by the initial clinical suspicion. Hypergastrinemia is the hallmark of ZES; however, hypergastrinemia might recognize several causes, which should be ruled out in order to make a final diagnosis. Gastrin levels > 1000 pg/mL and a gastric pH below 2 are considered to be diagnostic for gastrinoma; some specific tests, including esophageal pH-recording and secretin test, might be useful in selected cases, although they are not widely available. Endoscopic ultrasound is very useful for the diagnosis and the local staging of the primary tumor in patients with ZES, particularly in the setting of multiple endocrine neoplasia type 1. Some controversies about the management of these

tumors also exist. For the localized stage, the combination of proton pump inhibitory therapy, which usually resolves symptoms, and surgery, whenever feasible, with curative intent represents the hallmark of gastrinoma treatment. The high expression of somatostatin receptors in gastrinomas makes them highly responsive to somatostatin analogs, supporting their use as anti-proliferative agents in patients not amenable to surgical cure. Other medical options for advanced disease are super-imposable to other neuroendocrine neoplasms, and studies specifically focused on gastrinomas only are scant and often limited to case reports or small retrospective series. The multidisciplinary approach remains the cornerstone for the proper management of this composite disease. Herein, we reviewed available literature about gastrinoma-associated ZES with a specific focus on differential diagnosis, providing potential diagnostic and therapeutic algorithms.

Core Tip: As symptoms of Zollinger-Ellison syndrome are nonspecific and overlap with other gastrointestinal disorders, most of these patients are usually referred to general gastroenterologists, leading to a diagnostic delay. A better disease awareness together with the maintenance of a high index of suspicion are necessary to make the final diagnosis. The proper management of Zollinger-Ellison syndrome due to a gastrinoma include both the medical treatment for symptom's relief and surgery whenever feasible with curative intent; the multidisciplinary approach, with close cooperation between gastroenterologists and surgeons, and the referral to tertiary centers with great expertise in the neuroendocrine field are mandatory.

INTRODUCTION

Zollinger-Ellison syndrome (ZES) was firstly described in 1955 as associated with a neuroendocrine neoplasm (NEN) capable of ectopic gastrin secretion (namely gastrinoma)[1], resulting in gastric acid hypersecretion, which typically leads to gastroesophageal reflux disease (GERD), recurrent peptic ulcers, and chronic diarrhea. The terms gastrinoma and ZES have been frequently used as synonymous, although gastrinoma refers to the NEN secreting gastrin, whereas ZES refers to the clinical manifestations of the disease. ZES has an incidence of 1-1.5 cases/million per year[2]. Gastrinomas are NENs located in the duodenum (70%), pancreas (25%), and rarely (5%), in other sites, including stomach, liver, ovary, and lung. Gastrinoma is the most frequent functioning duodenal NEN and the second most frequently occurring functional pancreatic NEN (pNEN), following insulinoma; in turn, 15% of functioning pNENs is represented by gastrinoma. It may be sporadic, which is generally diagnosed between the ages of 50 and 70 years with a male to female ratio of 1.5-2:1 [3], whilst 20%-30% of the patients develop ZES in the context of a genetic syndrome known as multiple endocrine neoplasia type 1 (MEN-1)[4].

The diagnosis of ZES is not always straightforward due to both non-specific symptoms and confounding factors including proton pump inhibitor (PPI) therapy, which might temporarily relieve symptoms. Furthermore, as these patients tend to be referred to gastroenterologists because of diarrhea and/or reflux disease disorder, despite a better awareness of the disease, the diagnosis might be challenging for those

gastroenterologists with low experience in the neuroendocrine setting as well as for many oncologists who are less used to dealing with diarrhea and reflux disease. As a consequence, the average time between the onset of symptoms and the final diagnosis is often longer than 5 years[5,6], and nearly 25% of patients are metastatic at the first diagnosis and show a worse prognosis when compared to non-metastatic patients in whom the surgical management is associated with a promising 15-year survival rate of > 80%[7].

Furthermore, some controversies about the management of these tumors still exist, particularly regarding the exact role of surgery or medical treatment and the possible role of somatostatin analogs (SSAs)[3]. Given that gastrinoma and ZES need both a proper medical treatment for symptom relief and a surgical procedure whenever feasible, the multidisciplinary approach, with close cooperation between clinicians and surgeons, remains the cornerstone for proper management of this composite disease, which should be always referred to tertiary centers.

Herein, we review from a critical point of view current knowledge about gastrinoma-associated ZES, also providing potential diagnostic and therapeutic algorithms based on both evidence from literature and own personal experience.

METHODOLOGY

Bibliographical searches were performed in PubMed using the following keywords: Gastrinoma; Zollinger Ellison syndrome; neuroendocrine neoplasms; pancreatic neuroendocrine neoplasm; duodenal neuroendocrine neoplasm; diagnosis; therapy; guidelines. We searched for all relevant articles published over the last 10 years. The reference lists from the studies returned by the electronic search were manually searched to identify further relevant reports. The reference lists from all available review articles, primary studies, and proceedings of major meetings were also considered. Articles published as abstracts were included, whereas non-English language papers were excluded.

CLINICAL PRESENTATION

ZES is characterized by gastric acid hypersecretion and consequent hyperchlorhydria resulting in severe acid-related peptic disease and diarrhea. The symptoms usually resolve when gastric acid secretion is controlled pharmacologically with PPIs[8,9]; of note, the disappearance of diarrhea following PPI treatment is typical of ZES and represents one of the factors contributing to the diagnostic delay. According to data from the literature, common symptoms include abdominal pain (75%), diarrhea (73%), heartburn (44%), and weight loss (17%)[6,8,10]. As these symptoms are both not specific and often less severe due to concomitant PPI treatment, the final diagnosis is often delayed and patients are diagnosed with irritable bowel syndrome or reflux disease by gastroenterologists with low or no knowledge of the disease[8,11].

The endoscopic features are also not specific and might include erosions and ulcers [12], however, ZES patients often present with multiple ulcers located at unusual sites, *e.g.*, beyond the first or second portion of the duodenum[8,13]. Furthermore, enlarged gastric folds can be present in more than 90% of patients with ZES[11].

One should keep in mind that approximately 25% of gastrinomas occur in the context of MEN-1, which is characterized by the presence of parathyroid, pancreatic-duodenal, and pituitary tumors[14]; thus the occurrence of unexplained hypercalcemia might be a sign for possible MEN-1 syndrome-associated ZES[15,16], also taken into account that primary hyperparathyroidism is generally the presenting feature in the majority of cases of MEN-1 syndrome[8,16,17]. Of note, parathyroidectomy usually improves gastrin levels and basal acid output[16]. Finally, in ZES/MEN-1 patients, type 2 gastric NENs might occur[3].

DIFFERENTIAL DIAGNOSIS

Symptoms of ZES are nonspecific and overlap with other gastrointestinal (GI) disorders, which explains the frequent diagnostic delay.

As concerns chronic diarrhea in ZES, this is sustained by hyperchlorhydria and sodium and water malabsorption due to hypergastrinemia[18]. As afore-mentioned,

diarrhea is one of the most frequent symptoms in ZES; up to 75% of patients manifest diarrhea[19], and this could be the sole presenting symptom in 3%-10% of the patients [20]. Moreover, chronic diarrhea is one of the most frequent symptoms requiring gastroenterologist referral; its diagnostic workup could be challenging because many different causes could cooperate to diarrhea development and recurrence (*e.g.*, dietary habits, drugs). Recent British Society of Gastroenterology (BSG) guidelines for chronic diarrhea[21] tried to classify different causes of chronic diarrhea (Table 1) and to standardize a diagnostic work-up in these patients. Since hormone-secreting tumors are considered rare causes of chronic diarrhea, BSG guidelines suggest testing patients for these tumors only when other causes of diarrhea have been excluded. From a clinical point of view, the association between chronic diarrhea with both other ZES suggestive symptoms (*e.g.*, chronic peptic ulcer disease) and clinical response to PPIs may be helpful in diagnosing this challenging syndrome, taking into account that the delay in diagnosis of ZES remains between 6 to 9 years from the first clinical presentation[9,19].

Abdominal pain and heartburn are frequently reported as symptoms of ZES[9]. As well as diarrhea, they are sustained by hyperchlorhydria, which directly damages GI mucosa, causing ulcers and erosions. Abdominal pain could be associated with peptic ulcers, which, differently from *Helicobacter pylor*i or non-steroidal anti-inflammatory drug-related ulcers, are multiple, located at unusual locations (*e.g.*, the third part of the duodenum, small bowel) and complicated by bleeding, penetration, perforation, or strictures[8,13,19].

Similar to peptic ulcer disease, chronic GERD is one of the most frequent manifestations of ZES[13]. Heartburn and regurgitation are the most typical symptoms, which are super-imposable to symptoms associated with typical GERD; differently from the typical syndrome, patients with ZES often present esophageal strictures due to over-exposition to acid reflux.

Again, the association between these symptoms and chronic diarrhea, after exclusion of other common GI etiologies, might raise the suspicion of ZES, which requires specific tests in order to get the final diagnosis.

DIAGNOSIS

The diagnosis of ZES is quite challenging, also considered that the critical point is the initial suspicion of ZES. A suggested diagnostic algorithm is represented in Figure 1.

ZES is a clinical syndrome characterized by the following triad: (1) gastric acid hypersecretion, sustained by (2) fasting serum hypergastrinemia causing (3) peptic ulcer disease and diarrhea[1]. Hypergastrinemia is sustained by a gastrinoma, a rare NEN (located primarily in the duodenum or pancreas) that secretes gastrin.

Since ZES symptoms can be explained almost entirely by acid hypersecretion, PPIs, which significantly decrease acid secretion, can mitigate or resolve ZES symptoms, making ZES diagnosis even more challenging than in the past[9,22], but avoiding severe ZES complications.

Hypergastrinemia is the hallmark of ZES; however, hypergastrinemia might recognize several causes, which should be ruled out in order to make a final diagnosis of ZES[23]. In detail, it can be distinguished between (1) appropriate hypergastrinemia, due to atrophic gastritis (with or without pernicious anemia), anti-secretory therapy (PPIs or high-dose histamine H2-receptor antagonist, namely famotidine), chronic renal failure, *Helicobacter pylori*-related pan-gastritis, vagotomy, and (2) inappropriate hypergastrinemia that can be observed in ZES (sporadic or associated with MEN-1), antral-predominant *Helicobacter pylori* infection, retained-antrum syndrome, gastric-outlet obstruction, extensive small-bowel resection.

The diagnosis of ZES requires the demonstration of inappropriate gastrin secretion associated with gastric hyperchlorhydria, which corresponds to a gastric pH < 2[5]. Normal fasting gastrin levels are < 100 pg/mL; levels > 300 pg/mL are highly suspicious, and levels > 1000 pg/mL together with a gastric pH below 2 are considered to be diagnostic for gastrinoma[2,9,24]. Naso-gastric tube aspiration has classically been used to estimate gastric pH, but it can be uncomfortable for patients and can underestimate gastric acid output; alternatively, gastric pH can be measured during upper GI endoscopy, by aspiration of gastric juice for pH determination using either pH paper or a pH meter; while endoscopic sampling was shown to overestimate total acid volume, it provided more reproducible results and offered greater patient tolerance than nasogastric tube placement[5,23,25,26]. To avoid false-negative results, fasting serum gastrin levels and gastric pH should be measured after PPI withdrawal

Table 1 Differential diagnosis of chronic diarrhea[21]

Common	Infrequent	Rare
IBS-diarrhea	Small bowel bacterial overgrowth	Small bowel enteropathies (*i.e.* Whipple's disease, tropical sprue, amyloid, *etc.*)
Bile acid diarrhea	Mesenteric ischemia	Hypoparathyroidism
Diet (artificial sweeteners, caffeine, FODMAP malabsorption, *etc.*)	Lymphoma	Addison's disease
Colonic neoplasia	Surgical causes (small bowel resection, incontinence, *etc.*)	**Hormone secreting tumors** (*i.e.* VIPoma, gastrinoma, carcinoid)
IBD	Chronic pancreatitis	Autonomic neuropathy
Celiac disease	Radiation enteropathy	Factitious diarrhea
Drugs (antibiotics, NSAID, *etc.*)	Pancreatic carcinoma	Brainerd diarrhea
Overflow diarrhea	Hyperthyroidism	
	Diabetes	
	Chronic infections (*i.e.* giardiasis)	
	Cystic fibrosis	

FODMAP: Fermentable Oligosaccharides, Disaccharides, Monosaccharides, and Polyols; IBS: Irritable bowel syndrome; NSAID: Nonsteroidal anti-inflammatory drugs.
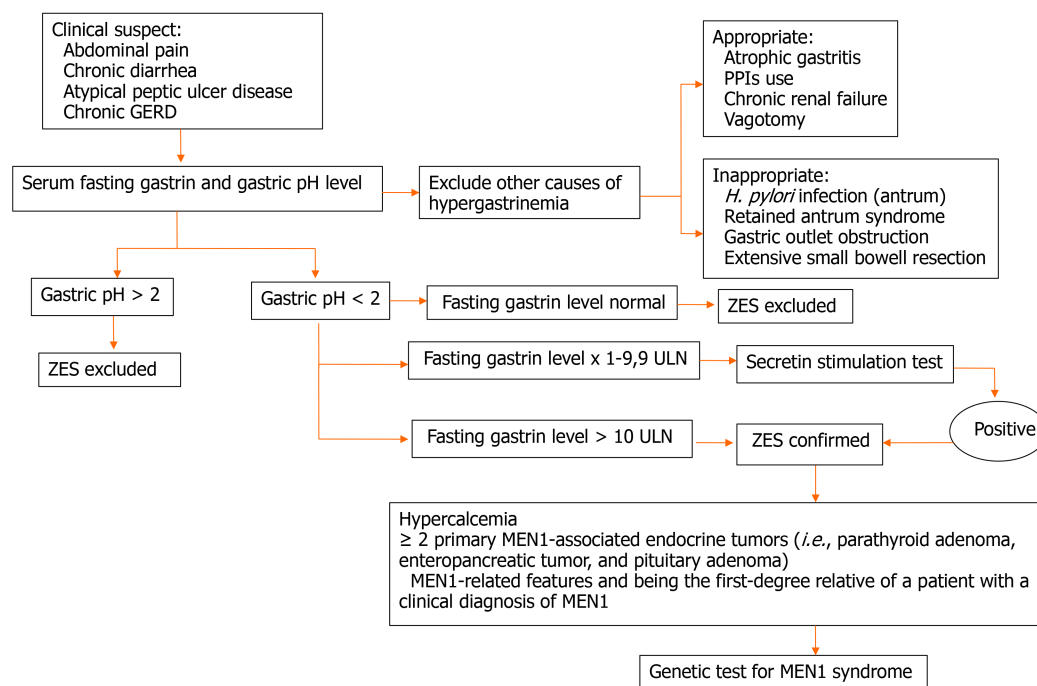
Figure 1 A suggested diagnostic algorithm is depicted. GERD: Gastroesophageal reflux disease; *H. pylori*: *Helicobacter pylori*; MEN-1: Multiple endocrine neoplasia type 1; PPIs: Proton pump inhibitors; ULN: Upper limit of normal; ZES: Zollinger Ellison syndrome.

[2,6,27]. However, PPI withdrawal could be dangerous for ZES patients, because it could bring a dramatic increase in gastric acid secretion, hence causing severe peptic ulcer disease and its complications[23], thus the decision to stop the treatment should be tailored to every single patient. Then, it is usually suggested to start histamine H2-receptor antagonists (*i.e.* famotidine) as soon as PPIs are stopped in order to prevent complications due to gastric acid hypersecretion. Having a shorter duration of action compared to PPIs, H2-antagonists could be used until the evening before serum gastrin and gastric pH tests[23].

Imaging and ultrasound endoscopy (endoscopic ultrasound)

Localization of the primary tumor and its metastases is the first diagnostic step when ZES associated with gastrinoma is suspected.

Contrast-enhanced computed tomography (CT) scan is useful to identify primary tumor > 1 cm, pancreatic head tumors, and liver metastases, with a sensitivity between 59% and 78% and a specificity between 95% and 98%, respectively. Conversely, sensitivity decreases for tumor size < 1 cm and extra-pancreatic locations[28,29].

Contrast-enhanced magnetic resonance imaging (MRI) showed high specificity (namely 100%) in detecting small pancreatic tumors and liver metastases, whereas sensibility is sub-optimal varying from 25% to 85%. Of note, MRI showed a higher sensibility for liver metastases detection when compared to CT scan[28,30].

Somatostatin receptor scintigraphy (Octreoscan®) has been used to localize gastrinomas[8,31]. This test involves the administration of indium radio-labeled octreotide, which binds selectively to somatostatin receptors found on gastrinoma cells. It showed quite good sensitivity (between 77% and 78%) and a good specificity (93%-94%) for primary tumor detection and its metastases, although sensitivity decreases for small tumors (< 1 cm)[32]. Diagnostic accuracy of somatostatin receptor scintigraphy (Octreoscan®) can be improved by performing it in combination with single-photon emission CT (SRS-SPECT)[28]. Different studies showed higher sensitivity and specificity in primary tumor detection, 78%-88% and 97%, respectively, when compared to Octreoscan® alone[33-35].

In more recent years, somatostatin receptor positron emission tomography (PET) techniques have shown great promise for improving the localization of gastrinomas as well as other NENs[36-39] and for the detection of distant metastases, including bone lesions. The radioisotope ^{68}Ga can be ligated to peptides that bind to somatostatin receptors found in abundance on the NEN surface[36]. This technique showed a higher sensibility and specificity (72%-100% and 83%-100%, respectively) when compared to the aforementioned diagnostic techniques in localizing the primary tumor, especially small size tumors[36,37,40]. Combining ^{68}Ga-radiotracers with traditional CT scans (PET/CT) further enhances diagnostic accuracy compared to PET alone, showing a sensitivity of 93% and a specificity of 96% in primary tumor detection [41]. Gallium-^{68}PET-scan should be always included in the diagnostic pathway of all NENs, including gastrinoma, in order to both identify the primary tumor and stage the disease.

Endoscopic ultrasound (EUS) has become an important diagnostic tool to localize gastrinomas, particularly small (*i.e.* < 2 cm) pancreatic lesions; its sensitivity and specificity are 75%-100% and 95%, respectively, for pancreatic tumors. Unfortunately, its sensitivity dramatically decreases in cases of duodenal localization, ranging from 38% to 63%[28,42]. A further advantage of this technique is the possibility of taking cytologic/histologic samples through a fine needle aspiration/biopsy (FNA/B) to confirm the diagnosis of NEN, even if false-negative results are possible mainly due to poor sampling adequacy. EUS-FNA/B is now considered the primary sampling technique for pancreatic tumors, with a sensitivity ranging between 80% and 90%, specificity at 96%[43], and a sampling adequacy rate of 83%-93%[44].

When used as a screening modality in asymptomatic patients with MEN-1, EUS has been reported to be more accurate than CT scan to detect smaller tumors[45]. Therefore, its diagnostic ability has led experts to recommend it as an annual screening modality for all patients with MEN-1, although recent evidence suggests that the growth rate of small pNENs (*i.e.* < 2 cm) is low and that EUS screening frequency can likely be extended[14,46].

Esophageal pH-recording

Since one of the most common symptoms of ZES is GERD, it could be argued that esophageal pH-monitoring could be a useful tool to diagnose ZES. Recent BSG guidelines for esophageal manometry and esophageal pH monitoring[47] stated indications to perform esophageal pH-monitoring, also including as an indication GERD symptoms that did not respond to double dose of PPIs. This technique allows to diagnose an increased acid exposure, to evaluate the association between symptoms and acid or non-acid reflux, and to identify different phenotypes of upper symptoms (*i.e.* non-erosive reflux disease, hypersensitive esophagus, and functional heartburn).

ZES is not usually included in diagnosis performed by esophageal pH-monitoring, and, consequently, ZES reference standard for esophageal pH-monitoring is lacking. However, evidence of a high number of acidic reflux episodes (*i.e.* esophageal pH < 4), a high number of long (*i.e.* > 5 min) reflux episodes, a high percentage of time with esophageal pH < 4, both on a double dose of PPIs and off PPIs, could raise the

suspicion of abnormal gastric acid secretion. This hypothesis should be confirmed by prospective studies; however, considering the rarity of this syndrome, it would be very difficult to obtain standard values to use in clinical practice; therefore, despite its potential utility, this test is not currently included in the standard diagnostic workup of gastrinoma.

Secretin provocative test

Secretin provocative test in ZES diagnosis founds its application in controversial cases, that is patients with suspected ZES, gastric pH < 2 but fasting serum gastrin < × 10 upper limit of normal[9]. To perform a secretin stimulation test, fasting gastrin levels are obtained before intravenous (IV) administration of secretin and then 2, 5, and 10 min after infusion[25]. Patients with gastrinomas exhibit an inappropriate increase in gastrin production in response to secretin infusion[9]. This mechanism can be explained in part by the fact that secretin receptors are expressed directly on the gastrinoma cell surface[48]. Different cut-offs for positive tests have been proposed, including an absolute increase in gastrin concentration ≥ 110 pg/mL or ≥ 200 pg/mL or a 50% increase in gastrin concentration[49]. However, previous data suggested that a positive secretin-provocative test (≥ 120 pg/mL increase) has a sensitivity of 94% and specificity of 100%, respectively[50]. According to data from the literature, a false-negative response can occur in 6% to 20% of patients[51,52], whereas false-positive responses, ranging from 15% to 39% in different studies[52,53], are found in patients with pernicious anemia or chronic PPI use.

In order to reduce the risk of false-positive results, PPI treatment should be withdrawn, but, again, the decision should be discussed in a case-by-case manner to limit the risk of severe complications (*e.g.*, perforation or bleeding). This might partially explain the reason why the secretin test can be difficult to be performed and should be reserved for strictly selected cases when the diagnosis is not straightforward.

MEN-1

MEN-1 is an autosomal dominant disorder, whose incidence has been estimated from random postmortem studies to be 0.25%, and to be 1%-18% in patients with primary hyperparathyroidism, 16%-38% in patients with gastrinomas, and less than 3% in patients with pituitary tumors[14]. From a clinical point of view, MEN-1 syndrome includes the occurrence of parathyroid adenoma (90%), entero-pancreatic tumor (30%-70%), being gastrinoma the most frequent (40%), and pituitary adenoma (30%-40%). Other tumors that might occur in MEN-1 patients are adrenal cortical tumor (40%), pheochromocytoma (< 1%), bronchopulmonary NEN (2%), thymic NEN (2%), gastric NEN (10%), lipomas, (30%), angiofibromas (85%), collagenomas (70%), and meningiomas (8%)[14].

In patients with an established diagnosis of gastrinoma-related ZES, MEN-1 syndrome might be present in approximately 25% of the cases. The presence of hypercalcemia due to hyperparathyroidism is one of the first signs. However, the diagnosis might be challenging in this specific setting as ZES does not usually develop in the absence of primary hyperparathyroidism, and hypergastrinemia has also been reported to be associated with hypercalcemia as a confounding factor[15]. Furthermore, parathyroidectomy leads to restoration of normocalcemia and improvement in clinical symptoms and biochemical abnormalities in as many as 20% of MEN-1 patients with ZES[14]. Moreover, staging and localization with CT or MRI is even more challenging in the setting of MEN-1 due to the presence of numerous small tumors < 1 cm in size[27,28]. A high index of suspicion must be maintained if a patient with chronic diarrhea and unexplained peptic ulcer disease presents with primary hyperparathyroidism. The genetic test for MEN-1 syndrome should be performed in a selected subgroup of patients, namely (1) in patients with two or more primary MEN-1-associated endocrine tumors (*e.g.*, parathyroid adenoma, entero-pancreatic tumor, and pituitary adenoma) or hypercalcemia associated with an endocrine tumor; and (2) patients showing MEN-1-related features and being the first-degree relative of a patient with a clinical diagnosis of MEN-1[14].

THERAPY

The management of gastrinoma and ZES includes both a proper medical treatment for symptom's relief and surgery with curative intent whenever feasible. A proposed therapeutic algorithm is represented in Figure 2.
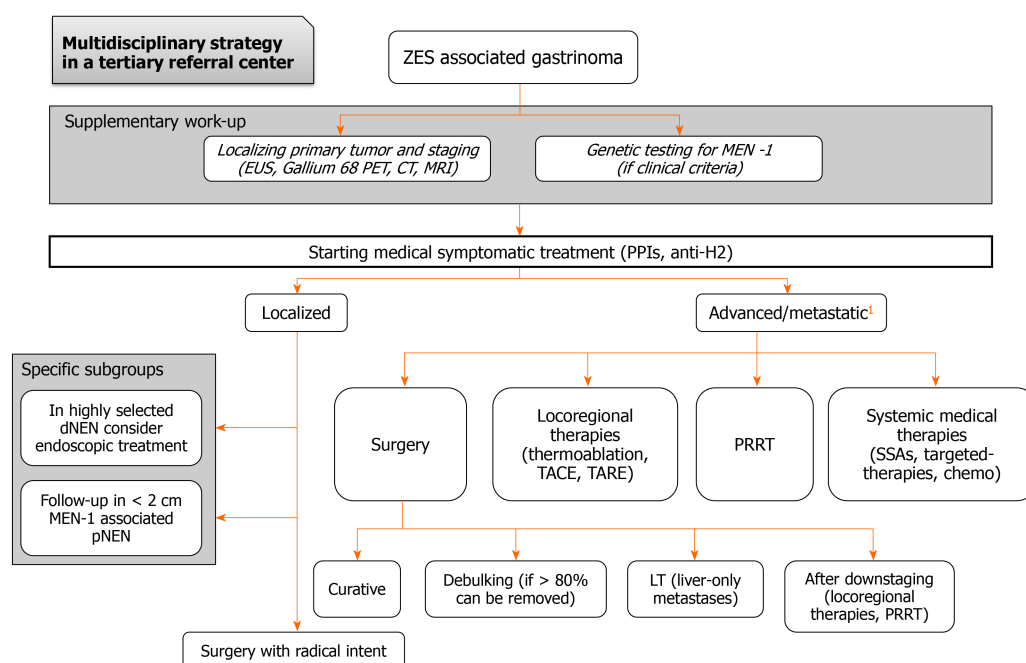
Figure 2 A proposed therapeutic algorithm is represented based on both evidence from literature and personal own experience. [1]Allocation driving prognostic factors are performance status, age, metastatic disease burden and pattern, comorbidities. CT: Computed tomography; dNEN: Duodenal neuroendocrine neoplasm; EUS: Endoscopic ultrasound; H2: Histamine receptor 2; LT: Orthotopic liver transplantation; MEN-1: Multiple endocrine neoplasia type 1; MRI: Magnetic resonance imaging; PET: Positron emission tomography; pNEN: Pancreatic NEN; PPIs: Proton pump inhibitors; PRRT: Peptide-radioreceptor therapy; SSAs: Somatostatin analogs; TACE: Transarterial chemoembolization; TARE: Transarterial radioembolization; ZES: Zollinger Ellison syndrome.

Surgery

The role of surgery in the treatment of gastrinoma has changed completely from the introduction of PPIs in the 1980s. In fact, before the advent of an effective anti-secretory therapy, surgery was performed to control acid hypersecretion, mainly removing the target cells of gastrin through total gastrectomy. These operations were, by the way, affected by a high mortality rate due to acid-related complications in the postoperative course. With the use of PPIs, gastric hypersecretion was no longer a problem, and the main determinant of prognosis became the gastrinoma itself because of its malignant potential and surgical excision started to be proposed as a potentially curative therapy. From 1981, the National Institute of Health began a prospective study recruiting patients with ZES for surgical therapy, with a well-designed surgical protocol in order to capture the long-term results of the best available surgical approach. The study reported a 10-year overall survival (OS) and disease-free survival (DFS) of 94% and 34%, respectively[54]. Therefore, surgery has gradually changed its role and gastrinoma resection has started to be increasingly proposed to patients eligible for resection. Currently, across the most important guidelines, surgical excision is generally recommended either for sporadic gastrinoma or for MEN-1 associated gastrinoma if complete tumor removal is possible[2,55-58]. Subsequent studies reported a 20 year OS of 58%-71%, a 20-year disease-related survival of 73%-88%[59], and a 10-year DFS of 25%-50%[60]. Surgery of the primary tumor also demonstrated to reduce the occurrence of liver metastases[61-63], which are one of the main determinants of prognosis, and to improve DFS in comparison with non-surgical management[62].

The majority of gastrinomas (from 60% to 90% depending on the series)[42,60] occur in the duodenum, and, since these are often very small lesions (less than 1 cm) and located at the submucosal layer, tumor detection is not so straightforward. Therefore, the surgical technique should follow a stepwise approach to search for the tumor even in case of negative preoperative imaging. In this context, surgery has firstly a diagnostic purpose, which is quite uncommon in modern surgery and, given the peculiarity of this technique and the rarity of the disease, it should be performed by experienced surgeons in tertiary referral centers. After a complete abdominal exploration, the duodenum and the pancreatic head are mobilized (Kocher maneuver) and carefully palpated. Intra-operative ultrasound with a linear probe is then performed on the duodenum and pancreas looking for the primary tumor and on the liver in search for liver metastases. Intra-operative endoscopy is performed thereafter

advancing the scope into the duodenum; duodenal gastrinomas may be found through trans-illumination of the bowel wall as non-trans-illuminated spots. If a lesion is identified, it should be marked with a suture and the duodenum opened around it for a full-thickness excision. If the described steps fail to reveal any lesion, a 3 cm longitudinal incision is made on the anterior aspect of the second portion of the duodenum, and the entire duodenal wall is palpated. Suspicious lesions are excised with a full-thickness rim of normal tissue and sent for pathology. The duodenum is then closed transversally, if possible, to minimize the risk of strictures[64,65]. In the hands of an experienced surgeon, lesions could be found in 98% of imaging-negative ZES patients, with a 50% curative rate[59], similar to that of imaging-positive patients. These findings suggest that surgery should be performed as soon as possible in sporadic ZES, despite negative imaging findings. Pancreatic gastrinomas should be enucleated if located 3 mm or farther from the main pancreatic duct. Conversely, lesions that are closer to the pancreatic duct require distal pancreatectomy with or without splenectomy if located in the body or tail of the gland and pancreaticoduodenectomy if located in the head/neck. Pancreaticoduodenectomy or distal pancreatectomy may be necessary also for local recurrence after enucleation[66].

Regional lymph nodes should always be removed because nodal metastases are present in almost half of the patients[54,67] and lymphadenectomy has been associated with increased DFS[68], as reported also for other pNENs[69-71]. The presence of primary gastrinoma located in a lymph node is controversial, however, several studies reported long disease-free survivors after resection of only a positive lymph node[72, 73], and this supports the role of routine lymphadenectomy.

Since pancreaticoduodenectomy provides complete removal of the regional lymph nodes of the pancreatic head, the results in terms of DFS are better with respect to enucleation because of the higher chance of radicality[54,67]. However, given the high postoperative morbidity and the good prognosis also of patients with small residual disease, pancreaticoduodenectomy is not recommended as the standard operation for these patients[2,55-58]. Generally, the indication for surgery should always follow a thorough risk/benefit assessment within a multidisciplinary tumor board aiming at maximum radicality and minimum morbidity. This is particularly the case for MEN-1 patients; in these patients, who have generally an earlier age of onset, pNENs should be resected in low-risk patients, and surgery is generally recommended for tumors larger than 2 cm[14,58]. However, according to most authorities, as well as all guidelines, surgical resection for an attempted cure should be performed in ZES patients whenever possible[2,27,58]. This is particularly true for functioning duodenal NENs, including gastrinomas, which have been reported to express a high metastatic potential[74], thus a radical surgical approach should be the first choice in this specific setting. However, in highly selected cases (*i.e.* duodenal lesions ≤ 1 cm, limited to the submucosal layer and without lymph nodal involvement), endoscopic resection might also be considered, although the risk of undetected micro-metastases might represent an issue.

Another controversial issue is laparoscopic surgery; while it is widely adopted for pNENs, its role for gastrinomas is limited to patients in whom preoperative imaging gives an accurate definition of tumor location. Unfortunately, as already mentioned, extensive exploration is often needed for diagnostic purposes. In these cases, laparoscopy is inadequate, and laparotomy is mandatory.

The role of surgical resection in ZES patients with advanced metastatic disease or even with extensive invasive localized disease is not well-defined. In this setting, the possibility of surgical removal of all resectable tumors (cytoreductive surgery, debulking surgery) should be considered, and surgery is generally recommended if ≥ 80% of all disease can be removed (generally feasible in 5%-15% of all metastatic gastrinomas), although only a few reports containing primarily gastrinomas treated with this approach are currently available[10,72].

Finally, in highly selected metastatic gastrinomas, with liver-only metastases and fulfilling strict inclusion criteria, liver transplantation might be considered, even if its use remains controversial and the risk of tumor recurrence represents an issue[58].

Liver-directed therapies

Studies specifically focused on liver-directed therapies in the context of gastrinomas are scant; however, as for other NENs, the embolization approaches in the setting of gastrinoma are generally reserved for patients with metastatic unresectable hepatic metastases either limited to the liver or with a liver-predominant disease, particularly if locally symptomatic[10,58]. Of note, liver-directed therapies are used less frequently in ZES than in other metastatic NENs, because in ZES, the hormone excess-state can be well-controlled medically.

Medical treatment

Among functioning NENs, gastrinoma is the most frequent type. There are two therapeutic goals in the management of patients with gastrinoma: The control of gastric acid hypersecretion and the treatment of the tumor itself.

Antisecretory medications

The therapy for syndrome control is based on PPI (*e.g.*, omeprazole, esomeprazole, lansoprazole, pantoprazole, *etc.*), which are highly effective drugs and considered the drugs of choice for suppressing acid secretion. PPIs effectively block gastric acid secretion by irreversibly binding to and inhibiting the hydrogen-potassium ATPase pump on the luminal surface of the parietal cell membrane. Theoretically, the choice and titration of anti-secretory therapy should be guided by the parameters of gastric acid secretions such as basal acid output (to reduce it below 10 mEq/h)[75], since using symptoms alone as a signal of efficacy might be misleading, even if in many centers these methods are not available. Therefore, in most cases, PPI therapy is started at an empirical maximized dosage. The recommended initial dose of omeprazole is 60 mg/daily or esomeprazole 120 mg/daily, lansoprazole 45 mg/daily, rabeprazole 60 mg/daily, pantoprazole 120 mg/daily, divided, twice-a-day[75-78]. The type of PPI used seems not to be of relevance and a systematic review of 12 randomized trials examining the relative effectiveness of different PPI doses and dosing regimens found no consistent differences in symptom resolution and esophagitis healing rates[79]. IV PPIs are indicated in patients with clinically significant upper GI bleeding from a suspected peptic ulcer. Omeprazole, pantoprazole, and esomeprazole are the only PPIs available as an IV formulation. The other patients can be treated with oral preparation.

As concerns efficacy, PPIs have significantly decreased the morbidity and mortality resulting from severe ulcer disease[80]. In 60% of patients, ulcer healing occurs within 2 wk; in 90%-100% of patients, healing occurs within 4 wk. PPIs are generally safe, even when used in high doses.

Once an effective clinical control of the peptic disease has been achieved, a gradual dose reduction is generally suggested[81,82]. In a study by Metz *et al*[83], 37 patients received high-dose omeprazole for almost 2 years, and nearly 50% were able to lower the dose down to 20 mg once daily, with 95% of patients experiencing safe long-term reductions in their medication dose. PPIs are generally well tolerated and can control hypergastrinemia in ZES for > 10 years (although some patients experience low vitamin B12 levels)[84].

No tachyphylaxis has been described. Therefore, the long duration of action, the fewer adverse effects, and the high potency make them superior to H2 blockers.

Regarding the use of H2-receptor antagonists in ZES, the dose usually is 4-8 times higher than the dose administered to patients with peptic ulcer disease. Although a good success rate exists, this treatment has been reported to fail in 50% of patients. Therefore, these drugs are never the first choice.

Only when PPIs are unable to control gastric acid secretion, SSAs can be considered, as they reduce gastrin secretion, even if they do not represent a first-line treatment at least for symptom control.

Even if this is not a treatment currently approved in localized gastrinoma, it is worth mentioning that in animals, the cholecystokinin-2 receptor antagonist YF476 has been shown to inhibit the development of enterochromaffin-like cell-tumors in susceptible animals with induced hypergastrinemia. Therefore, this drug could represent a potential option in ZES, not only to inhibit hypergastrinemia but also to prevent gastric NEN type 2 (*e.g.*, associated with ZES/MEN-1). Furthermore, there continues to be interest in the development of cholecystokinin-2 receptor antagonists as anti-secretory agents[85]. However, strong evidence supporting the role of these molecules in this specific setting is lacking.

Anti-proliferative treatment

Approximately one-third of ZES patients present with metastatic disease to the liver [10,86]. There are several systemic therapeutic options for advanced gastrinoma, not substantially different from the ones for other NENs, however, studies evaluating specific response rates in gastrinomas alone are limited.

SSAs like octreotide and lanreotide are highly effective in controlling the symptoms associated with hormone hypersecretion in all functioning tumors[87,88]; furthermore, they can reduce gastrin levels and their anti-proliferative effect has been demonstrated in PROMID and CLARINET studies[89,90]. However, in these studies only a few cases of gastrinoma were included, and, even if different case reports and case series suggested the role of SSAs in controlling gastrin secretion and symptoms in ZES

patients[91-94], to date only a few studies with a very low number of patients invest-igated specifically the role of SSAs in ZES[3].

The multitargeted tyrosine kinase inhibitor, sunitinib, has demonstrated an improved progression-free survival from 5.5 mo to 11.4 mo in metastatic pNENs[95]. Moreover, based on the results of two randomized, double-blind, prospective, placebo-controlled studies, the mammalian target of rapamycin-inhibitor everolimus has been approved in advanced both pancreatic[96] and extra-pNENs[96]. However, there are no specific studies on the effects of sunitinib/everolimus in the specific setting of gastrinomas.

Streptozocin, 5-fluorouracil, and doxorubicin have been used, with the response rate reported to be as high as 69%[97]. Despite these reported response rates, the true radiologic response rate is more probably between 10% and 40%[98,99]. More recently, anti-proliferative activity has also been shown for temozolomide. Data came from retrospective studies[100] as well as from a prospective randomized study comparing capecitabine plus temozolomide to temozolomide alone in pNENs that revealed a median progression-free survival longer in the combination arm (22.7 mo *vs* 14.4 mo, hazard ratio 0.58, P = 0.023), but satisfactory in both[101]. Moreover, a recent real-world analysis confirmed the combination of capecitabine and temozolomide as an active treatment for metastatic NENs[102]. Because of these studies, the use of capecitabine plus temozolomide has become routine for advanced pNENs, including gastrinomas.

Lastly, peptide receptor radionuclide therapy (PRRT) may be the most promising systemic therapy, and it has been repeatedly reported as particularly useful for symptom relief in functioning forms, even if this aspect might be less important in the setting of gastrinomas due to concomitant PPI treatment which is considered to be the first-line approach for symptoms' control[10]. Two different isotopes have been used in most studies: ^{90}Yttrium (^{90}Y)- or ^{177}Lutetium (^{177}Lu)-labeled SSAs[103]. The approval of PRRT treatment comes from the promising results of a double-blinded, control phase 3 trial (NETTER-1)[104] in patients with advanced unresectable, midgut carcinoids and the results of treatment of 510 patients with advanced pNENs and other NENs[105,106]. According to data from the literature, gastrinomas are one of the malignant pNENs that were most responsive to PRRT; however, they also had one of the highest recurrence rates leading to a poorer prognosis[103,105]. In detail, in one study including 11 patients with metastatic ZES[107] treated with either ^{90}Y-and/or ^{177}Lu-labeled SSAs, the mean serum gastrin decreased by 81%, complete response occurred in 9%, partial tumor response in 45%, tumor stabilization in 45%, with a persistence of the antitumor effect for a median period of 14 mo in 64% of the cases. Another study[108] involving 30 gastrinoma patients treated with ^{90}Y-labeled SSAs reported a partial response rate of 33% with a mean OS time of 40 mo.

CONCLUSION

As the diagnosis of ZES is challenging; the maintenance of a high index of suspicion is necessary to get the final diagnosis. Better disease awareness is useful to reduce the diagnostic delay, particularly due to the improper referral of patients to physicians with low or no expertise in the neuroendocrine field. The association between typical symptoms including chronic diarrhea, reflux disorder, and recurrent peptic disease particularly at unusual sites should raise the suspicion of ZES after exclusion of alternative and more common GI etiologies. The possibility of an underlying MEN-1 syndrome should be always considered, particularly in young patients with concomitant hypercalcemia suggestive of hyperparathyroidism and/or familiar history of MEN-1. A fasting gastrin level is generally the first step and confounding factors such as PPI use need to be considered. Gastric pH, esophageal pH-recording, and possibly a secretin stimulation test might be necessary as well, although the decision to perform them should be tailored to every single patient, considered both the need to withdraw PPI treatment and the limited availability of these tests in routine clinical practice. Tumor localization must be performed and EUS with the possibility of getting a sampling through FNA is considered to be a more accurate technique than conventional imaging for small lesions. Given the high expression of STTRs in gastrinomas, gallium-68PET-scan should be always included in the diagnostic pathway of all NENs, including gastrinoma, in order to both identify the primary tumor and to stage the disease.

Regarding the treatment of the localized disease, the two milestones are represented by PPIs for symptoms' control and surgery with curative intent. The role of surgery in

the treatment of gastrinoma has changed completely from the introduction of PPIs. In the past, total gastrectomy represented the sole effective treatment to treat ZES by removing the end-organ target of gastrin. With the use of PPIs, gastric hypersecretion was no longer considered a problem and surgical excision started to be proposed as a potentially curative therapy. Surgical removal of the primary tumor (and possibly its metastases) with curative intent should be, indeed, always performed. Unfortunately, the diagnosis is often made when the disease is too advanced for a surgical approach. The first step, again, is represented by syndrome control, based on PPIs, which are considered to be the drugs of choice for suppressing acid secretion. In order to achieve tumor growth control, SSAs constitute a viable option; studies specifically focused on advanced gastrinomas are scanty and often retrospective, however, according to data from the literature, treatments for the advanced disease are super-imposable to other NENs and include targeted therapies, chemotherapy, and PRRT. As there is a need for both a proper medical treatment for symptom's relief and a surgical procedure whenever feasible with curative intent, the multidisciplinary approach, with close cooperation between clinicians and surgeons, remains the cornerstone for proper management of this composite disease. Due to the risk of overlapping ZES with other GI common disorders, referral to tertiary centers with great expertise in the neuroendocrine field is mandatory.

ACKNOWLEDGEMENTS

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MINIREVIEWS

Optical diagnosis of colorectal polyps using convolutional neural networks

Rawen Kader, Andreas V Hadjinicolaou, Fanourios Georgiades, Danail Stoyanov, Laurence B Lovat

ORCID number: Rawen Kader 0000-0001-9133-0838; Andreas V Hadjinicolaou 0000-0002-6520-443X; Fanourios Georgiades 0000-0003-0440-2720; Danail Stoyanov 0000-0002-0980-3227; Laurence B Lovat 0000-0003-4542-3915.

Rawen Kader, Danail Stoyanov, Laurence B Lovat, Wellcome/EPSRC Centre for Interventional and Surgical Sciences, University College London, London W1W 7TY, United Kingdom

Rawen Kader, Laurence B Lovat, Division of Surgery and Interventional Sciences, University College London, London W1W 7TY, United Kingdom

Andreas V Hadjinicolaou, MRC Cancer Unit, Department of Gastroenterology, University of Cambridge, Cambridge CB2 0QQ, United Kingdom

Fanourios Georgiades, Department of Surgery, University of Cambridge, Cambridge CB2 0QQ, United Kingdom

Danail Stoyanov, Department of Computer Science, University College London, London W1W 7TY, United Kingdom

Corresponding author: Rawen Kader, BMed, MBBS, MRCP, Research Fellow, Wellcome/EPSRC Centre for Interventional and Surgical Sciences, University College London, Charles Bell House, 43-45 Foley Street, Fitzrovia, London W1W 7TY, United Kingdom. r.kader@nhs.net

Abstract

Colonoscopy remains the gold standard investigation for colorectal cancer screening as it offers the opportunity to both detect and resect pre-malignant and neoplastic polyps. Although technologies for image-enhanced endoscopy are widely available, optical diagnosis has not been incorporated into routine clinical practice, mainly due to significant inter-operator variability. In recent years, there has been a growing number of studies demonstrating the potential of convolutional neural networks (CNN) to enhance optical diagnosis of polyps. Data suggest that the use of CNNs might mitigate the inter-operator variability amongst endoscopists, potentially enabling a "resect and discard" or "leave in" strategy to be adopted in real-time. This would have significant financial benefits for healthcare systems, avoid unnecessary polypectomies of non-neoplastic polyps and improve the efficiency of colonoscopy. Here, we review advances in CNN for the optical diagnosis of colorectal polyps, current limitations and future directions.

Key Words: Artificial intelligence; Deep learning; Convolutional neural networks; Computer aided diagnosis; Optical diagnosis; Colorectal polyps

Core Tip: A convolutional neural network (CNN) is a specific type of artificial intelligence deep learning. These networks may play an important role in the coming years in assisting endoscopists to optically diagnose colorectal polyps. CNNs can mitigate the inter-operator variability amongst endoscopists, potentially enabling a "resect and discard" or "leave in" strategy to be adopted. This would improve the efficiency of colonoscopy, reduce healthcare costs and reduce adverse events for patients by avoiding unnecessary resections of non-neoplastic polyps. In this article, we expand on the most relevant studies in this field and discuss limitations and future directions that will determine fulfilment of the potential of CNN in the optical diagnosis of colorectal polyps.

INTRODUCTION

Colorectal cancer (CRC) is the third most commonly diagnosed cancer worldwide[1] and thus, a significant burden on global healthcare systems. Most CRCs develop in a relatively predictable, stepwise sequence from mutation-accumulating neoplastic polyps, such as adenomas and sessile serrated lesions (SSL)[2]. Current evidence-based societal guidelines unequivocally accept colonoscopy to be the gold standard tool for screening of CRC[3]. Colonoscopy offers the opportunity to both detect and resect neoplastic polyps[4] and its implementation, especially as part of bowel cancer screening programs, has been linked to a significant reduction in the incidence of the CRC and CRC-related mortality[5].

Over 90% of polyps detected at colonoscopy are either small (6-9 mm) or diminutive (≤ 5 mm), entities that are thought to harbour a very low risk for developing into CRC [6]. Furthermore, almost half of these polyps are non-neoplastic in nature; and frequently hyperplastic[7]. Accurate differentiation of neoplastic from non-neoplastic polyps can prevent the unnecessary resection of the latter, avoiding an intervention which is not cost-effective and which carries risks of significant morbidity[8].

Recent years have seen significant research activity in the use of artificial intelligence (AI), particularly convolutional neural networks (CNN), to optically diagnose colorectal polyps. The field is gaining increasing momentum. The aim of this review article is to summarise and critically appraise the available medical literature related to advances in CNN for optical diagnosis of colorectal polyps and highlight the field's current limitations and future directions.

OPTICAL DIAGNOSIS

The term "optical diagnosis" refers to the use of advanced imaging techniques for real-time, *in-vivo* polyp characterisation and evaluation to guide therapeutic decisions[9]. Accurate optical diagnosis of diminutive polyps would enable identification of hyperplastic polyps in the rectosigmoid region, where they are commonly found, and allow the endoscopist to confidently take a "diagnose and leave" approach instead of resecting the lesion. Equally, for diminutive adenomas, accurate optical diagnosis would prompt the endoscopist to remove the lesion on the spot and discard the specimen without the need for histological assessment ("resect and discard" strategy) [9].

The American Society of Gastrointestinal Endoscopy established the Preservation and Incorporation of Valuable endoscopic Innovations (PIVI) to provide thresholds that are required of endoscopic technology in order to implement a "resect and discard" (PIVI 1) and "diagnose and leave" (PIVI 2) strategy[9]. PIVI 1 requires ≥ 90%

concordance in post-polypectomy surveillance intervals when comparing the combination of optical diagnosis for diminutive adenomas with histopathology assessment of all other polyps against decisions based solely on histopathology evaluation of all identified polyps[10]. PIVI 2 requires a technology to achieve a negative predictive value (NPV) of ≥ 90% for diminutive adenomatous polyps in the rectosigmoid region[9].

There has been extensive research in image enhanced endoscopy (IEE), such as narrow band imaging (NBI), to assist endoscopists in optical diagnosis to characterise diminutive polyps[11-13]. Using IEE, expert endoscopists in academic centres have consistently demonstrated an optical diagnosis accuracy that exceeds PIVI thresholds [14-16], however, studies have often found community and non-expert endoscopists to fall short of these minimal thresholds[17]. An example is the multi-centre DISCARD-2 study which evaluated the optical diagnosis accuracy of 28 community endoscopists using NBI. Disappointingly, the endoscopists' optical diagnosis derived colonoscopy surveillance intervals only matched 68% of the histopathology derived intervals[18]. Although widely available, technologies for optical diagnosis has not been incorporated into routine clinical practice with one of the main barriers being the inter-operator variability amongst endoscopists[19].

WHAT IS A CONVOLUTIONAL NEURAL NETWORK?

AI is the ability of computers to perform tasks that traditionally require human intelligence (Figure 1)[20]. Machine learning (ML) is a subset of AI, whereby computers continuously learn from data without explicit human programming[21]. This can be used to predicate a polyp's histology. ML models can be trained using unsupervised or supervised techniques. Unsupervised learning is when the input and output data are not paired. Supervised ML is more labour intensive as it requires paired input and output data for training. An example of a supervised ML model for optical diagnosis is to annotate a bounding box around a polyp (input data), commonly referred to as a region of interest, and label it with the histology of the polyp (output data). The model automatically learns to extract features that allow it to differentiate polyp subtypes and output a diagnosis based on the histology classification system it was trained with but the annotation process is time consuming for the clinician.

Deep learning is a subset of ML, whereby algorithms use multiple layers within a neural network[22], mimicking the human brain, to extract high level features from input data. CNNs are the most commonly used network in the application of deep learning to optically diagnose polyps. They provide an objective output, bypassing the human inter and intra-operator variability, and can develop classification algorithms without exhaustive effort as they do not require human-crafted feature extraction or extensive pre-processing of data[23].

Building a CNN model typically involves three separate datasets; a training set, a validation set and a test set[24]. The training set is used to develop the model so that it predicts a label (*e.g.*, adenomatous or hyperplastic polyp for polyp characterisation) based on features extracted from the endoscopic image by the algorithm itself. The validation set is used to avoid over-fitting into the training dataset through fine tuning of the hyperparameters of the model. Finally, the testing set is used as an independent dataset to evaluate the generalisability of the CNN. With smaller datasets, cross-validation can be used to assess the model's robustness. In cross-validation, the data is split into equal parts (*e.g.*, 4 parts), with one part held out as a validation dataset. This process is repeated multiple times, with the results of each split eventually pooled together to decide how robust the model is[24]. CNNs evaluated using cross-validation should still be assessed against an independent test set to examine their generalisability[24].

CONVOLUTIONAL NEURAL NETWORKS AND OPTICAL DIAGNOSIS

It is only in the last few years that the use of CNNs in optical diagnosis of colorectal polyps has been extensively investigated, with various studies emerging (Table 1). Many of these studies have in fact demonstrated the capability of CNNs to surpass the PIVI 2 threshold in order to support a "leave in" strategy for rectosigmoid hyperplastic polyps (Table 2). This was first demonstrated by Chen *et al*[25], who used a single centre, retrospective, still image dataset of 2157 polyps to train a CNN and reported a

Table 1 Summary of the studies on convolutional neural network algorithms for the optical diagnosis of colorectal polyps

Ref.	Study design (training/testing)	Multi-centre study	Dataset	Image quality	Classification system	Lesion number (training/testing)	SSL excluded	Endoscopic processor	Image modality (training)	Real-time capability
Komeda *et al* [37]	Retrospective	Single	Video	Not specified	Adenoma/non-adenoma	Not specified/10	No	Not specified	WLI, NBI, chromoendoscopy	Not specified
Chen *et al*[25]	Retrospective/prospective	Single	Still	HQ	Hyperplastic/neoplastic	2157/284	Yes	Olympus 260 + 290	Magnified NBI	Real-time (approximately 450 ms)
Byrne *et al* [23]	Retrospective/prospective	Single	Video	All images	NICE Type 1/NICE Type 2	220/125	Yes	Olympus 190	NBI-NF	Real-time (approximately 50 ms)
Zachariah *et al*[26]	Prospective	Two	Still	Adequate and HQ	Adenomatous/serrated polyp	5278/634	No	Olympus 190 (90%), 180 (7%), Pentax i10(3%)	WLI, NBI, i-SCAN	Real-time (approximately 13 ms)
Ozawa *et al* [38]	Retrospective/prospective	Single	Still	HQ	Hyperplastic/adenomatous/SSL/CRC/other	WLI: 17566/783 NBI: 2865/290	No	Olympus 260 + 290	WLI, NBI	Real-time (approximately 20 ms)
Jin *et al*[31]	Retrospective/prospective	Single	Still	HQ	Hyperplastic/adenomatous	2150/300	Yes	Olympus 290	NBI-NF	Real-time (approximately 10 ms)
Song *et al*[39]	Retrospective/prospective	Single	Still	HQ	Serrated polyp/benign adenoma/MSM/DSMC	624/545	No	Olympus 290	NBI-NF	Real-time (approximately 20-40 ms)
Rodriguez-Diaz *et al*[28]	Retrospective/prospective	Two	Still	Not specified	Neoplastic (adenomas, CRC)/non-neoplastic (hyperplastic, normal)	607/280	Training: Yes Testing: No	Olympus 190	NBI-NF, NBI (digital magnification)	Real-time (approximately 100 ms)
van der Zander *et al* [27]	Retrospective/prospective	Not specified	Still	HQ	Benign (hyperplastic)/pre-malignant (adenomatous, SSL, T1 CRC)	398/60	No	Fujifilm, Pentax	WLI, BLI, i-SCAN	Real-time (approximately 14.8 ms)

SSL: Sessile serrated lesion; WLI: White light imaging; BLI: Blue light imaging; NBI: Narrow band imaging; NBI-NF: Narrow band imaging–near focus; NICE: NBI International Colorectal Endoscopic; HQ: High-quality; CRC: Colorectal cancer; MSMC: Mucosal or superficial submucosal cancer; DSMC: Deep submucosal cancer.

sensitivity for identifying adenomas of 96.3% , specificity 78.1%, and NPV of 91.5% when evaluating a test set of 284 colonic and rectal diminutive adenomatous and hyperplastic polyps. Using colonic diminutive polyps is a common strategy to assess against PIVI 2 due to difficulties in obtaining large datasets of diminutive rectosigmoid polyps. An important limitation of this study is that it used magnified narrow-band

Table 2 Summary of the per-polyp results of studies on convolutional neural network algorithms for the optical diagnosis of colorectal polyps (cross-validation results not included)

Ref.	Image Modality (testing)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy for neoplasia (%)	PIVI 1 achieved (%)	PIVI 2 achieved (%)
Komeda *et al*[37]	Not specified	-	-	-	-	70	-	-
Chen *et al*[25]	Magnified NBI	96.3	78.1	89.6	91.5	90.1	-	Yes (91.5)
Byrne *et al*[23]	NBI-NF	98	83	90	97	94	-	Yes (97)
Zachariah *et al* [26]	NBI	-	-	-	96.5	93.1	Yes (98.3)	Yes (96.5)
	WLI	-	-	-	88.9	92.8	Yes (90.8)	No (88.9)
Ozawa *et al*[38]1	NBI	97	-	84	88	-	-	-
	WLI	98	-	85	88	-	-	-
Jin *et al*	NBI-NF	83.3	91.7	93.3	78.6	86.7	-	-
Song *et al*[39]	NBI-NF (test set 1)	84.1	74	88.3	67.7	-	-	-
	NBI-NF (test set 2)	88.5	72.1	88.6	84.7	-	-	-
Rodriguez-Diaz *et al*[28]	NBI-NF (90%) + NBI (10%)	95	88	-	93	-	Yes (94 (20/90 LC))	Yes (98 (6/68 LC))
van der Zander *et al*[27]	WLI + BLI	95.6	93.3	97.7	87.5	95.0	-	No (87.5)

1Per frame analysis reported only.

WLI: White light imaging; BLI: Blue light imaging; NBI: Narrow band imaging; NBI-NF: Narrow band imaging–near focus; PIVI: Preservation and Incorporation of Valuable endoscopic Innovations; PPV: Positive predictor value; NPV: Negative predictor value; LC: Low-confidence.
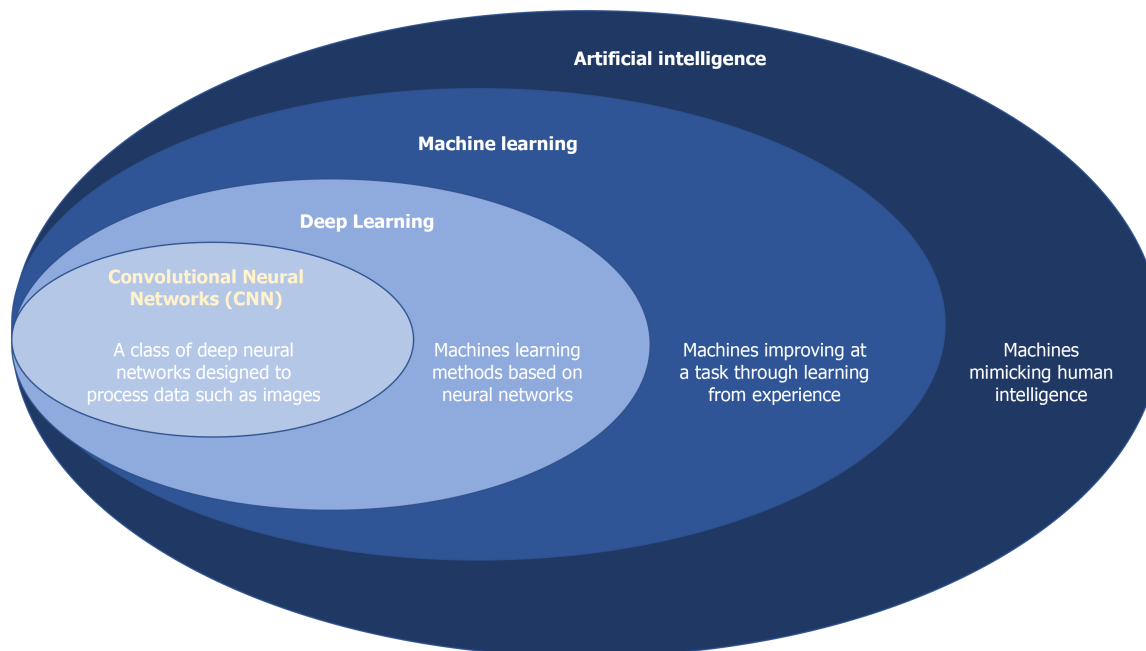
Figure 1 The relationship between convolutional neural networks, deep learning, machine learning and artificial intelligence.

imaging (NBI) data. This recently developed modality is not yet readily available in most endoscopy departments, although it will become more widely used with time.

Byrne *et al*[23] further advanced the field by training a CNN with NBI-near focus (NBI-NF) which is more commonly used in Europe and North America. It was trained with 220 polyp positive videos and when tested against 125 diminutive polyps which were collected prospectively, the model diagnosed 106 polyps with high confidence, achieving a sensitivity for identifying NBI International Colorectal Endoscopic (NICE)

type 1 polyps of 98%, specificity 83% and NPV of 97%. A novelty worth highlighting in this study was the use of images derived from videos, an approach that reduces selection bias compared to retrospective still images as endoscopists usually capture high quality polyp views that are free from motion blur and surface artifact. An additional advantage of this CNN is that it simplified the clinical workflow as it automatically diagnoses polyps without requiring a still image of the polyp to be captured. Limitations of the study are that SSLs, normal tissue and lymphoid aggregates were excluded from the final analysis and the videos used to train and test the CNN were captured from colonoscopies performed by a single expert endoscopist and hence, potentially less generalisable to novice users.

The most commonly used imaging modalities amongst community endoscopists are white light imaging (WLI) and NBI without magnification. Using a large retrospective still image training set of 5278 polyps and tested against 634 polyps, Zachariah *et al*[26]'s CNN fell short of *PIVI* 2 in WLI (NPV of 88.9% and accuracy 92.8%) but achieved the threshold in NBI without magnification (NPV of 90.8% and accuracy 93.1%). This study advanced the field as it demonstrated the capabilities of CNNs to optically diagnose polyps in standard NBI modality and also to differentiate adenomas from serrated polyps through the inclusion of SSLs in its dataset.

Whilst the majority of CNNs have been trained and tested using Olympus data, studies are emerging using data from other manufacturers. van der Zander *et al*[27] recently developed a CNN using Fujifilm data in high definition white light (HDWL) and blue light imaging (BLI). The CNN was more efficacious when it used a unique multimodal imaging approach where it combined both HDWL and BLI images of the same polyp in its decision process compared to a single imaging modality. When evaluated against 60 prospectively collected diminutive polyps, it did not reach the *PIVI* 2 threshold with a NPV of 87.5% but did achieve an optical diagnosis accuracy of 95% (sensitivity for identifying pre-malignant polyps 95.6% and specificity 93.3%) and demonstrated superiority to both expert and novice endoscopists in human benchmark testing.

In comparison to PIVI 2, there are fewer studies evaluating the performance of CNNs against PIVI 1. The CNN presented in Zachariah *et al*[26] reached PIVI 1 thresholds in both WLI and NBI with normal magnification, achieving concordance with histology-based colonoscopy surveillance intervals in 90.9% and 98.3% of patients, for each respective modality. Rodrigues-Diaz *et al*[28] used a single centre retrospective still image dataset to train a CNN with 607 polyps and tested against 90 diminutive polyps where it achieved a high confidence diagnosis in 78% of cases, with a 94% agreement with histology-based colonoscopy surveillance intervals. Tested against 68 rectosigmoid polyps, the model diagnosed 88% of polyps with high confidence, achieving PIVI 2 thresholds with a NPV of 97%.

There is also potential to expand the use of optical diagnosis CNNs outside of the "resect and discard" and "leave in strategy". A dilemma that can complicate issuing post-polypectomy surveillance intervals is discrepancies between endoscopic and histological diagnosis and classification of polyps with tissue fragmentation in the specimen retrieval process playing an important role. Shahidi *et al*[29]'s proof of concept study used a CNN to resolve discrepancies in polyps ≤ 3 mm in size. Tested against 900 polyps that were ≤ 3 mm and optically diagnosed as adenomatous by an expert endoscopist, the CNN diagnosed the adenomas with high confidence in 644 polyps, with 256 polyps deemed to be of sub-optimal imaging quality. However, of these high confidence diagnoses, the pathologists diagnosed 15.4% as normal mucosa, 13.2% as hyperplastic polyp and 0.3% as SSL. In this context, a CNN could help to mitigate against the risk of under-surveillance.

Whilst CNN's diagnostic accuracy excels in many studies, without real-time capabilities, they would have no clinical utility. Prior to the era of deep learning, computer aided diagnosis algorithms lacked real-time capability, but most CNNs do not share this problem and often process data at a rate that exceeds the 25 frames per second that is generated in a video recording of a colonoscopy procedure. Given the excellent performance in ex-vivo studies and the real-time capabilities displayed by CNNs, the future appears promising for their integration in colonoscopy.

TRANSPARENCY OF CONVOLUTIONAL NEURAL NETWORKS

The complexity of CNN models' decision process is often referred to as a "black box" and represents an important barrier to its acceptance by both clinicians and patients [30]. Opening the ''black box'' to display the raw features which informed the CNN's
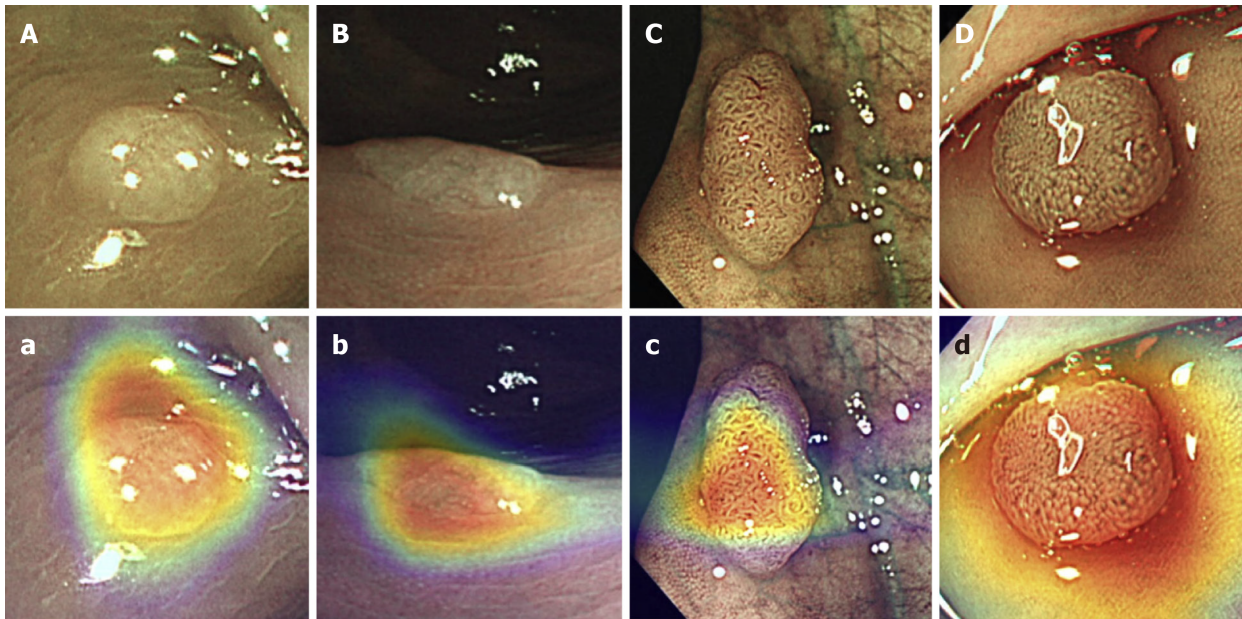
Figure 2 Illustration of coloured heatmaps, overlaid to the polyp, which demonstrates the regions that most likely contributed to the convolutional neural networks's diagnosis. A, B, C, D: Original narrow band imaging (NBI) of polyps; a, b, c, d: Coloured heatmap overlaid on the NBI image; Red: Higher probability that this region informed the convolutional neural networks (CNN)'s diagnosis; Blue: Lower probability that this region informed the CNN's diagnosis. Images adapted and modified with permission from the publisher[31]. Citation: Jin EH, Lee D, Bae JH, Kang HY, Kwak MS, Seo JY, Yang JI, Yang SY, Lim SH, Yim JY, Lim JH, Chung GE, Chung SJ, Choi JM, Han YM, Kang SJ, Lee J, Chan Kim H, Kim JS. Improved Accuracy in Optical Diagnosis of Colorectal Polyps Using Convolutional Neural Networks. *Gastroenterology* 2020; 158(8): 2169-2179. Copyright© The Authors 2020. Published by Elsevier.

decision is important for transparency especially from a safety standpoint[28]. Transparency can help identify biases within the neural network and aid root-cause analyses in cases of patient harm, for example, if a neoplastic polyp that subsequently develops into a CRC is originally misdiagnosed as non-neoplastic by the CNN model.

For polyp characterisation, important steps have been taken to open the black box. Jin *et al*[31] developed a CNN that generated a coloured heat map, overlaid to the polyp, to help the endoscopist comprehend the specific aspects of the image that contributed to the CNN's prediction (Figure 2). This could help the endoscopist to decide which information is relevant and which decisions are truly based on appropriate image analysis. If, for example, the heatmap is overlaid to normal mucosa, then the endoscopist would quickly be able to appreciate this and disregard the CNN's diagnosis.

More recently, in order to further enhance CNN transparency, Rodriguez-Diaz *et al* [28] developed a colour coded segmentation model (Figure 3). In this model, the CNN divides the polyp into distinct segments to allow the endoscopist to identify the specific regions within the image that is informing the CNN's decision. The CNN predicts the histology of each subregion of the segmented polyp, with high confidence neoplastic diagnoses coloured in red, high confidence non-neoplastic in green, and low confidence/indeterminate diagnoses in yellow, with the final predication resulting from an aggregate of all the analysed regions. The end result is a detailed spatial colour coded histology map of the polyp surface, which the endoscopist can visualise and incorporate into their decision process[28], enhancing the interpretability of this CNN model in comparison to others. However, an important limitation to this advanced CNN is that it currently lacks the ability to operate at a video rate.

Further research in the interpretability of CNN models is required to improve its acceptance[32] and accelerate its translation to clinical practise.

LIMITATIONS AND FUTURE DIRECTIONS

Despite the promise shown by CNNs this far, it is crucial to recognise that there are various limitations that need to be overcome before they can become part of the endoscopic clinical workflow. The most significant limitations are the reliance on retrospective datasets[33], which are inherently subject to selection bias, and the lack of prospective studies and randomised controlled trials[34]. Most studies train and test
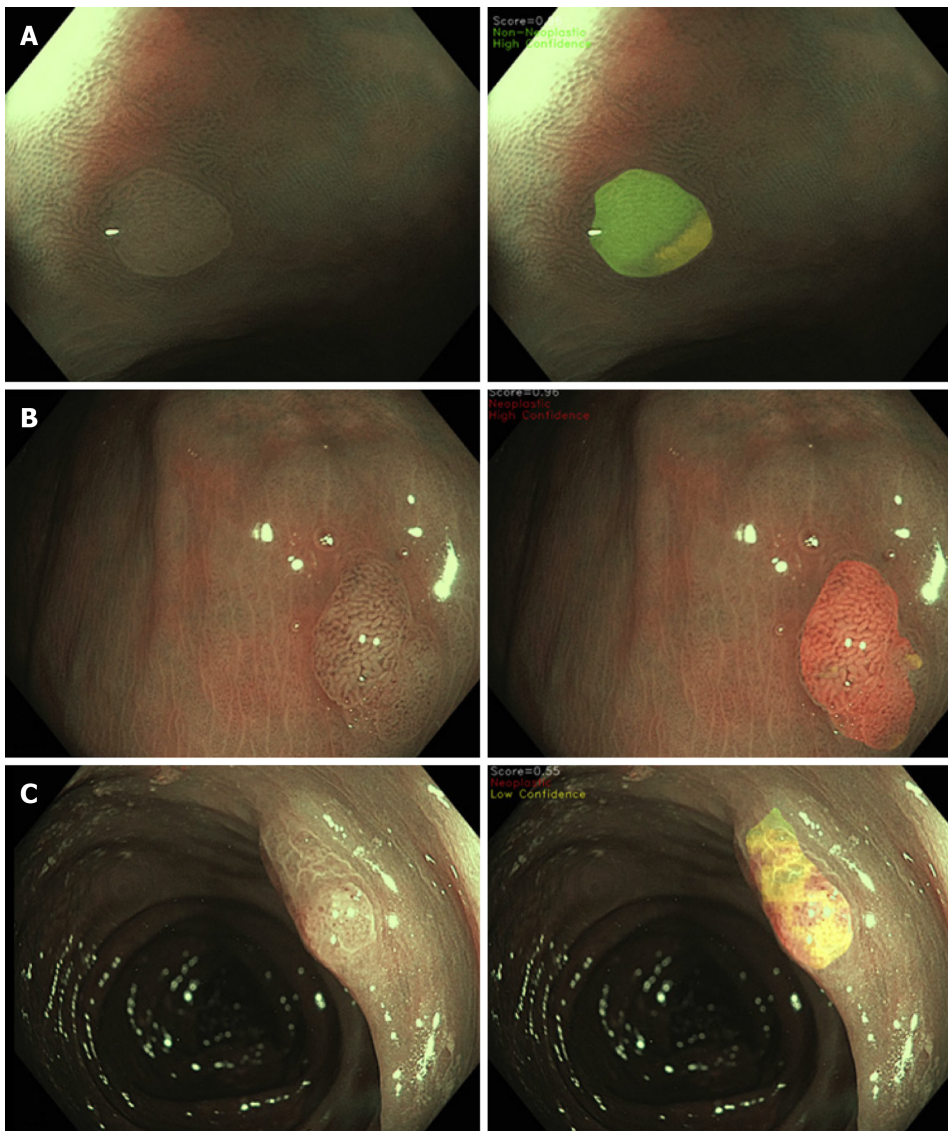
Figure 3 Spatial colour coded histology map which allows the user to visualise the sub-regions of the polyp surface that contributed to the convolutional neural networks's decision process. A: Hyperplastic polyps; B: Adenomatous polyps; C: Sessile serrated lesions; Red: High-confidence neoplastic diagnosis; Green: High-confidence non-neoplastic diagnosis; Yellow: Indeterminate or low-confidence diagnosis. Adapted from Ref. [28]. Citation: Rodriguez-Diaz E, Baffy G, Lo WK, Mashimo H, Vidyarthi G, Mohapatra SS, Singh SK. Real-time artificial intelligence-based histologic classification of colorectal polyps with augmented visualization. *Gastrointest Endosc* 2021; 93: 662-670. Copyright© The Authors 2021. Published by Elsevier.

CNNs using high quality images of polyps, free from "noise" such as motion blur and polyp surface artifact (*e.g.*, mucus, stool or blood). The extent to which CNNs pre-clinical results are reproducible in the real-world setting, where 'noise' is frequently encountered, remains to be seen.

To the best of our knowledge, there have been no prospective randomised controlled clinical trials evaluating optical diagnosis CNN *in-vivo*. This is partly due to clinical trials being time consuming and expensive, and an alternative pragmatic approach could be the use of a benchmark test in the form a publicly available external dataset to compare different CNN models[35]. No such datasets currently exist for polyp characterisation and therefore the generalisability of CNN models remains poorly understood. Generalisability refers to the CNN performance with different endoscope models and clinical settings from the site that the data was generated to train the CNN. To date, only one study[36] has evaluated generalisability, and this was limited to a small testing set of 69 polyp images from two population cohorts (Australian and Japanese) using two separate endoscope manufactures (Olympus and Fujifilm). Despite the small test-set, this study highlighted the concerns of generalisability as the operator area under the curve fell from 94.3% for the internal set, to 84.5% and 90.3% for the external testing sets (NBI and BLI respectively).

Another important limitation is that studies often exclude polyps that are not adenomas or hyperplastic polyps, restricting the possible classification outputs of CNNs. This, in turn, limits their clinical utility as polyps such as SSL and inflammatory polyps would be misclassified due to limitations in the initial training phase of the CNNs when the categorisation system is established.

Research in this field is likely to continue to expand and future directions to consider include: (1) Guidelines to identify the role of CNNs in the clinical workflow, specifically, whether it is a second reader, a concurrent reader or a provider of an independent diagnosis[30]; (2) Prospective multi-centre randomised clinical trials; (3) Publicly available external datasets for benchmark testing and evaluation of the generalisability of CNN models in different clinical settings and population cohorts; and (4) Acquiring datasets inclusive of all polyp sub-types to advance CNN classification systems.

CONCLUSION

In summary, this is an exciting time for the endoscopy community. CNNs diagnostic performance has excelled in ex-vivo studies and in human benchmarking testing. CNNs are likely to be a key adjunct in optically diagnosing polyps and have renewed optimism that implementation of a "resect and discard" and "leave in" strategy is feasible due to the potential to alleviate the inter-operator variability amongst endoscopists. This would bring significant financial benefits to healthcare systems, avoid unnecessary polypectomies of non-neoplastic polyps and improve the efficiency of colonoscopy. However, prospective multi-centre randomised controlled trials and publicly available datasets for benchmark testing are required to further evaluate the efficacy and generalisability of CNNs. Furthermore, with these models now emerging in endoscopy units, it's imperative that guidelines are developed to establish their role in the clinical workflow.

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Liver-spleen axis dysfunction in COVID-19

Sara Cococcia, Marco Vincenzo Lenti, Giovanni Santacroce, Giovanna Achilli, Federica Borrelli de Andreis, Antonio Di Sabatino

Sara Cococcia, Marco Vincenzo Lenti, Giovanni Santacroce, Giovanna Achilli, Federica Borrelli de Andreis, Antonio Di Sabatino, First Department of Internal Medicine, San Matteo Hospital Foundation, University of Pavia, Pavia 27100, Italy

Sara Cococcia, Department of Gastroenterology, Royal Free Hospital, London NW3 2QG, United Kingdom

Corresponding author: Marco Vincenzo Lenti, MD, Academic Research, Research Assistant Professor, First Department of Internal Medicine, San Matteo Hospital Foundation, University of Pavia, Viale Golgi 19, Pavia 27100, Italy. marco.lenti@unipv.it

Abstract

Coronavirus disease 2019 (COVID-19), caused by the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is an acute infectious disease that spreads mainly through the respiratory route. Besides interstitial pneumonia, a number of other clinical manifestations were noticed in COVID-19 patients. In particular, liver and spleen dysfunctions have been described both as complications of COVID-19 and as potential predisposing factors for severe COVID-19. Liver damage is rather common in COVID-19 patients, and it is most likely multifactorial, caused by the direct insult of SARS-CoV-2 to the liver by the cytokine storm triggered by the virus, by the use of hepatotoxic drugs, and as a consequence of hypoxia. Although generally mild, liver impairment has been found to be associated with a higher rate of intensive care unit admission. A higher mortality rate was reported among chronic liver disease patients. Instead, spleen impairment in patients with COVID-19 has been poorly described. The main anatomical changes are the architectural derangement of the B cell compartment, white pulp atrophy, and reduction or absence of lymphoid follicles, while, from a functional point of view, the IgM memory B cell pool is markedly depleted. The outcome of COVID-19 in asplenic or hyposplenic patients is yet to be defined. In this review, we will summarise the current knowledge regarding the impact of SARS-CoV-2 on the liver and spleen function, as well as the outcome of patients with a pre-existent liver disease or defective spleen function.

Key Words: Asplenia; Chronic liver disease; IgM memory B cell; Liver transplantation; Transaminase

Core Tip: The severe acute respiratory syndrome coronavirus 2 has rapidly spread worldwide, primarily causing interstitial pneumonia, although many other organs can be involved. Here, we will discuss the current knowledge regarding the liver and spleen involvement caused by this infection.

INTRODUCTION

In December 2019, a novel coronavirus-related pneumonia was detected in a Chinese group of patients[1]. The pathogen was later named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)[2], and on 30th January 2020 the World Health Organization publicly declared the outbreak of the new virus-related disease, the so-called coronavirus disease 19 (COVID-19)[3].

The most common clinical manifestations of SARS-CoV-2 infection include fever, dry cough, dyspnoea, fatigue, and myalgia[4,5], but the increasing information in published literature reported a wide spectrum of extrapulmonary symptoms and signs, especially arising from the gastrointestinal tract[6]. Hepatic involvement in COVID-19 patients has been largely documented in several observational studies, highlighting a significant prevalence of liver impairment in hospitalized individuals and a correlation with the severity of the disease[7,8]. COVID-19 implications for individuals with a pre-existent chronic liver disease (CLD) have also been evaluated, and a few studies have focused on the management and prognosis of post-transplant patients[9,10].

Little is known about the splenic involvement in COVID-19 patients. The spleen plays a fundamental role in the immune system modulation, regulating the T and B cell responses to the antigenic targets in the blood, and the tropism of the coronaviruses for the spleen has been documented[11]. Although splenic alterations in autoptic specimens have already been shown, and these anatomical changes might contribute to the abnormal immune reaction occurring in COVID-19[12], data on prognosis of COVID-19 individuals with splenic function impairment have been poorly investigated so far.

In this review, we aim at elucidating the pathological role of SARS-CoV-2 in patients with hepatic and splenic involvement, ranging from specific biochemical alterations to any histopathological modifications. Secondly, our purpose is to evaluate the impact of COVID-19 in individuals with a pre-existent diagnosis of hepatic disease or defective spleen function or asplenia.

MATERIALS AND METHODS

From January to March 2021 we searched on MEDLINE (PubMed) by using the medical subject heading terms "liver", "hepatic", "spleen", "splenectomy", "hyposplenic" matched with "coronavirus", "COVID-19", "SARS-CoV-2" for all articles published since database inception. More than 3000 papers were found with this search strategy, most of which were not strictly related to the subject of this review. Hence, we selected human studies exploring relationships between COVID-19 and liver or spleen function, as well as the outcomes of COVID-19 patients with CLD or spleen hypofunction/asplenia. Given the high number of papers and senior authors (SC, MVL, ADS), after a careful review, we selected the most important or representative ones, summarising current evidence. We also searched for additional papers in the reference lists of review articles, and they were included if deemed appropriate.

LIVER IMPAIRMENT IN COVID-19

Pathogenesis

Since the most recent studies reporting clinical manifestation of COVID-19 were carried out, the alteration of liver function tests (LFTs) has been reported[4,13-15]. These abnormalities, which still have an unclear clinical significance, have been repeatedly reported in patients suffering from a more severe disease[4,16-18]. The exact cause of liver damage during SARS-CoV-2 infection is partly unknown and most likely multifactorial (Figure 1)[18]. One of the possible explanations has been found in the direct insult of SARS-CoV-2 to the liver through the binding of the virus to the angiotensin-converting enzyme 2 (ACE2) receptor, which represents the main cell entry receptor for the virus[19-21]. ACE2 receptors, which are key players in regulating arterial blood pressure, are expressed in almost any tissue of the human body, especially in the lungs, kidneys, gut, liver and brain. Their polymorphisms may lead to a cardiovascular disease and a stroke[22]. However, the ACE2 receptor is highly expressed by cholangiocytes rather than hepatocytes; therefore, the hepatic damage would be channelled through the bile duct dysfunction, which might alter the immune responses and liver regeneration[20]. Nonetheless, it should be noted that alkaline phosphatase (ALP) is not constantly raised in these patients[23].

In addition to the aforementioned mechanisms, the cytokine storm resulting from the excessive immune response triggered by the virus could be another factor leading to liver damage[23,24]. An excessive increase in pro-inflammatory cytokines has been found in a high percentage of critically-ill COVID-19 patients, alongside with a reduction in T cells and an increase in the neutrophilic count. The hypothesis that the lymphocytopenia and C-reactive protein (CRP) levels are independently correlated to the presence of liver damage has been proposed, suggesting a role of the cytokine storm in causing liver dysfunction[25]. This hypothesis has also been proved with regard to organs other than the liver, including heart and kidneys[26], supporting the idea that the cytokine storm may cause shock and tissue damage. Another contributing factor is the use of potentially hepatotoxic drugs, including antibiotics (*e.g.*, macrolides), antiviral agents especially used during the first wave of the pandemic, corticosteroids, and paracetamol[23,24]. Lastly, liver damage can be caused by hypoxia, as a result of severe respiratory failure[23,24,27].

Clinical findings

Liver abnormalities are rather common in COVID-19 patients. The proportion of COVID-19 inpatients with an elevated alanine aminotransferase (ALT) has been found to be as high as 36%, and a higher proportion (46%) also had raised aspartate aminotransferase (AST)[18,28]. On the contrary, ALP or gamma-glutamyl transpeptidase (GGT) alterations were reported more rarely[18,29]. Although rather common, in most cases liver injury is mild and it usually manifests in more critically-ill patients[18,30,31]. A mild-to-moderate increase of ALT was reported in 43/87 patients (49.4%), and a higher mortality rate was observed among those with deranged LFT who had developed acute respiratory distress syndrome (ARDS)[29]. Similarly, Richardson *et al*[7], who enrolled more than 5000 patients with liver involvement, showed that acute hepatic injury, although rare, was associated with higher mortality. Liver involvement was reported in 2700 patients (39%), and 1% of the whole cohort developed acute liver injury. Another study enrolling more than 2000 patients confirmed these findings, reporting acute liver injury in a quarter of the included patients and severe liver injury in only 6.4% of the patients. However, this small proportion had a more complex clinical course, including intensive care admission and intubation need in more than 60% of the cases, renal replacement therapy in a third, and mortality as high as 42%[31]. A low incidence of severe liver injury (9%) was also reported by a German study enrolling 44 patients of which 6 had deranged ALT. Also, the German cohort reported AST to be more commonly deranged than ALT[28].

Although generally mild, liver impairment has been found to be associated to a higher rate of intensive care unit admission[30,32], as well as to a longer hospital stay [33]. Ponziani *et al*[30] reported liver involvement in 161 out of 515 patients enrolled (31.3%) and no cases of severe acute liver injury. Moreover, although liver involvement led to a higher need for intensive care, no increase in mortality was recorded among those patients. However, conflicting data have been published on the role of liver impairment in increasing mortality in patients without pre-existing liver disease. Medetalibeyoglu *et al*[32] reported that AST/ALT ratio was a good predictor of mortality (area under the curve [AUC]: 0.713; *P* = 0.0001) in a cohort of 554 individuals enrolled in Turkey, and that AST and ALT levels were independently
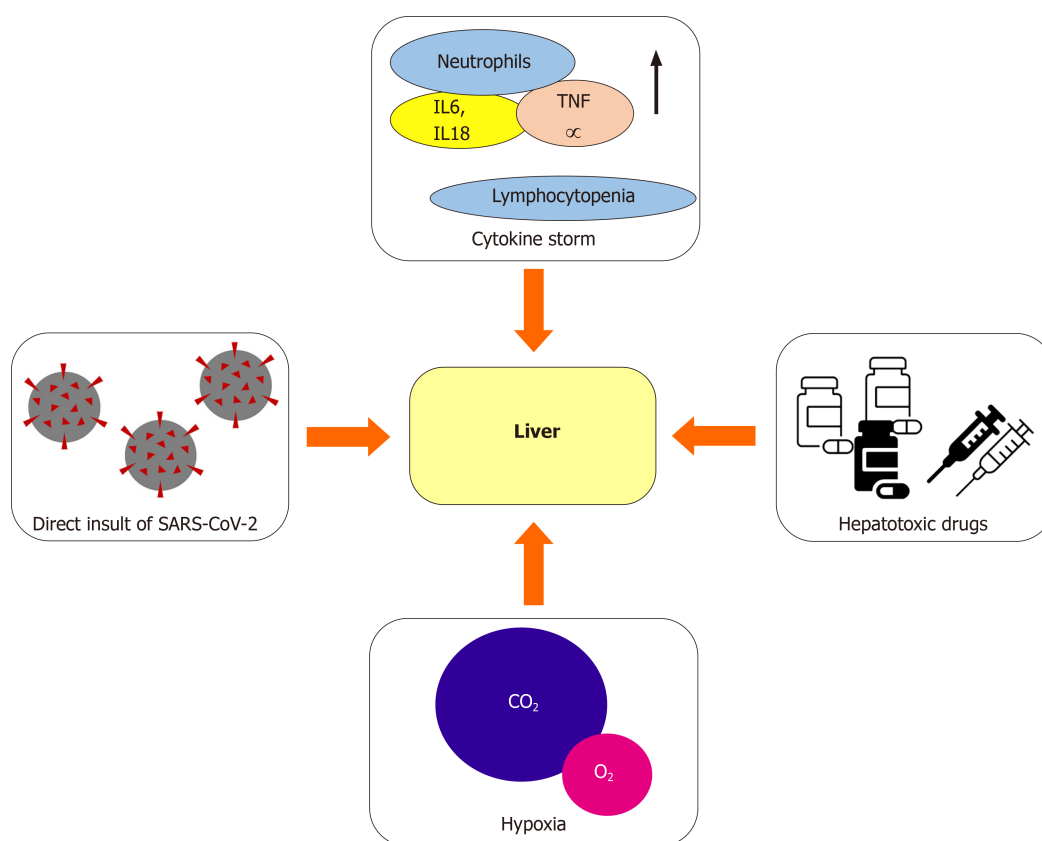
Figure 1 Putative mechanisms of liver damage in coronavirus disease 2019. IL: Interleukin; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; TNF: Tumour necrosis factor.

associated with an increased need for intensive care and with mortality (P = 0.001). Table 1 reports the main studies focusing on liver abnormalities in COVID-19 patients.

Histological features

Limited data are available about histological liver findings in COVID-19 patients. Lagana *et al*[34] reported the histological features of 40 patients who died of COVID-19-related complications and who had liver biochemical abnormalities. Two-thirds of the included patients presented macrovesicular steatosis, which was most commonly panlobular, while 2 patients (7%) showed active steatohepatitis. Half of the included patients had mild lobular necroinflammation and, therefore, active hepatitis, which was mild in 80% of the cases and moderate in the remaining 20%. Similarly, portal inflammation was reported in 20 patients, 3 of which had interface hepatitis. Lobular mild and focal cholestasis changes were observed in 15 (38%) cases. Although the ACE2 receptor is mainly expressed by cholangiocytes in the liver, ductopenia was not reported. Vascular alterations (*i.e.* phlebosclerosis, portal arteriolar muscular hyperplasia, focal fibrinoid necrosis, and sinusoidal thrombus) were less common (15%). Interestingly, no significant correlation was found between laboratory and histological findings. Wang *et al*[35] demonstrated, in 2 deceased COVID-19 patients, that SARS-CoV-2 can infect the liver causing direct cytopathy. They also reported massive hepatic apoptosis as well as binuclear hepatocytes. However, due to the small sample size, further studies are needed to confirm these preliminary findings.

COVID-19 IN PATIENTS WITH A PRE-EXISTING CLD

Immune dysregulation is known to affect people with CLD or cirrhosis, leading to the concern that these patients are at higher risk of having a more severe form of COVID-19[36]. A limited number of studies have investigated the role of COVID-19 in patients with a pre-existing CLD and most of them only included a limited number of patients from a restricted geographical area. It is to be noted that all these studies reported a higher mortality rate among CLD patients[37-43]. Marjot *et al*[43] conducted one of the largest studies of CLD cases (745 patients) from 29 different countries. They showed

Ref.	Country	Patients	Liver involvement criteria	Patients with liver involvement, *n* (%)	Main findings
Table 1 Main studies reporting liver involvement in patients without pre-existing liver disease					
Fan *et al*[33]	China	148	ALT > 40 U/L or AST > 35 U/L	55 (37.2)	Abnormal liver function is common in COVID-19 inpatients, leading to a longer hospital stay
Goyal *et al*[17]	United States	375	ALT > 40 U/L	120 (32)	Mechanically ventilated patients more likely to have liver involvement.
Lenti *et al*[29]	Italy	100	ALT or GGT > 50 U/L	58/93 (62.4)	Liver involvement correlate to higher mortality and ICU need in those who develop ARDS
Medetalibeyoglu *et al*[32]	Turkey	554	ALT or AST > 40 U/L	153 (27.6)	Higher rate of moderate-to-severe pneumonia and ICU admission need in patients with liver involvement
Phipps *et al*[31]	United States	2273	ALT > 50 U/L	537 (24)	Severe liver involvement was rare (6.4%) and led to worse outcomes (ICU admission, higher mortality)
Ponziani *et al*[30]	Italy	515	AST > 45 U/I orALT > 45 U/I orGGT > 61	161 (31.3)	No cases of severe liver injury in this cohort. Liver involvement was generally mild and, although correlated to a higher need of ICU care, not associated to higher mortality
Richardson *et al*[7]	United States	5700	ALT > 60	2176 (39)	Acute liver injury occurred in 1% of the included patients and was associated with higher mortality
Schattenberg *et al* [28]	Germany	44	ALT >50 U/L	6/38 (15.8)	Severe liver involvement was rare (9%), with AST more commonly deranged than ALT

ALT: Alanine aminotransferase; ARDS: Acute severe respiratory distress syndrome; AST: Aspartate aminotransferase; COVID-19: Coronavirus disease 2019; GGT; Gamma-glutamyl transpeptidase; ICU: Intensive care unit.

that CLD was associated with increased mortality according to the Child-Pugh class. They reported an increase in mortality, ranging from 19% in Child-Pugh-A patients to 51% in Child-Pugh-C patients. Although mortality has consistently reported to be increased in CLD patients, respiratory failure was found to be the main cause of death in these patients. Interestingly, alcohol-related liver disease was found to be independently associated to higher mortality. Liver decompensation was also reported to be common in cirrhotic patients (46%) with half of them having acute-on-chronic liver failure[44].

Among patients with CLD, liver transplant recipients were thought to represent a high risk category due to their frailty, comorbidities, and immunosuppressant therapy. Only few studies evaluated their clinical outcomes, showing conflicting results. Additionally, the majority of these studies are small case series in which patients did not always have the confirmation of SARS-CoV-2 infection[45-49]. Complying with preventive measures (*i.e.* frequent hand washing/sanitisation, use of surgical mask in public places and avoidance of public or crowded places) has been found effective to reduce the infection rate in this population[49]. A large multinational registry-based study[50], including 151 transplanted patients with laboratory-confirmed infection, showed that liver transplantation was not independently associated with higher mortality, hospitalisation rate or intensive care unit admission, whereas age and comorbidities were[47,50]. Tables 2 and 3 report the main studies focusing respectively on the outcome of COVID-19 in patients with CLD and in those with a transplanted liver.

SPLEEN IMPAIRMENT IN COVID-19

Spleen impairment in patients with COVID-19 has been poorly described. It is assumed that it may be driven by several mechanisms, including direct organ attack by the virus, cytokine-mediated immune pathogenesis, microvascular dysfunction, and lymphocyte apoptosis (Figure 2)[51].

Coronavirus detection in biopsies and autopsies has shown a tropism of this virus family for the spleen. The first available evidence is related to studies carried out on patients infected with SARS-CoV[11,52] and in experimental models of the Middle East respiratory syndrome[53]. In 2020, through immunohistochemistry techniques and the real-time reverse-transcript polymerase chain reaction assay, the SARS-CoV-2 nucleocapsid protein and the RNA were detected in the spleen tissue[54-56].

Table 2 Main studies reporting outcomes in patients with pre-existing chronic liver disease

Ref.	Country	Patients	Patients with CLD, *n* (%)	Main findings
Bajaj *et al*[40]	United States	272	37 (13.6)	Higher mortality in cirrhotic COVID-19 positive patients
Hashemi *et al* [41]	United States	363	69 (19)	CLD patients had higher ICU admission and mechanical ventilation rate. CLD was a predictor of mortality
Iavarone *et al* [42]	Italy	50	50 (100)	COVID-19 infection led to liver function deterioration. CLD patients had increased mortality
Marjot *et al*[43]	International	1365	745 (54.6)	CLD correlate to higher mortality rate according to the CPT class. ALD was an independent risk factor for mortality
Qi *et al*[39]	China	21	21 (100)	Respiratory failure was the cause of death in most patients
Singh *et al*[37]	United States	250	60 (46.1)	Pre-existing CLD patients had higher hospitalisation and mortality rates
Sarin *et al*[38]	International	228	228 (100)	Decompensation of pre-existing CLD occurred in one fifth of cirrhotic patients

CLD: Chronic liver disease; COVID-19: Coronavirus disease 2019.

Table 3 Main studies reporting outcomes in liver transplant patients

Ref.	Country	Patients	Patients with LT	Main findings
Bhoori *et al* [45]	Italy	151 (COVID status unknown)	151 (100)	3 deaths recorded in long-term LT recipient on low immunosuppressant dose
Belli *et al* [47]	International	103	103 (100)	Mortality might correlate with age and longer time since LT
Donato *et al* [49]	Italy	640 (8 COVID positive)	640 (100)	Low prevalence of infection in LT patients who adhere to preventive measures
Lee *et al*[48]	United States	38	38 (100)	High mortality in LT patients regardless of time since transplant
Pereira *et al* [46]	United States	90	14 (15)	Solid organ transplant recipient had more severe outcomes
Webb *et al* [50]	International	778	151 (19.4)	LT patients did not have a higher mortality, ICU admission or hospitalisation rate; age and comorbidities correlated with outcomes

COVID: Coronavirus disease; ICU: Intensive care unit; LT: Liver transplant.

This tropism of coronaviruses for the spleen, as for other organs, seems to be mediated by the presence of the ACE2 receptor. In fact, a study published in 2004 already described ACE2 receptors in the red pulp sinus endothelium[57]. More recent studies have confirmed the expression of ACE2 receptor in the splenic tissue, although at lower levels compared to others (*i.e.* small intestine, testis, kidneys, heart, thyroid, and adipose tissue)[58,59]. These studies also highlighted no difference, according to sex and age, in ACE2 receptor expression. Further immunohistochemical studies detected this receptor in tissue-resident CD169+ macrophages[54].

Autopsy studies have revealed interesting anatomical changes in the spleen during SARS-CoV-2 infection[60-63], including a reduction in the splenic cellular composition, with a specific depletion of T and B lymphocyte pools. Some authors assumed that this lymphocytopenia was linked to SARS-CoV-2-induced apoptosis, *via* Fas/Fas-ligand signalling, as well as increased interleukin (IL)-6 secretion by macrophages[54]. Furthermore, other frequent histopathological features were the white pulp atrophy and the reduction or absence of lymphoid follicles, with increased red pulp to white pulp proportion. In addition, spleen autopsies frequently showed a congested and haemorrhagic appearance. Microscopic studies of splenic vessels revealed, in many cases, a splenic infarction due to arterial thrombosis and proliferation of fibrous tissue in the sinuses.

Table 4 Summary of the main studies reporting coronavirus disease 2019 related spleen dysfunction

Ref.	Country	Patients	Patients with spleen involvement, *n* (%)	Main findings
Feng *et al*[54]	China	6	6 (100)	ACE2 expression on tissue-resident CD169+ macrophages in spleen; viral NP antigen found in ACE2+ cells in spleen; direct damage of spleen tissue (lymph follicle depletion, splenic nodule atrophy, lymphocyte reduction, *etc*.)
Remmelink *et al*[55]	Belgium	17	11 (65)	SARS-CoV-2 RNA detected in spleen autopsy samples by RT-PCR assay
Sekulic *et al* [56]	United States	2	2 (100)	SARS-CoV-2 RNA detected at high level in spleen FFPE samples by RT-PCR assay
Han *et al*[58]	China	7356	NA	Expression of ACE2 in spleen tissue (lower than in other tissues), without difference according to sex
Li *et al*[59]	China	31	NA	Expression of ACE2 in spleen tissue (lower than in other tissues), without difference according to sex and age
Xu *et al*[60]	China	10	10 (100)	Decrease in spleen cell composition with decrease in lymphocyte components, white pulp atrophied, lymphoid follicles decreased or absent, increase in red pulp to white pulp ratio
Menter *et al* [61]	Switzerland	21	6 (29)	Acute splenitis and/or septic neutrophilic leucocytosis of the red pulp, suggesting vascular disfunction in patients with COVID-19
Lax *et al*[62]	Austria	11	10 (90)	White pulp atrophy due to lymphocyte depletion, areas of haemorrhage with acute or chronic congestion
Duarte-Neto *et al*[63]	Brazil	5	5 (100)	Lymphoid hypoplasia in 100%, red pulp haemorrhages in 60%, splenitis in 40%, extramedullary haematopoiesis in 50%, endothelial changes in 80%, vasculitis and arterial thrombus in 20%
Lenti *et al*[29]	Italy	63	55 (87.3)	IgM memory B cell depletion that correlates with increased mortality and superimposed infections
Kaneko *et al* [65]	United States	11	11 (100)	Loss of spleen germinal centres due to depletion of Bcl-6+ germinal centre B cells and Bcl-6+ germinal centre T follicular helper cells, resulting in a dysregulated humoral immune response

COVID-19: Coronavirus disease 2019; FFPE: Formalin-fixed paraffin-embedded; NA: Not available; NP: Nucleocapsid protein; RT-PCR: Real-time reverse-transcript polymerase chain reaction; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2.

The functional impact of these anatomical damages has been poorly investigated. A recent study[12] assessed the splenic immunological function through the detection of circulating IgM+ IgD+ CD27+ B lymphocytes, also known as IgM memory B cells, a unique B cell population in the marginal zone of the spleen which plays a major role in early inflammatory responses, including those caused by viral and bacterial infections [64]. A high prevalence of persistent IgM memory B cell depletion was demonstrated in patients with COVID-19, resulting in a higher mortality rate and an increased risk of developing superimposed bacterial infections. Other molecular studies have suggested that the loss of germinal centres may be due to the depletion of Bcl-6+ germinal centre B cells and Bcl-6+ germinal centre T follicular helper cells, resulting in a dysregulated immune response during the SARS-CoV-2 infection[65]. Although further studies are needed, it can be assumed that splenic involvement could be one of the causes of immune perturbations associated with severe COVID-19[66]. It still has to be ascertained whether the spleen immunological defect is reversible or not. Conversely, the haemocateretic function, assessed by counting pitted red cells (PRCs; red cells with membrane abnormalities [pits] visible under interference phase microscopy[67]) was preserved in patients with acute COVID-19, contrary to what happens in asplenia and spleen hypofunction[12]. The long average life span of circulating erythrocytes (approximately 120 d) might explain the lack of PRC increase in the acute phase of COVID-19.

COVID-19 IN ASPLENIC OR HYPOSPLENIC PATIENTS

It is well known that asplenic or hyposplenic patients are predisposed to a greater risk of developing serious infections or overwhelming post-splenectomy infections, due to the defect in mounting the immune response against encapsulated bacteria[67,68].
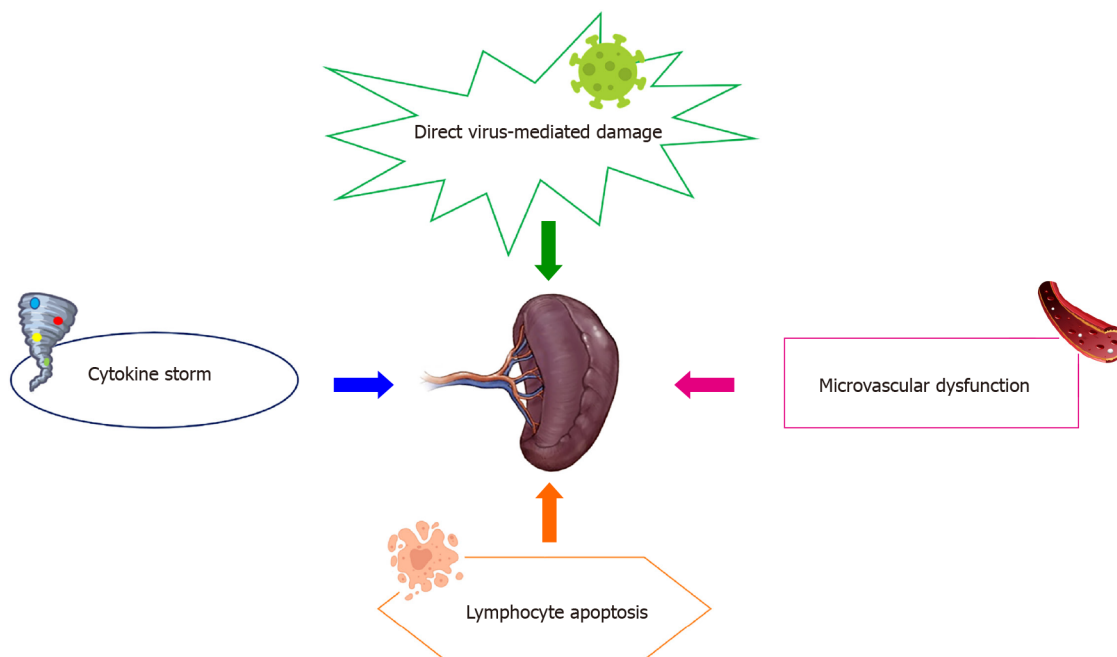
Figure 2 Putative mechanisms of spleen damage in coronavirus disease 2019.

Starting from these premises, it would be interesting to know whether patients with asplenia or spleen dysfunction could be more susceptible to Sars-CoV-2, both in terms of severity and incidence of the disease. Indeed, apart from a document drafted by the British Society of Haematology, stating that asplenic and hyposplenic patients are not exposed to a major risk of COVID-19, data regarding this population are completely missing[69]. Moreover, it is unknown whether patients who might develop spleen hypofunction as a consequence of COVID-19 could be more exposed to infections sustained by encapsulated bacteria and less responsive to vaccine immune-prophylaxis.

According to a single-centre, longitudinal, prospective, study conducted in an academic, tertiary referral hospital from Northern Italy, asplenic/hyposplenic patients did not seem to have an increased risk of developing COVID-19. The study had the purpose of characterising the spleen function, through circulating IgM memory B cell and PRC detection, in patients with COVID-19, in relation to their clinical outcome. Overall, 66 COVID-19 patients (mean age: 74 ± 16.6 years; 29 females) were enrolled; three patients had been splenectomised for trauma, all of them having IgM memory B cell depletion, and one of them died. Most COVID-19 patients had marked IgM memory B cell depletion, and this was associated to a higher mortality rate and a higher risk of developing superimposed infections[12]. Another important study conducted to identify, quantify, and analyse factors associated with COVID-19-related death in one of the largest cohort studies on this topic conducted so far (primary care records of 17278392 adults were linked to 10926 COVID-19-related deaths), considered asplenia as a comorbidity of interest. The results showed that 0.2% of the study population were affected by asplenia, and that the proportion of COVID-19-related death was 0.14%. In addition, asplenic COVID-19 patients had a 1.62 higher risk of death than individuals with a normal spleen function[70].

Indeed, further studies are needed to clarify the impact of SARS-CoV-2 in patients without a spleen or with spleen dysfunction. Table 4 reports the main studies reporting COVID-19 related spleen dysfunction.

CONCLUSION

While the involvement of the respiratory system in SARS-CoV-2 infection is well established, the impact on the liver and spleen has not been explored much. Some studies have shown a direct tropism of the virus for these organs, and this may be one of the mechanisms underlying their damage, in association with the systemic inflammatory response. Regarding the liver, its involvement seems to be quite common, especially in more severe cases of infection, resulting in a worse prognosis for these

patients. The spleen involvement, on the other hand, has been poorly investigated. The splenic immune function appears to be defective in COVID-19 patients, resulting in a higher mortality rate and superimposed infections. Further studies may lead to a better diagnostic and therapeutic approach in SARS-CoV-2-infected patients, especially those with pre-existing liver and spleen diseases, who seem to be at higher risk of a worse outcome.

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Primary gastric non-Hodgkin lymphomas: Recent advances regarding disease pathogenesis and treatment

Michael D Diamantidis, Maria Papaioannou, Evdoxia Hatjiharissi

ORCID number: Michael D Diamantidis 0000-0002-0041-5930; Maria Papaioannou 0000-0002-1999-9622; Evdoxia Hatjiharissi 0000-0002-8233-0542.

Author contributions: Diamantidis MD performed the literature review and wrote the manuscript; Hatjiharissi E wrote the manuscript, performed the literature review and corrected the manuscript; Papaioannou M wrote and corrected the manuscript.

Michael D Diamantidis, Department of Hematology, Thalassemia and Sickle Cell Disease Unit, General Hospital of Larissa, Larissa 41221, Thessaly, Greece

Maria Papaioannou, Evdoxia Hatjiharissi, Division of Hematology, First Department of Internal Medicine, AHEPA General Hospital, Aristotle University of Thessaloniki, Thessaloniki 54636, Greece

Corresponding author: Evdoxia Hatjiharissi, MD, PhD, Consultant Hematologist, Division of Hematology, First Department of Internal Medicine, AHEPA General Hospital, Aristotle University of Thessaloniki, St. Kiriakidis 1, Thessaloniki 54636, Greece. ehatjiharissi@gmail.com

Abstract

Primary gastric lymphomas (PGLs) are distinct lymphoproliferative neoplasms described as heterogeneous entities clinically and molecularly. Their main histological types are diffuse large B-cell lymphoma (DLBCL) or mucosa-associated lymphoma tissue. PGL has been one of the main fields of clinical research of our group in recent years. Although gastric DLBCLs are frequent, sufficient data to guide optimal care are scarce. Until today, a multidisciplinary approach has been applied, including chemotherapy, surgery, radiotherapy or a combination of these treatments. In this minireview article, we provide an overview of the clinical manifestations, diagnosis and staging of these diseases, along with their molecular pathogenesis and the most important related clinical published series. We then discuss the scientific gaps, perils and pitfalls that exist regarding the aforementioned studies, in parallel with the unmet need for future research and comment on the proper methodology for such retrospective studies. Aiming to fill this gap, we retrospectively evaluated the trends in clinical presentation, management and outcome among 165 patients with DLBCL PGL who were seen in our institutions in 1980-2014. The study cohort was divided into two subgroups, comparing the main 2 therapeutic options [cyclophosphamide doxorubicin vincristine prednisone (CHOP) *vs* rituximab-CHOP (R-CHOP)]. A better outcome with immunochemotherapy (R-CHOP) was observed. In the next 2 mo, we will present the update of our study with the same basic conclusion.

Key Words: Primary gastric lymphoma; Extranodal non-Hodgkin's lymphoma; Diffuse large B-cell lymphoma; Mucosa-associated lymphoid tissue; Immunochemotherapy; Rituximab-cyclophosphamide doxorubicin vincristine prednisone

Core Tip: A few small, heterogeneous, retrospective studies have attempted to determine the optimal treatment for gastric diffuse large B-cell lymphoma, investigating the role of chemotherapy +/- rituximab, surgery and radiation in patient outcomes. Our retrospective research suggests that a better outcome is observed for these patients after the introduction of immunochemotherapy (rituximab-cyclophosphamide doxorubicin vincristine prednisone). Because statistical analysis might differ among various studies, it is crucial to correctly define the terms freedom from progression and lymphoma-specific survival. The latter provides information on whether the patients died from lymphoma or from other causes.

INTRODUCTION

Primary gastric lymphomas (PGLs) are a diverse group of lymphoproliferative disorders that originate from the stomach and comprise many different histologic types. Either of diffuse large B-cell lymphoma (DLBCL) subtype or mucosa-associated lymphoma tissue (MALT) histology, PGL is the second most common gastric malignancy globally, following the adenocarcinoma of the stomach[1,2]. The latter is the most common form of gastric cancer and the fifth most common malignancy in the world[3]. Despite the fact that prevention and treatment of *Helicobacter pylori* infection (*H. pylori* I) has led to a decrease in its overall incidence, gastric cancer remains the 3rd most deadly cancer, with an estimated 783000 deaths in 2018 worldwide[4,5]. Therefore, accurately recognizing and diagnosing gastric cancer from gastric lymphomas is important, as these diseases are treated differently, and any confusion may result in inappropriate treatment management.

The gastrointestinal tract (GIT) is the most common site for the development of extranodal lymphomas. The incidence of these neoplasms has been increasing in recent years[2,6]. The stomach represents 30%-40% of all extranodal lymphomas and 55%-65% of all GI lymphomas. The incidence of PGL varies from 4% to 20% of extranodal non-Hodgkin lymphomas (NHLs) and reaches up to 5% of primary gastric neoplasms[2]. The incidence of PGL is estimated to be 1 per 100000 in Western countries[7]. B-cell lymphomas are more frequent in these countries than in Eastern countries[1].

To date, the term PGL was originally used to describe lymphomas that arise from the stomach. However, within the medical literature, controversy exists regarding the definition, staging and treatment of this entity. Most cases of PGLs are B-cell subtypes of NHLs. The majority of these subtypes have DLBCL histology and are classified as DLBCL of the stomach, not otherwise specified (NOS).

PGLs are histologically heterogeneous neoplasms. This contributes to a different biology, clinical presentation and prognosis and subsequently determines special therapeutic needs for each subtype[8,9]. For example, certain subtypes of PGLs, such as DLBCL, are more aggressive than others and require immediate therapy[8], whereas for patients with MALT histology, unique management is usually applied ranging from watch and wait to antibiotic-based treatment[9].

As stated above, PGLs are histologically, biologically and clinically heterogeneous neoplasms. Although gastric DLBCL is an extranodal high-grade lymphoma, it is considered less aggressive than its nodal counterpart and other extranodal DLBCL locations. Its appropriate treatment has not been satisfactorily determined, and treatment choices vary considerably. Human immunodeficiency virus, Epstein-Barr infection, hepatitis B virus, human T-cell lymphotropic virus 1, immunosuppression, celiac disease, inflammatory bowel disease, and *H. pylori* I have all been implicated in the factors predisposing patients to PGLs, increasing the risk of developing the disease [1,2,10]. PGL usually occurs in patients older than 50 years. There are many older

patients over 80 years of age. Males are more prone to be diagnosed with PGL with a 2-3-fold higher risk than females[2].

This review mainly focuses on DLBCL gastric lymphoma, which is one of the main fields of our clinical research and comments briefly on MALT lymphoma. The molecular etiology and pathophysiology of DLBCL gastric lymphomas and the available clinical data for their optimal management will be discussed. In parallel, a brief review of the MALT subtype that represents almost 50% of PGLs will also be provided. This review aims to meet the therapeutic needs of those who are involved and/or interested in the treatment of GI-DLBCL lymphomas and extensively focuses on the role of rituximab, the first in class anti-CD20 monoclonal antibody (mAb), in the outcome of patients with PGL of the DLBCL subtype.

CLINICAL MANIFESTATIONS AND DIAGNOSIS

The stomach is the most common site for the development of extranodal lymphomas in the GI tract, accounting for 60% of cases, followed by the small bowel, ileum, cecum, colon and rectum[7]. Distinguishing PGL from secondary dissemination of the stomach due to primary nodal lymphoma can be difficult. No peripheral and mediastinal lymphadenopathy at the time of diagnosis, no spleen or liver infiltration and normal blood counts are in contrast to the presence of a secondary gastric lymphoma[11].

The diagnosis of PGL can be delayed for many years due to the presence of nonspecific symptoms, mimicking peptic ulcer disease, gastritis, functional gastric or even pancreatic disorder. The main symptoms include nausea, vomiting, anorexia, abdominal distention, fullness or pain, indigestion, dyspepsia and weight loss, whereas weakness, night sweats, fever, jaundice, hematemesis or melena are less common[2,7]. An obvious epigastric mass or perforation is rare as an initial presentation[7,10,12].

An appropriate endoscopic evaluation with generously sized tissue samples is the hallmark of diagnosis. The diagnostic accuracy of endoscopic biopsy is very high, reaching 90%. Endoscopic ultrasonography can improve this diagnostic accuracy. The diagnosis becomes difficult when there is deep infiltration and preservation of the mucosa. Computed tomography (CT), magnetic resonance imaging (MRI) and 18F-fluorodeoxyglucose positron emission tomography (FDG-PET) assist in the diagnosis and staging of PGL[1,7]. Sporadically, PGL might present as multifocal, clonally identical foci surrounded by macroscopically unaffected tissue. Thus, gastric mapping of unaffected mucosa is strongly recommended[13]. Bone marrow infiltration, B symptoms and elevated lactate dehydrogenase (LDH) are more frequently encountered in nodal lymphomas than in gastric lymphomas[13].

Different staging systems have been proposed for PGLs. The Ann Arbor staging system, which is widely used for primary nodal lymphomas, is considered unsatisfactory as PGLs originate from the lining of the stomach instead of the lymph nodes[7,13]. In recent years, a more specific Lugano staging system for PGLs was proposed and applied based on the Lugano score[14,15], which includes the following stages: Stage IE — Lymphoma is confined to the GIT (single lesion or multiple noncontiguous lesions): IE1 = mucosa, submucosa; IE2 = muscularis propria, serosa; Stage II — Lymphoma extends into the abdomen from the primary site within the GI tract: II1 = local nodal involvement; II2 = distant nodal involvement; Stage IIE — Penetration of serosa to involve adjacent organs or tissues; Stage IV — Disseminated extranodal involvement or concomitant supra diaphragmatic nodal involvement. Note: Stage III does not exist because gastric lymphoma is always below the diaphragm.

A complete staging work-up includes the following: Biochemical examinations, chest, abdomen and pelvis CT scan, bone marrow biopsy, thorough endoscopy including biopsies from the stomach, duodenum and gastroesophageal junction, endoscopic ultrasound, evaluation of the Waldeyer ring, investigation for *H. pylori* I, routine histology and immunohistochemistry. Cytogenetic studies and even fluorescence in situ hybridization (FISH) can all be used in biopsies to provide the appropriate information needed for optimal treatment. PET/CT scans have documented diagnostic and prognostic value only for DLBCL lymphomas, in contrast to MALT gastric lymphomas, which can be reported as false-negative because of the small tumor burden of the disease and their indolent behavior[13]. However, there is an unmet need regarding the use of PET scans in the clinical setting to guide treatment. Usually, this examination is performed before and after the end of treatment to guide therapeutic decisions as a standard of care. Because the stomach is

an abdominal organ, it is unclear how PET scans can assist in the aforementioned necessary clinical decisions.

In general, the prognosis of extranodal lymphomas varies according to the affected organ; it is poor in the testis, central nervous system (CNS) and intestine, whereas it is quite good in the stomach, mediastinum and bone. Nevertheless, PGL is an aggressive malignancy characterized by rapid growth. However, the prognosis of DLBCL PGL is relatively good, with a 5-year overall survival (OS) higher than 80%[7].

COMPARISON AMONG CLINICAL STUDIES/TREATMENT

The optimal treatment for DLBCL PGLs is not clear, because prospective clinical studies are missing. In the past, a spectrum of treatment approaches was applied, ranging from gastrectomy or radiotherapy alone to chemotherapy (cyclophosphamide doxorubicin vincristine prednisone, CHOP) or the combination of chemotherapy plus radiotherapy and surgery. Wang *et al*[16] compared surgery over conservative treatment in a retrospective study. Conservative treatment in this study included chemotherapy (CHOP) or radiotherapy alone, chemotherapy plus radiotherapy or *H. pylori* I eradication (HPE). The authors found superiority of surgery alone compared with conservative treatment in the DLBCL type regarding prognosis, but not in the MALT type[16]. Currently, the role of surgical resection has been minimized, even in cases of extreme intestinal obstruction, as immunochemotherapy can induce rapid and complete resolution of large obstructing tumor masses. Gastrectomy is restricted to the management of major complications, including perforation or hemorrhage of DLBCL PGLs.

In contrast, other studies demonstrated that DLBCL PGL is a potentially curable disease with rituximab-CHOP (R-CHOP)-like treatment, leading to long-term survival [17]. Investigators found that surgical treatment did not offer survival benefits when compared with chemotherapy for 5-year progression-free survival (PFS) and OS estimates and that no significant differences were noted in these endpoints for patients treated with R-CHOP or conventional chemotherapy[18].

Sohn *et al*[19] directly compared CHOP *vs* R-CHOP as a front-line approach in 93 patients with DLBCL PGL. With a median follow-up of 48 mo, no differences were noted among the 2 groups regarding OS, EFS and CR. High serum levels of β2-microglobulin were associated with worse OS and EFS in patients who received R-CHOP[19]. In a retrospective analysis of 95 Japanese patients, the clinical outcomes of gastric DLBCL were extremely favorable for localized-stage patients in the rituximab era. Conversely, these treatments were poor for advanced-stage patients[20]. Interestingly, an effective approach in treating deeply infiltrated DLBCL PGL patients by switching fractioned R-CHOP (rituximab d0, 50% dose of CHOP d1 and d5) to standard R-CHOP cycles guided by endoscopic ultrasonography has been proposed [21].

The following factors were identified as having a negative impact on survival: age above 65, Eastern Cooperative Oncology Group 2-3, B symptoms, bulky disease, IPI 3-4, more than 3 treatment lines, and absence of response to first-line treatment[17].

Conversely, other factors were considered negative for prognosis in the subsequent study: elevated LDH levels, chemotherapy or radiotherapy alone or the combination of chemotherapy plus radiotherapy[16]. The non-germinal center B-cell-like lymphoma (GCB) subtype has also been associated with shorter OS[18]. *H. pylori* I negativity, advanced Lugano stage and elevated LDH levels have been reported as adverse prognostic factors in gastric DLBCL[22].

Low serum albumin at diagnosis was the only risk factor for developing gastric complications, such as bleeding and stenosis, in patients with gastric DLBCL who received R-CHOP[23]. Furthermore, a low CD4:CD8 ratio at diagnosis is an independent poor prognostic factor for subsequent OS and EFS24 (24 mo after diagnosis) in patients with gastric DLBCL[24]. Finally, the microRNA miR-150 is reportedly a negative independent prognostic biomarker for primary GI DLBCL[25].

Some patients with DLBCL PGL also have a MALT component. The 5-year PFS and OS estimates were similar when de novo DLBCL patients were compared with DLBCL/MALT patients, suggesting that patients with a MALT component, along with DLBCL, might have the same biological type of lymphoma as de novo DLBCL patients [18]. In such DLBCL/MALT cases, an important deregulation of Bcl-2 and an upregulation of p53 protein of uncertain clinical significance have been observed[26]. A synopsis of the studies comparing R-CHOP *vs* CHOP for DLBCL PGLs is shown in Table 1. Indeed, there is a lack of a head-to-head comparison between CHOP and R-

Table 1 Studies comparing rituximab-cyclophosphamide doxorubicin vincristine prednisone *vs* cyclophosphamide doxorubicin vincristine prednisone for diffuse large B-cell lymphoma primary gastric lymphomas

Ref.		Number of Pts	R-CHOP OS	CHOP OS	R-CHOP PFS	CHOP PFS	Comments
Sohn *et al* [19], 2012	Double-arm Retrospective Study (R-CHOP *vs* CHOP as 1st line treatment)	93 (55 R-CHOP, 38 CHOP)	3-yr 84.7% (*P* > 0.05)	3-yr 94.7% (*P* > 0.05)	3-yr 81.7% (EFS) (*P* > 0.05)	3-yr 86% (EFS) (*P* > 0.05)	CR: (CHOP: 93.9%), (R-CHOP: 92.5%)
Liu *et al* [62], 2018	Double-arm Retrospective Study (diagnosis: 1973-2000 era *vs* 2001-2014 era of immuno-CT)	SEER Database 7051 [(4186, 1973-2000), (2865, 2001-2014)]	5-yr 53% (*P* = 0.001)	5-yr 47% (*P* = 0.001)			
Tanaka *et al*[20], 2012	Single-arm Retrospective Study (R-CHOP)	95	3-yr 91% (localized disease); 3-yr 95% (localized disease); 3-yr 64% (localized disease)		3-yr 91% (localized disease); 3-yr 92% (localized disease); 3-yr 43% (localized disease)		6c. R-CHOP; 3-4 c. R-CHOP plus radiotherapy; R-CHOP ± radiotherapy
Couto *et al*[17], 2021	Single-arm Retrospective Study (R-CHOP)	101	Not reached		Not reached		80% CR (after 1st line); 54% CR (3 yrs FU)

R-CHOP: Rituximab-cyclophosphamide doxorubicin vincristine prednisone; CHOP: Cyclophosphamide doxorubicin vincristine prednisone; OS: Overall survival; PFS: Progression-free survival; EFS: Event free survival; CR: Complete remission; CT: Computed tomography; FU: Follow-up.

CHOP in PGLs.

Regarding the role of radiotherapy, more data are available for patients with gastric MALT lymphoma or early-stage gastric lymphoma. When there is an unsatisfactory response to HPE, recurrence after HPE or in MALT cases negative for *H. pylori* I, gastric radiotherapy of the entire stomach plus irradiation of the pathological and perigastric lymph nodes (30-440 Gy, 15-20 fractions) has been proposed. However, it is less clear whether radiotherapy should be applied in cases of DLBCL PGLs. However, involved-field radiotherapy has a role, especially for patients with DLBCL PGL of advanced stage who achieve partial remission (PR) after immunochemotherapy (R-CHOP)[27]. R-CHOP plus additional local treatment for gastric lesions (*e.g.*, consolidative radiotherapy or surgical resection) has also been recommended[28]. Alternatively, several studies have found that in the era of immunochemotherapy (R-CHOP), radiotherapy does not improve OS[29-31]. The side effects of radiotherapy should always be taken into account in clinical decision making[27].

Despite the presence of several clinical series involving primary gastric DLBCL lymphomas mainly addressing the issue of selecting the optimal treatment, there are sporadic single cases in the literature[22,32-34]. Some very rare, more aggressive cases of DLBCL lymphoma originating from the stomach and infiltrating the adrenals bilaterally have been reported[32,34]. The first patient presented with nausea, vomiting, abdominal pain and hypotension, was treated with glucocorticoids and died after developing respiratory failure, severe hypotension refractory to vasopressors and severe metabolic acidosis[34]. The second case was a DLBCL, PGL of the non-germinal center (non-GC) type. This patient received 8 cycles of rituximab therapy, 6 cycles of CHOP and 3 cycles of prophylactic intrathecal chemotherapy. The patient maintained a CR for approximately 14 mo after the completion of the aforementioned treatment. The latter is in favor of the hypothesis that DLBCL lymphomas of the stomach have a better prognosis than other DLBCL nodal and extranodal lymphomas. In contrast to the very dismal prognosis of primary adrenal lymphomas (PALs)[35], this patient survived, likely because the primary neoplasm was gastric DLBCL, which has better biological and clinical behavior for unknown molecular reasons (even though it is considered an aggressive neoplasm, being DLBCL).

Regarding the role of HPE, Nakamura *et al*[36] studied 420 patients with gastric MALT lymphoma and found a significant responsiveness to HPE therapy (77%), with treatment failure (relapse or progressive disease) occurring in only 9% of the patients. However, this primary refractory disease was not associated with a dismal outcome, as the subsequent therapy still yielded a 90% OS rate after 10 years[36].

Nevertheless, even though HPE has already been established as an optimal strategy for the management of gastric MALT lymphoma, there are conflicting results, either in favor of or against HPE for patients with DLBCL PGLs. Thus, HPE has been reported to be a suitable strategy for patients with DLBCL PGLs[37,38]. The concept of a less

aggressive biological behavior for *H. pylori* I-dependent gastric DLBCL has been proposed with the suggestion to apply HPE in such cases[39]. However, it is not clear how accurately these lymphomas can be distinguished. Alternatively, high-grade gastric lymphomas can rapidly progress if they do not respond to HPE. The loss of *H. pylori* I dependency and the possible high-grade lymphomatic evolution/transformation are separate and distinct events in the natural history of PGL[38,40]. The description of defined molecular markers linked to *H. pylori* I dependency of PGLs is beyond the scope of this article.

Moreover, a substantial portion of early-stage *H. pylori* I-positive gastric de novo DLBCLs remain *H. pylori* I-dependent and respond to antibiotic treatment (HPE). Prospective studies to validate these findings are needed[41]. Our personal opinion is that HPE should not be applied as monotherapy, even in the early stage of *H. pylori* I-positive DLBCL PGLs.

MOLECULAR PATHOGENESIS

Extranodal lymphomas are distinct types of lymphomas that show a predilection for anatomical sites harboring extranodal lymphoid tissue, such as the CNS, testis, mediastinum, bone and GIT, in contrast to the typical pattern of the nodal counterpart in the lymph nodes for nodal lymphomas[42]. Extranodal lymphomas can even appear in immune-privileged (sanctuary) sites (CNS, testis) or arise in sites of chronic inflammation, effusions or other closed spaces within the body. The complex mechanisms of local immune evasion leading to extranodal lymphoproliferations have not been fully elucidated[43]. The capacity of mature lymphocytes to recirculate between blood and lymphoid tissue and to migrate to extranodal anatomical sites is crucial for the pathogenesis of the disease. During this process, lymphocytes interact with endothelial venules, mediated by receptor molecules (integrins and lymphocytes)[44].

The role of specific B-cell receptor (BCR) antigens has been proposed in the process of lymphomagenesis. Oncogenic translocations during BCR development and generation (VDJ rearrangement), the activation of mature B-cells and the germinal center reaction, the mechanisms of loss of immunological self-tolerance, and the role of infectious agents and autoantigens are all hallmarks and basic elements of lymphomagenesis, a complex multifactorial process, in both aggressive and indolent lymphomas[45]. Gastric DLBCL is a high-grade lymphoma compared to low-grade MALT lymphomas. Whether DLBCL transforms from low-grade MALT lymphoma or whether it arises de novo in the stomach is unknown. DLBCL gastric lymphoma has been associated with a lower CR and shorter survival than MALT lymphoma[2,46]. Nevertheless, transformed DLBCLs from MALTs are CD10- and Bcl-2-negative, while de novo DLBCLs are CD10- and Bcl-2-positive[31,46].

The oncogene Bcl-6 is located on chromosome 3q27 and is frequently present in the majority of extranodal high-grade lymphomas. Conversely, Bcl-2 oncogene expression was significantly lower in gastric lymphomas than in other primary extranodal high-grade B-cell lymphomas (HGBCLs). p53 protein expression did not differ significantly between these 2 groups[2].

Primary gastric DLBCL
DLBCL is described by diffuse proliferation of large, atypical cells, with vesicular nuclei, prominent nucleoli, and basophilic cytoplasm. These cells typically express CD19, CD20, CD22 and CD79a (pan-B-cell markers). Bcl-6 is expressed in 60% of cases. FISH can identify poor prognostic subtypes of DLBCL, such as double-hit (DH) or triple-hit (TH) lymphomas (high-grade, B-cells), characterized by translocations of MYC and Bcl-2 and/or Bcl-6[47,48]. DH or TH lymphomas are defined by their genetic aberrations, irrespective of their morphology. Genetic variability has been documented for DLBCL PGL[47]. Gene expression profiling distinguishes DLBCL into GCB and non-GCB or activated B-cell-like (ABC) subtypes based on the cell of origin profile. ABC lymphomas show a worse prognosis than GCB lymphomas[49]. In routine diagnostic practice, this screening is conducted by immunohistochemistry based on the assessment of three markers (CD10, bcl-6 and MUM1)[50] (Figure 1).

More analytically, ABC DLBCLs are characterized by nuclear factor kappa beta (NF-κB) activation, showing a higher frequency of Bcl-2 amplifications, Bcl-6 rearrangements and recurrent mutations of MYD88, PRDM1 and CD79B, whereas GCB-like DLBCLs are enriched for activating EZH2 and Bcl-2 mutations, defined by perturbations/molecular defects in the JAK/STAT and PI3K/AKT signaling pathways[48]. EZH2 overexpression has been associated with inferior outcomes in patients with
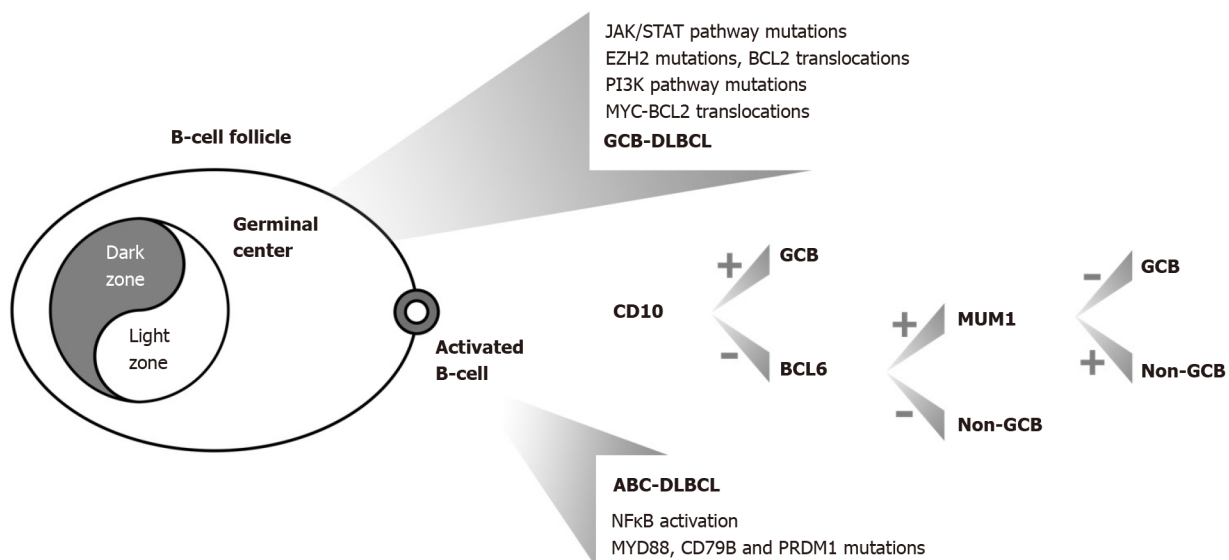
Figure 1 Primary gastric diffuse large B-cell lymphoma lymphomas and related molecular lesions. GCB: Germinal center B-cell lymphoma; ABC: Activated B-cell-like lymphoma; the combination of MYC plus BCL2 translocations corresponds to 'double hit lymphomas'; DLBCL: Diffuse large B-cell lymphoma; NF-κβ: Nuclear factor κappa beta.

DLBCL PGL[51] (Figure 1).

Interestingly, 2 HGBCLs were included in the recent revised WHO classification of lymphoid neoplasms. These entities are clinically and biologically distinct from DLBCL NOS and Burkitt lymphoma (BL). The HGBCL, NOS entity includes cases previously termed 'unclassifiable, with features intermediate between DLBCL and BL', or showing blastoid morphology but lacking DH/TH translocations[49].

High levels of Bcl-6 expression were found in GCB gastric lymphomas, whereas in the non-GCB cases, a high Bcl-6 expression level correlated importantly with mutations producing Bcl-6 deregulation, even if in the latter cases no correlation was found between survival rates[2].

Clinical studies addressing the role of programmed cell death 1 (PD-1) and its ligand (PD-L1) have shown promising results. PD-1 blockade in patients with PD-L1 expression on tumor cells has been linked with clinical responses. Investigators from Japan evaluated the role of PD-L1 expression on neoplastic and non neoplastic immune cells in the microenvironment (miPD-L1) in a retrospective study of patients with GI DLBCL lymphoma. They found that elevated miPD-L1 expression had a favorable impact on the outcome of these DLBCL patients, regardless of the anatomical site of the disease[52].

Gastric MALT lymphoma

MALT lymphoma is a low-grade B-cell NHL, and the majority of cases (approximately 90%) are directly related to *H. pylori* I. However, 10% of gastric MALT lymphomas are *H. pylori* I negative[53]. Chronic *H. pylori* I of the gastric mucosa and the accompanying inflammation have been strongly linked to MALT lymphomagenesis. Moreover, abnormalities in the expression of various miRNAs contribute to the neoplastic gastric phenotype[54,55].

H. pylori I expresses proteins related to the corresponding genes, contributing to the related lymphomagenesis from the bacterium. These are cytotoxin-associated gene A (CagA), vacuolization cytotoxin A (VacA) and heat shock proteins (Hsps). The Cag pathogenicity island (a common gene sequence considered responsible for the pathophysiology of the infection) contains over 40 genes, which mainly code for a complex type IV secretion system. This pathogenicity island is usually absent from *H. pylori* I strains isolated from asymptomatic human carriers. The CagA protein is frequently co-expressed with the vacuolating cytotoxin VacA[56].

Hamoudi *et al*[57] established the connection between abnormal NF-κB signaling due to the chromosomal translocations, t(11;18)(q21;q21)/*API2-MALT1*, t(1;14) (p22;q32)/BCL10-IGH, t(14;18) (q32;q21)/IGH-MALT1 and t(3;14) (p13;q32)/FOXP1-IGH,in gastric MALT lymphomas[57,58] (Figure 2).

MALT1 and BCL10 proteins are involved in surface immune receptor-mediated activation of the NF-κB transcription factor; chromosomal translocations involving
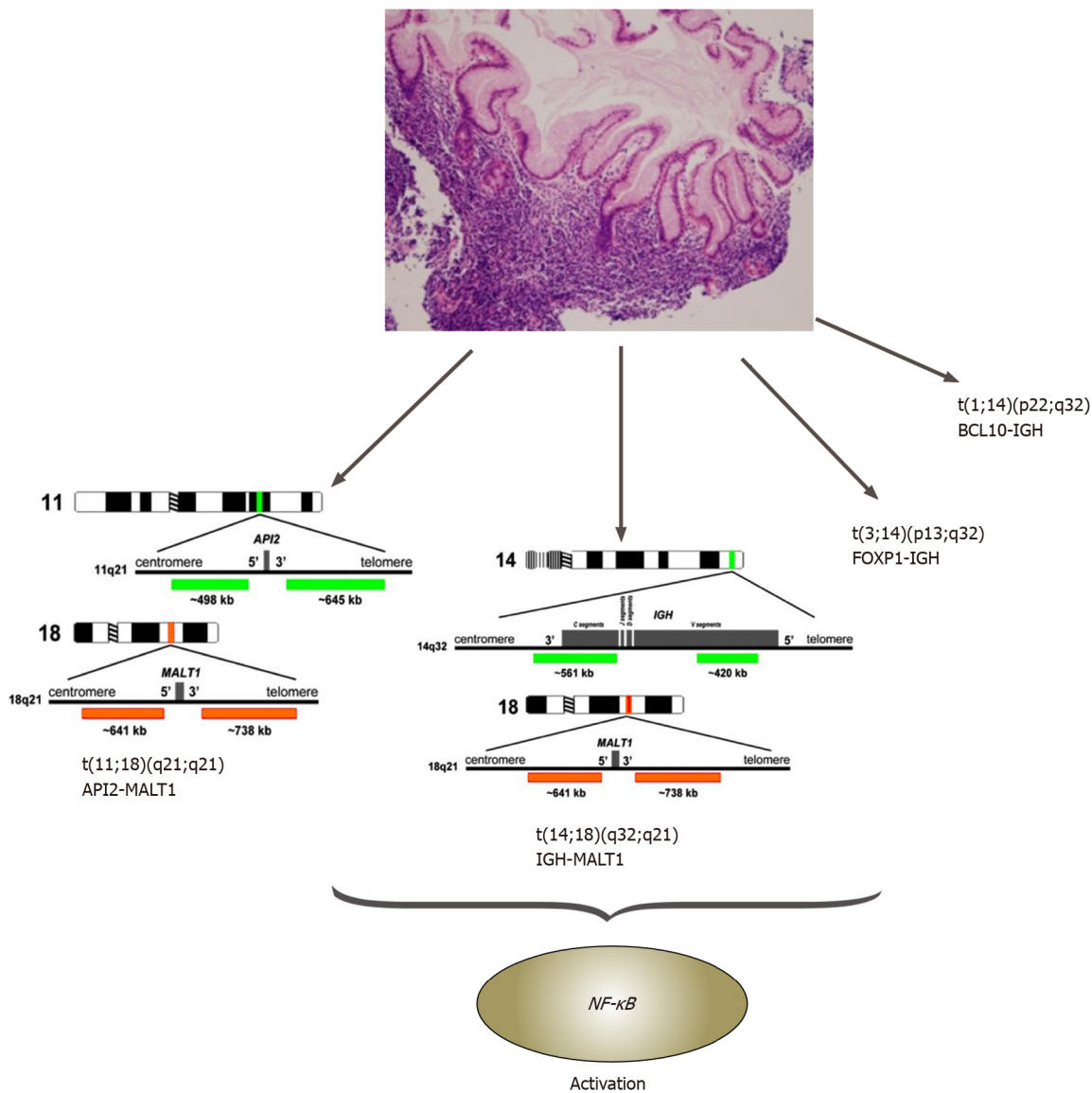
Figure 2 Gastric mucosa-associated lymphoid tissue lymphomas and related chromosomal translocations. BCL: B-cell lymphoma; FOXP: Forkhead box protein; IGH: Immunoglobulin heavy (chain); MALT: Mucosa-associated lymphoid tissue; NF-κB: Nuclear factor kappa beta.

these genes are believed to exert their oncogenic activities through constitutive activation of the NF-κB pathway, leading to the expression of numerous genes important for cell survival and proliferation[40,55,57,58] (Figure 2).

In gastric MALT lymphoma, t(11;18)/*API2-MALT1* is the most frequent translocation, detected in 20% of cases. This translocation fuses the N-terminal region of *API2* to the C-terminal region of *MALT1* and generates a functional chimeric fusion, which can activate the NF-κB pathway. Clinically, t(11;18) is more frequently associated with the absence of *H. pylori* I, and the majority of translocation-positive cases do not respond to HPE therapy. Interestingly, t(11;18)-positive cases rarely transform to DLBCL[55,58].

Gastric MALT lymphoma is indirectly influenced by *H. pylori* I through T-cell stimulation, and recent studies have shown that *H. pylori*-triggering chemokines and their receptors, *H. pylori*-associated epigenetic changes, *H. pylori*-regulated miRNA expression and tumor infiltration by CD4+ CD25+ regulatory T cells contribute to lymphomagenesis of gastric MALT lymphoma (Figure 3). Recent studies have also demonstrated that the translocation of CagA into B lymphocytes inhibits apoptosis through p53 accumulation, BAD phosphorylation and the upregulation of Bcl-2 and Bcl-XL expression (Figure 3). In gastric MALT lymphoma, CagA may stimulate lymphomagenesis directly through the regulation of signal transduction, and intracellular CagA is associated with *H. pylori* I dependence. These findings represent a substantial paradigm shift compared with the classical theory of *H. pylori*-reactive T cells contributing indirectly to the development of MALT lymphoma[40].
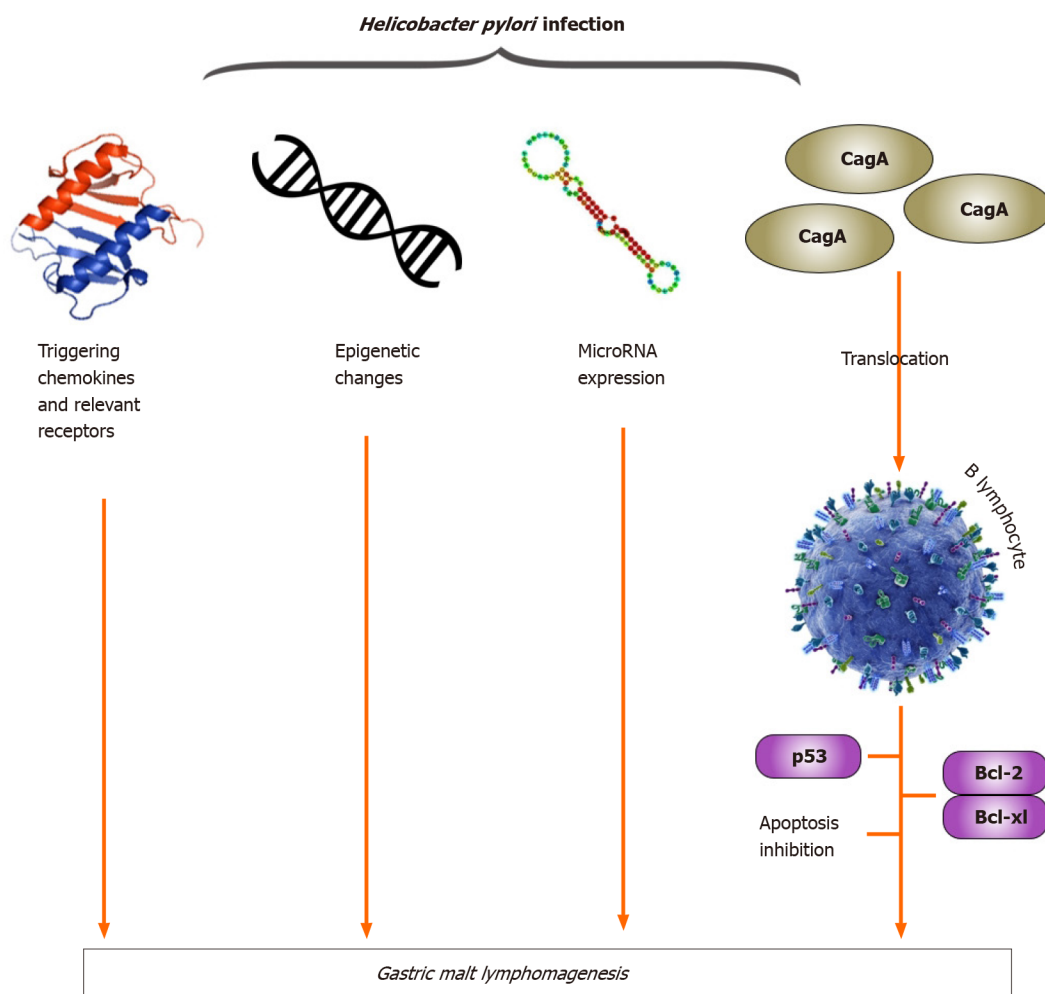
Figure 3 *Helicobacter pylori* infection, molecular mechanisms and gastric mucosa-associated lymphoid tissue lymphomagenesis. BCL: B-cell lymphoma; Bcl-XL: B-cell leukemia XL; CagA: Cytotoxin-associated gene A.

Other cytogenetic aberrations, often associated with one of the four main chromosomal translocations described above, include trisomies 3, 12 and/or 18, which can also present as a sole abnormality in one-fifth of the total cases. Somatic missense mutations in PIM1 and cMYC have been reported in 46% of MALT gastric lymphomas and in 30% of transformed MALT lymphomas. The majority of these genetic lesions are not MALT lymphoma specific. Aberrant somatic hypermutation can still be encountered in indolent lymphomas, such as MALT, but not at the extent noted in DLBCL lymphomas[40,55,58]. Interestingly, the loss of the chemokine receptor CXCR4 and the upregulation of CXCR7 have been associated with the progression of gastric MALT lymphoma to DLBCL lymphoma[59]. Furthermore, lower expression of the microRNA miR-34a has also been linked to the transition from MALT to DLBCL lymphoma[54]. Finally, among the proposed pathogenetic etiologies for *H. pylori*-negative MALT lymphoma cases, genetic alterations in NF-κB signaling are the main hypothesis[53].

SCIENTIFIC GAPS

While gastric DLBCLs are frequent, sufficient data to guide optimal care are still limited. In the past, gastrectomy was the treatment of choice for these patients. Nevertheless, due to the observed high morbidity rates linked with this procedure, novel therapeutic approaches have emerged, such as radiation and combination chemotherapy. Hence, until today, a multidisciplinary approach has been applied, including chemotherapy, surgery, radiotherapy or a combination of these modalities.

Today, immunochemotherapy with R-CHOP is the most acceptable option for treating gastric DLBCL, as for nodal DLBCL. R-CHOP was established as a standard approach for DLBCL patients; in the study of patients aged 60-80 years, the rate of

complete response (CR) was significantly higher in the group that received R-CHOP *vs* CHOP[60]. Since then, a few small, heterogeneous, retrospective studies have attempted to determine the optimal management of gastric DLBCL, investigating the role of immunochemotherapy, surgery and radiation in patient outcomes[16-21,23,61-63].

Significant advances in diagnosis, treatment and response assessment options over the last years have been made in the field of high-grade lymphomas. Molecular characterization of DLBCL has also described 3 major lymphoma subgroups that correlate with distinct biological and clinical behavior (ABCs, GCBs, double hit lymphomas), supporting the rationale for distinct therapeutic options[48]. However, these advances were extracted from nodal DLBCL, while the intrinsic pathogenesis of primary gastric DLBCL is unclear, and similar studies on this particular type of lymphoma are lacking.

The heterogeneity of the various clinical retrospective studies investigating the outcomes of patients with DLBCL PGLs is impressive. For example, these studies differ in the number of patients, in the time intervals when each therapeutic approach was applied, or in the type of therapeutic approaches compared. Other studies calculate surgery alone and other surgeries with chemotherapy and/or radiotherapy, without separating treatment subgroups of patients. Some researchers place all DLBCL patients together into the statistical analysis, regardless of the anatomical site (stomach, intestine). Hence, comparisons are difficult and not head-to-head. Thus, evidence-based conclusions cannot be drawn, and these results should be regarded with caution.

Finally, the use of various staging systems combined with the variability in the applied procedures for staging make the application of meaningful comparisons among the published series difficult.

CURRENT AND FUTURE RESEARCH — FRONTIER PERSPECTIVE

We retrospectively evaluated the clinical profile and the patterns of outcome among patients who were treated after the diagnosis of aggressive, B-cell, primary endocrine lymphoma (another type of extranodal lymphoma). The patients were diagnosed with either primary testicular lymphoma, primary thyroid lymphoma (PTHL), or PAL. Better outcomes were observed in patients with PTHL for whom the median OS had not been reached until the end date of the study, whereas the PAL group had the worst prognosis[35].

To better understand the nature and outcome of extranodal DLBCL PGL, we described patients' and disease characteristics and assessed trends in treatment options, management and outcome among 159 newly diagnosed patients with primary gastric DLBCL who were seen in our institutions in the years 1971-2017.

Previously, we retrospectively evaluated the trends in clinical presentation, management and outcome among 165 consecutive patients with biopsy-proven primary gastric DLBCL who were seen in 1980-2014. The study cohort was divided into two subgroups based on the era of treatment (CHOP *vs* R-CHOP, before and after the initiation of rituximab). A better outcome after immunochemotherapy (R-CHOP) was observed comparatively[64].

Our novel manuscript and update of the same cohort of patients will be sent for peer review within the next 2 mo (under preparation). We have been preparing and analyzing it for years, focusing on the proper methodology and aiming to correct the perils and pitfalls seen in other relevant studies in the past. We will still have the same conclusion that a better outcome has been noted for the R-CHOP patient cohort, as in the past[64]. However, there are individual variations of the results regarding the OS and freedom from progression (FFP) time intervals, which will be analyzed accordingly, now that a longer follow-up of the patients has been achieved.

The term FFP is based on the strict scientific definition for this type of lymphoma and is preferable to define the aforementioned important endpoint for retrospective clinical studies. PFS has disadvantages in nonrandomized studies because in such studies, there is a lack of specific or concrete criteria for the comparison between time intervals (fixed check points), necessary for the re-evaluation of the disease and the definition of relapse in a similar way (for example, with CT or MRI). However, the term PFS is more widely used in the literature in an equivalent meaning for these lymphomas without being absolutely accurate or to the point in a strict scientific sense. We especially focused on defining FFP accurately, as this is crucial for this novel study. FFP for our novel update will be measured from the initiation of the first treatment until relapse or until death or until the last day of the study for the non

relapsed patients or until the day of the last follow-up for the censored patients (lost to follow-up).

Per-protocol analysis will be used in our clinical research compared to intention-to-treat analysis. The latter is considered a better marker of treatment efficacy for prospective, randomized studies and not for retrospective studies.

Finally, lymphoma-specific survival, another important endpoint, will be measured from diagnosis until the time of death from lymphoma. The number of patients who died from causes other than lymphoma was not calculated at this endpoint. As the long-year follow-up continued, we noted a proportion of our patients dying from lymphoma but also other patients dying from causes other than lymphoma. This analysis is important because it attributes the specific hazard ratio to DLBCL gastric lymphoma (death risk) and separates causes of death other than lymphoma for patients who have survived longer. Importantly, when a patient died from another cause in addition to lymphoma, there was no relapse because the patient was in follow-up. Thus, the possible drug might have protected the patient from relapse, and these patients contributed to the studied time-to-event analysis.

CONCLUSION

In conclusion, retrospective studies, despite their limitations, if conducted with the correct methodology, can provide useful clinical information for treating patients. Our research in recent years has shown that immunochemotherapy (R-CHOP) is the optimal treatment for patients with DLBCL PGLs, as it is associated with a better outcome.

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ORIGINAL ARTICLE

Basic Study

Proteomics identifies a novel role of fibrinogen-like protein 1 in Crohn's disease

Xue-Liang Sun, Li-Chao Qiao, Jing Gong, Ke Wen, Zhi-Zhong Xu, Bo-Lin Yang

ORCID number: Xue-Liang Sun 0000-0001-8520-6937; Li-Chao Qiao 0000-0002-0292-2936; Jing Gong 0000-0001-8423-3706; Ke Wen 0000-0003-3925-6567; Zhi-Zhong Xu 0000-0002-1725-7190; Bo-Lin Yang 0000-0002-2474-4085.

Xue-Liang Sun, Li-Chao Qiao, Jing Gong, Bo-Lin Yang, First Clinical Medical College, The Affiliated Hospital of Nanjing University of Chinese Medicine, Nanjing 210029, Jiangsu Province, China

Xue-Liang Sun, Ke Wen, Zhi-Zhong Xu, Department of Colorectal Surgery, Suzhou TCM Hospital Affiliated to Nanjing University of Chinese Medicine, Suzhou 215000, Jiangsu Province, China

Bo-Lin Yang, Department of Colorectal Surgery, Jiangsu Province Hospital of Chinese Medicine, Nanjing 210029, Jiangsu Province, China

Corresponding author: Bo-Lin Yang, MD, Chief Doctor, First Clinical Medical College, The Affiliated Hospital of Nanjing University of Chinese Medicine, No. 155 Hanzhong Road, Nanjing 210029, Jiangsu Province, China. yfy0051@njucm.edu.cn

Abstract

BACKGROUND

Crohn's disease (CD) is an incurable intestinal disorder with unclear etiology and pathogenesis. Currently, there is a lack of specific biomarkers and drug targets for CD in clinical practice. It is essential to identify the precise pathophysiological mechanism of CD and investigate new therapeutic targets.

AIM

To explore a new biomarker and therapeutic target for CD and verify its role in the CD pathological mechanism.

METHODS

Proteomics was performed to quantify the protein profile in the plasma of 20 CD patients and 20 matched healthy controls. Hub genes among the selected differentially expressed proteins (DEPs) were detected *via* the MCODE plugin in Cytoscape software. The expression level of one hub gene with an immunoregulatory role that interested us was verified in the inflamed intestinal tissues of 20 CD patients by immunohistochemical analysis. After that, the effects of the selected hub gene on the intestinal inflammation of CD were identified in a CD cell model by examining the levels of proinflammatory cytokines by enzyme-linked immunosorbent assays and the expression of the NF-κB signalling pathway by quantitative real-time PCR analysis and Western blot assays.

RESULTS

Thirty-five DEPs were selected from 393 credible proteins identified by proteomic analysis. Among the DEPs, fibrinogen-like protein 1 (*FGL1*), which attracted our attention due to its function in the regulation of the immune response, had 1.722-fold higher expression in the plasma of CD patients and was identified as a hub gene by MCODE. Furthermore, the expression of *FGL1* in the intestinal mucosal and epithelial tissues of CD patients was also upregulated ($P < 0.05$). *In vitro*, the mRNA levels of *FGL1* and *NF-κB*; the protein expression levels of FGL1, IKKα, IKKβ, p-IKKα/β, p-IκBα, and p-p65; and the concentrations of the proinflammatory cytokines IL-1β, IL-6, IL-17, and TNF-α were increased ($P < 0.05$) after stimulation with lipopolysaccharide, which were reversed by knockdown of FGL1 with siRNA transfection ($P < 0.05$). Conversely, FGL1 overexpression enhanced the abovementioned results ($P < 0.05$).

CONCLUSION

FGL1 can induce intestinal inflammation by activating the canonical NF-κB signalling pathway, and it may be considered a potential biomarker and therapeutic target for CD.

Key Words: Crohn's disease; Fibrinogen-like protein 1; Proteomics; NF-κB pathway

Core Tip: In this study, fibrinogen-like protein 1 (FGL1) was identified to be significantly upregulated in the plasma and intestinal mucosa of Crohn's disease (CD) patients. *In vitro*, silencing FGL1 downregulated the levels of the proinflammatory cytokines IL-1β, IL-6, IL-17, and TNF-α. Furthermore, FGL1 knockdown suppressed the mRNA expression of *NF-κB* and the protein levels of IKKα, IKKβ, p-IKKα/β, p-IκBα, and p-p65. These results could be reversed by the overexpression of FGL1. Taken together, these data suggest that FGL1 may induce intestinal inflammation by activating the canonical NF-κB signalling pathway and has the potential to be a therapeutic target for CD.

INTRODUCTION

Crohn's disease (CD) is a chronic, idiopathic intestinal inflammatory disease affecting any segment of the gastrointestinal tract. Although CD is believed to be a result of an imbalanced interaction among genetic susceptibility, environmental factors, the intestinal microflora, and the immune system, the precise pathogenesis is still not entirely clear[1]. Consequently, CD remains incurable even though great advancement has been achieved in medical therapy. Symptoms evolving in a relapsing and remitting manner indicate that CD has a progressive disease course that may induce complications, such as abscess, fistula, and stricture development. Eventually, up to 70% of CD patients require at least one intestinal surgery over their lifetime[2]. Targeted therapy is anticipated to change the natural course of CD, and even to cure it.

Currently, anti-tumor necrosis factor (TNF) agents (infliximab, adalimumab, and certolizumab pegol) are the most potent drugs for inducing and maintaining remission of CD. Unfortunately, anti-TNF treatment failure is common. Primary non-response occurs in 21.9% of infliximab-treated CD patients and 26.8% of adalimumab-treated patients[3]. More than 60% of patients treated with infliximab or adalimumab do not achieve deep remission[3]. These data indicate that increased TNF-α levels may be the result of an immunoinflammatory response instead of the cause. Vedolizumab blocking the α4β7 integrin can induce endoscopic remission in approximately one-

third of CD patients at week 52[4]. The decreased long-term efficacy of biologic medications makes it urgent to investigate new therapeutic targets for CD.

Omics techniques, including genomics, metabolomics, and proteomics, have been applied to explore potential biomarkers and targets for CD in recent years. It is widely known that cellular function and biological behaviour are primarily regulated by proteins. The protein domain is likely the most ubiquitously affected in disease development, treatment response, and physical recovery. Hence, it is promising to reveal the crucial changes in CD pathogenesis and discover novel drug targets by proteomics directly profiling protein expression. Proteomic techniques are classified into three major stages: Discovery, verification, and validation. Currently, the application of proteomics in CD remains in the initial discovery phase[5].

In the present study, we applied proteomics to identify differentially expressed proteins (DEPs) in the plasma of CD patients in an attempt to discover a potential biomarker and therapeutic target for CD. Our data showed that fibrinogen-like protein 1 (FGL1) was significantly upregulated in the plasma of CD patients. FGL1, also known as hepassocin or hepatocyte-derived fibrinogen-related protein 1 (HFREP1), is a hepatocyte-secreted protein that belongs to the fibrinogen family[6]. However, FGL1 lacks a platelet-binding site, a cross-linking region, and a thrombin-sensitive site, which are crucial for fibrin clot formation. Several studies have demonstrated that FGL1 can regulate immune systems to induce inflammatory response and tumor immune evasion[7,8]. To date, whether FGL1 is correlated with the development of CD remains unclear. Therefore, we further verified the expression of FGL1 in intestinal tissues of CD patients and validated its crucial role in the pathogenesis of CD *in vitro*.

MATERIALS AND METHODS

Clinical samples
Plasma samples were collected from 20 treatment-naive patients with CD and 20 age- and sex-matched healthy individuals between July 2017 and August 2018. The protein profiles in the plasma were analysed by tandem mass tag (TMT)-based quantitative proteomics. Paraffin-embedded mucosal biopsy specimens from an additional 20 treatment-naive patients with active CD and 20 matched healthy individuals undergoing colonoscopy screening were obtained for immunohistochemical examination. The protocols of this study were approved by the ethics committee of the Affiliated Hospital of Nanjing University of Chinese Medicine (2018NL-171-02). All patients provided informed consent.

TMT-based quantitative proteomics
Plasma samples were homogenized in sodium dodecyl sulfate (SDS) lysis buffer. Centrifugation was performed to collect the supernatant. Total protein concentrations were quantified using a bicinchoninic acid (BCA) assay (Thermo Scientific, United States). Protein extracts were reduced with reducing buffer (10 mmol/L dithiothreitol, 8 mol/L urea, and 100 mmol/L tetraethylammonium bromide (TEAB), pH 8.0) at 60 °C for 1 h. All samples were alkylated with iodoacetamide for 40 min at room temperature in the dark. After centrifugation, the protein pellets were digested with TEAB (100 mmol/L) and sequencing-grade trypsin (1 µg/µL) at 37 °C for 12 h.

For TMT labelling, 100 µL of protein sample was incubated with a mixed solution of 41 µL of TMT labelling reagent (Thermo Fisher Scientific, United States) and 41 µL of anhydrous acetonitrile for 1 h at room temperature. The reaction was terminated with 8 µL of 5% hydroxylamine. The samples from the CD patients were labelled with TMT-130 and TMT-131, while those from the healthy controls were labelled with TMT-126 and TMT-127.

The TMT-labelled peptides were eluted by using an Agilent Zorbax Extend-C18 column (2.1 mm × 150 mm, 5 µm) and fractionated with a high-performance liquid chromatography (HPLC) system at a flow rate of 300 µL/min. The elution gradient was set to 98%, 95%, 75%, 60%, and 10%. The collected peptides were loaded on a reverse-phase trap column (C18, 100 µm × 20 mm, Thermo Fisher Scientific, United States) and enriched on an analysis column (C18, 75 µm × 150 mm, Thermo Fisher Scientific, United States) following redissolution in nano-HPLC buffer (HPLC water containing 0.1% formic acid). The flow rate was 300 nL/min, and the linear elution gradient was set as 5%, 30%, 50%, and 100%.

For mass spectrometry (MS) survey scans, the ion spray voltage, interface heating temperature, MS resolution, and ion population were set to 1,800 V, 250 °C, 70000, and $1 × 10^6$, respectively. The precursor ion was acquired at 300-1600 m/z. A maximum of

10 precursors were selected for higher-energy collisional dissociation with analysis in an LTQ Orbitrap Velos Pro (Thermo Fisher Scientific, United States), and the normal chemical energy was 32%. For MS/MS detection, the tandem MS resolution, ion population, ion maximum injection time, and dynamic exclusion time were set to 17500, 2×10^5, 80 ms, and 30 s, respectively.

Quantitative proteomic analysis

The raw proteomic data were analysed using Proteome Discoverer software (version 2.2, Thermo Fisher Scientific, United States) and searched against the UniProtKB database (Hunam, 2015-09, 88473 sequences). Andromeda was used as the search engine with the following parameters: (1) *Homo sapiens* taxonomy; (2) Q Exactive plus as instrument type; (3) Trypsin as the proteolytic enzyme, with two missed cleavages allowed; (4) TMT 6 plex and cysteine carbamidomethylations as fixed modifications; (5) Oxidation of methionine as a variable modification; (6) 20 ppm as the MS tolerance; and (7) Seven amino acids as minimum cut-off for peptide length. A false discovery rate (FDR) of less than 1% was set to refine the results.

For quantitative analysis, the TMT reporter ion intensity of each protein was analysed using Proteome Discoverer software. Proteins with empty values were discarded. Student's *t* test was performed to examine the difference in each protein between the two groups with Perseus software. Proteins with a fold change > 1.5 or < 0.67 and a *P* value < 0.05 were considered to be DEPs.

Bioinformatics analysis

Genes of DEPs were visualized in Cytoscape software (version 3.7.2), in which the MCODE plugin was used to select significant modules for identification of hub genes. Subsequently, the hub genes were input into the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (https://string-db.org/) to construct a protein-protein interaction (PPI) network. The Database for Annotation, Visualization and Integrated Discovery (DAVID) database (https://david.ncifcrf.gov/) was applied for gene ontology (GO) enrichment analysis. Reactome pathway analysis (https://www.reactome.org/) was performed for pathway enrichment analysis.

Immunohistochemical staining

Immunohistochemical staining was implemented to detect the expression of FGL1 in inflamed intestinal tissues of CD patients and normal intestinal biopsies. Mucosal biopsy specimens were fixed in 10% neutral formalin for 24 h. Afterwards, they were embedded in paraffin and cut into 5 μm sections. The sections were deparaffinized and rehydrated and then incubated in citrate buffer (pH 6.0) for antigen retrieval. After endogenous peroxidase activity was quenched with 3% hydrogen peroxide, the samples were incubated in 1% bovine serum albumin (BSA) to block non-specific immunoglobulin binding. Subsequently, the slides were incubated with an anti-FGL1 antibody (1:200 dilution, 16000-1-AP, Proteintech, United States) at 4 °C overnight. Following washing with phosphate buffered saline (PBS), the slides were incubated with a secondary IgG antibody (1:1000 dilution, ab6721, Abcam, United Kingdom) at room temperature for 1 h, counterstained with haematoxylin, and stained with a diaminobenzidine kit (DAB, Beyotime, China). All the sections were visualized under a light microscope (Nikon 80i, Japan). ImageJ software (version 1.52) was used to calculate the integrated optical density (IOD) values.

Cell culture and treatment

The human colonic adenoma cell line HT-29 (ATCC, United States) was cultured with Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum, 100 μg/mL penicillin, and 100 U/mL streptomycin at 37 °C with 5% CO_2. The HT-29 cells were stimulated with 100 ng/mL lipopolysaccharide (LPS, Sigma, United States) to establish a cell model of intestinal inflammation. To uncover the impact of FGL1 on intestinal inflammation, the HT-29 cells were transfected with *FGL1* siRNA and plasmid DNA (Nanjing KeyGen Biotech Co., Ltd., China) before stimulation with LPS. The transfection efficiency was determined by examining the mRNA expression of *FGL1*.

Quantitative real-time PCR analysis

Total RNA in HT-29 cells was extracted using a TRIzol reagent kit (Takara, Japan) according to the manufacturer's instructions. A PrimeScript RT reagent kit (Takara, Japan) was used for reverse transcription of the extracted RNA into cDNA. Quantitative real-time PCR (qRT-PCR) was conducted to detect the mRNA expression

of *FGL1* and *NF-κB* by using a SYBR green kit (Takara, Japan). The housekeeping gene β-actin was used for normalization to an endogenous reference. The relative gene expression was evaluated by using the $2^{-\Delta\Delta Ct}$ method. The sequences of the PCR primers are as follows: *FGL1*-forward: 5'-ATGGCAAAGGTGTTCAGTTTCA-3', reverse: 5'-ACAATCTGCATACTGCCTCTTG-3'; *NF-κB*-forward: 5'-GAAGCACGAATGACAGAGGC-3', reverse: 5'-GCTTGGCGGATTAGCTCTTTT-3'; and β-actin-forward: 5'-CATGTACGTTGCTATCCAGGC-3', reverse: 5'-CTCCTTAAT-GTCACGCACGAT-3'.

Enzymelinked immunosorbent assay

The levels of the proinflammatory cytokines IL-1β, IL-6, IL-17, and TNF-α (Sigma, United States) in the culture medium collected after 48 h were examined by enzyme linked immunosorbent assay (ELISA) according to the manufacturer's protocol.

Western blot assay

Cells lysed with radioimmunoprecipitation assay (RIPA) lysis buffer were centrifuged at 12000 *g* for 20 min at 4 °C. Protein concentrations in the collected supernatant were quantified with a BCA assay kit. After equal amounts of protein (20 μg/well) were loaded and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), they were transferred onto polyvinylidene difluoride (PVDF) membranes and incubated in 5% BSA for 1 h at room temperature. The membranes were washed with Tris-borate saline containing 0.1% Tween-20 (TBST) and were incubated with primary antibodies against FGL1 (1:1000 dilution, 16000-1-AP, Proteintech, United States), IKKα (1:1000 dilution, ab32041, Abcam, United Kingdom), IKKβ (1:1000 dilution, ab32135, Abcam), p-IKKα/β (1:1000 dilution, ab194528, Abcam), IκBα (1:1000 dilution, ab32518, Abcam), p-IκBα (1:1000 dilution, ab133462, Abcam), NF-κB (p65, 1:1000 dilution, ab32536, Abcam), p-p65 (1:1000 dilution, ab76302, Abcam) and β-actin (1:1000 dilution, 20536-1-AP, Proteintech) at 4 °C overnight. The membranes were washed with TBST again and incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature. The blots were imaged using enhanced chemiluminescence (ECL). ImageJ software was used to calculate the protein signal grey values.

Statistical analysis

All data were statistically analysed with SPSS 22.0 (SPSS Inc., United States). Continuous variables with a normal distribution are summarized using the mean ± SD, which in a skewness distribution are expressed as the median with range. The Mann-Whitney *U* test, Student's *t* tests, and chi-square test were performed to compare numerical variables and categorical variables as appropriate. One-way analysis of variance was used for multi-group comparisons. A two-sided *P* value < 0.05 was considered statistically significant.

RESULTS

Patients' characteristics

Twenty treatment-naive CD patients and 20 healthy controls were recruited for plasma proteomic analysis. The diagnostic criteria for CD referred to the clinical guidelines of the American College of Gastroenterology (ACG)[9]. Thirteen males and seven females with a median age of 20.5 (14-43) years were included in the CD group, and eleven males and nine females with a median age of 24.5 (18-46) years were included in the normal control group. Baseline demographic characteristics were comparable between the two groups (*P* > 0.05).

Colonoscopic biopsy specimens from an additional 20 treatment-naive patients with active CD and 20 healthy controls were used for immunohistochemical staining. There was no significant difference in sex distribution, age, or biopsy site between the two groups (*P* > 0.05). The baseline clinical characteristics of patients for plasma proteomic detection and immunohistochemical analysis are presented in Tables 1 and 2, respectively.

FGL1 is significantly upregulated in plasma proteomic analysis

Plasma samples in each group were randomly divided into four subclusters. A total of 393 credible proteins were identified by proteomic analysis, among which 35 had differential expression between the two groups (Figure 1A). Among the DEPs, FGL1

Table 1 Clinical characteristics of patients for plasma proteomic analysis

Item	Crohn's disease (*n* = 20)	Normal control (*n* = 20)	*P* value
Sex			
Male	13	11	0.519
Female	7	9	
Median age (range), yr	20.5 (14-43)	24.5 (18-46)	0.069
Disease location in the endoscopy			
Ileum	6	N/A	
Colon	6	N/A	
Ileocolon	8	N/A	

N/A: Not applicable.

Table 2 Clinical characteristics of patients for immunohistochemical assay

Item	Crohn's disease (*n* = 20)	Normal control (*n* = 20)	*P* value
Sex			
Male	15	13	0.731
Female	5	7	
Age (mean ± SD), yr	27.1 ± 7.9	25.6 ± 4.5	0.465
Biopsy site			
Terminal ileum	8	5	0.832
Ascending colon	2	1	
Transverse colon	2	2	
Descending colon	2	4	
Sigmoid colon	4	6	
Rectum	2	2	

SD: Standard deviation.

attracted our attention because of its function in the regulation of the immune response. The expression level of FGL1 in the plasma of CD patients was 1.722-fold greater than that in healthy people (Figure 1B). Three MCODE modules were established to screen hub genes *via* Cytoscape software. As *FGL1* was contained in the 3rd module, the genes in this module were used for further bioinformatics analysis. Figure 1C shows the PPI network of the genes. GO enrichment analysis showed that the genes were involved in the biological processes of platelet degranulation, acute phase response, platelet activation, and negative regulation of endopeptidase activity, and the molecular functions of heparin binding and serine-type endopeptidase inhibitor activity. Reactome pathway analysis demonstrated that the genes were related to the common pathway of fibrin clot formation and the pathways of platelet degranulation, peptide ligand-binding receptors, haemostasis, G alpha (i) signalling events, and innate immune system.

FGL1 expression is increased in intestinal tissues of CD patients

Immunohistochemical analysis was performed to verify the expression of FGL1 in the intestinal tissues of CD patients. The results demonstrated that the FGL1 levels in the intestinal mucosal and epithelial tissues were higher than those in the normal intestinal tissues ($P < 0.01$, Figure 2).
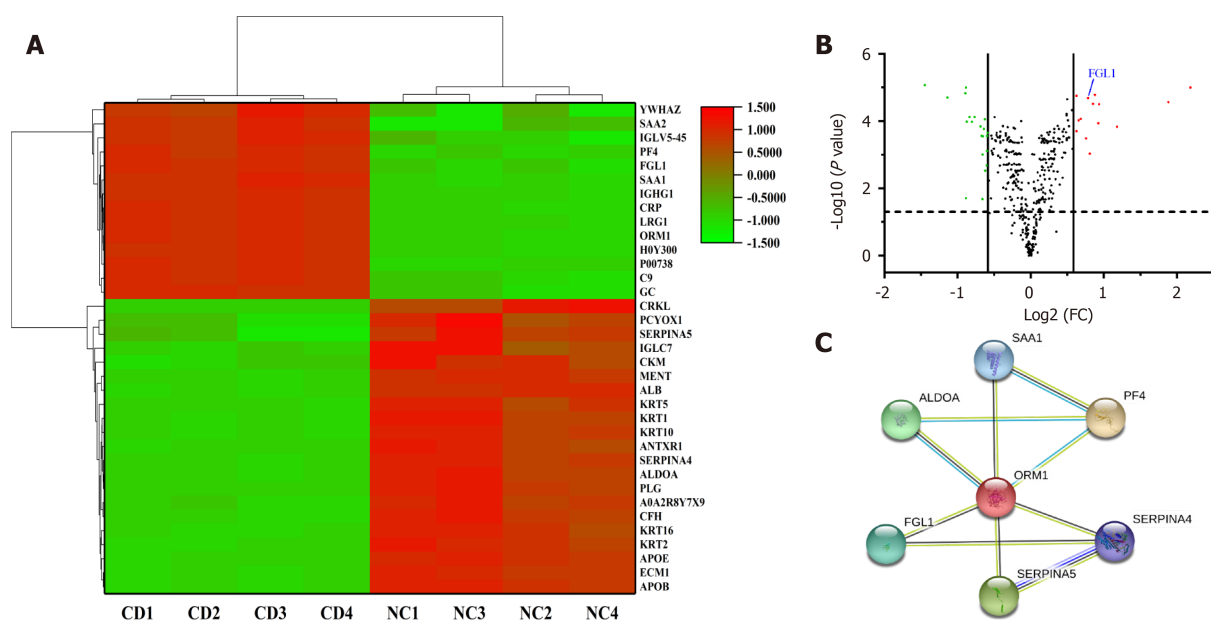
Figure 1 Fibrinogen-like protein 1 expression in the plasma of Crohn's disease patients. A: Heat map showing 35 differentially expressed proteins between Crohn's disease (CD) patients and healthy individuals, among which fibrinogen-like protein 1 (FGL1) expression was upregulated in the CD group; B: The FGL1 expression level in the plasma of CD patients was 1.722-fold greater than that in healthy people; C: Protein-protein interaction network of an MCODE module containing *FGL1* as a hub gene. CD: Crohn's disease; NC: Normal control; FGL1: Fibrinogen-like protein 1.
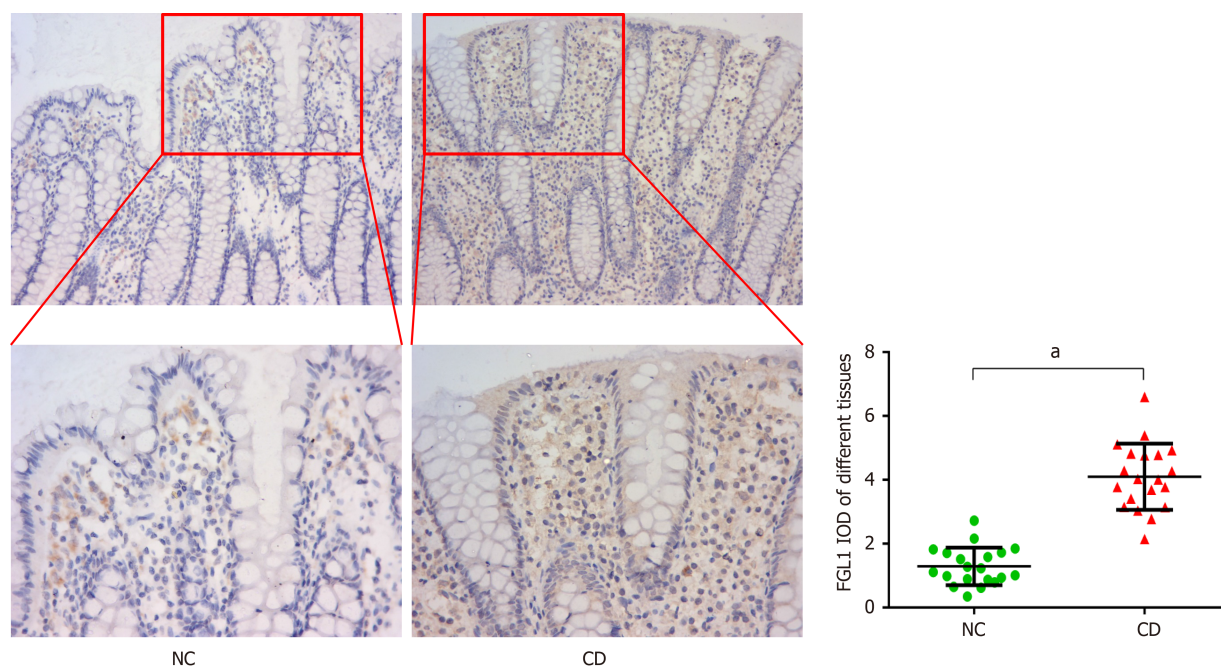
Figure 2 Fibrinogen-like protein 1 expression, as determined by immunohistochemical analysis (× 200, × 400), was increased in the intestinal mucosal and epithelial tissues of Crohn's disease patients. [a]*P* < 0.01 *vs* normal control group. NC: Normal control group; CD: Crohn's disease group; FGL1: Fibrinogen-like protein 1.

FGL1 mediates the expression of proinflammatory cytokines in intestinal epithelial cells

To investigate the regulation of intestinal inflammation by FGL1, proinflammatory cytokines were detected by ELISA after *FGL1* siRNA and plasmids were transfected into HT-29 cells. After LPS stimulation, the IL-1β, IL-6, IL-17, and TNF-α levels were significantly upregulated. FGL1 knockdown reversed the expression of IL-1β, IL-6, IL-17, and TNF-α, while overexpression of FGL1 elevated the levels of the four proinflammatory cytokines (*P* < 0.05, Figure 3).
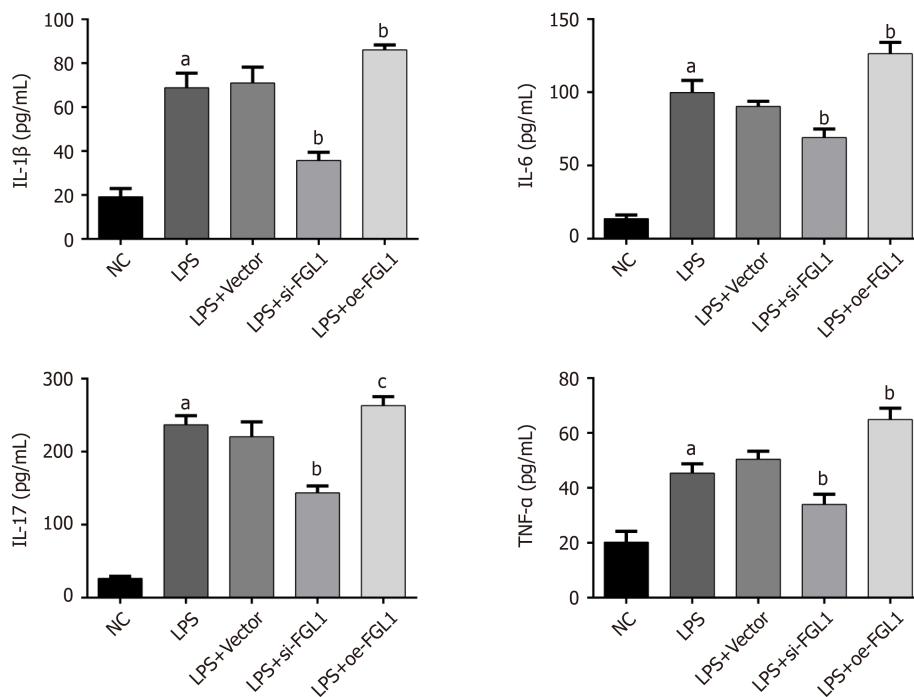
Figure 3 Expression of proinflammatory cytokines in different groups, as determined by enzyme linked immunosorbent assay. [a]*P* < 0.01 *vs* normal control group; [b]*P* < 0.01, [c]*P* < 0.05 *vs* lipopolysaccharide. NC: Normal control group; LPS: Crohn's disease (CD) model induced by lipopolysaccharide (LPS); LPS+Vector: Transfected with an empty vector based on the CD model; LPS+si-FGL1: Transfected with FGL1 siRNA based on the CD model; LPS+oe-FGL1: Transfected with the FGL1 plasmid based on the CD model; FGL1: Fibrinogen-like protein 1.

FGL1 activates the NF-κB signalling pathway

Given that NF-κB plays a fundamental role in the intestinal inflammation of CD, the modulation of the NF-κB signalling pathway by FGL1 was investigated. The mRNA expression levels of *FGL1* and *NF-κB* were increased by LPS stimulation. After intervention with *FGL1* siRNA, the mRNA expression levels of *FGL1* and *NF-κB* were both downregulated, while the mRNA levels were enhanced following FGL1 overexpression with plasmid transfection (*P* < 0.01, Figure 4A and B).

The exact mechanism by which FGL1 regulates the NF-κB signalling pathway was revealed by Western blot assay. The protein levels of FGL1, IKKα, IKKβ, p-IKKα/β, p-IκBα, and p-p65 were upregulated in HT-29 cells stimulated with LPS (*P* < 0.05). *FGL1* gene knockdown inhibited the protein expression of FGL1 and downregulated the protein expression of IKKα, IKKβ, p-IKKα/β, p-IκBα, and p-p65 (*P* < 0.05). Conversely, the overexpression of the *FGL1* gene enhanced the protein expression of FGL1, IKKα, IKKβ, p-IKKα/β, p-IκBα, and p-p65 (*P* < 0.05, Figure 4C).

DISCUSSION

In the present study, we took advantage of proteomics for a large-scale screen of DEPs between the plasma of CD patients and healthy people. The expression of *FGL1*, a hub gene among the DEPs, was increased in the plasma of CD patients, which was verified in intestinal mucosal and epithelial tissues. Furthermore, FGL1 was validated to exacerbate the inflammatory response in intestinal epithelial cells by activating the NF-κB signalling pathway.

At present, knowledge about the etiology and pathogenesis of CD is limited, which makes it incurable. Hence, it is essential to detect potential drug targets for CD. As proteins are directly involved in nearly all pathophysiological processes, proteomics has become a hotspot tool for the discovery of novel biomarkers or therapeutic targets for CD, differential diagnosis between CD and ulcerative colitis, and disease stratification by examining and quantifying thousands of proteins encoded by the genome in a holistic manner[10-13]. To our knowledge, the present study revealed for the first time by proteomics that FGL1 may be a key contributor to CD onset and progression.
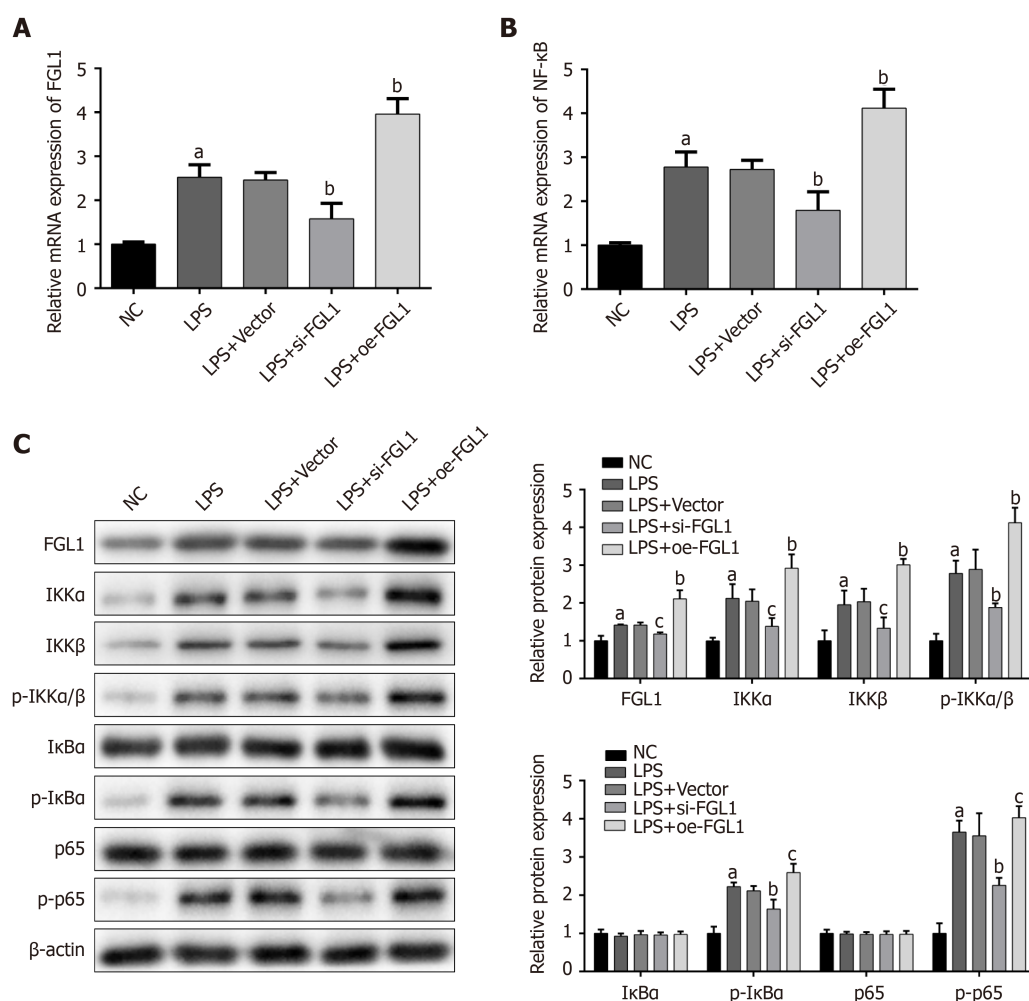
Figure 4 Impact of fibrinogen-like protein 1 on the NF-κB signalling pathway. A and B: Effect of knockdown or overexpression of fibrinogen-like protein 1 (FGL1) on the mRNA expression of *FGL1* and *NF-κB*, as determined by quantitative real-time PCR assay; C: Effect of FGL1 on the protein expression of related proteins in the canonical NF-κB signalling pathway, as determined by Western blot assay. $^aP < 0.01$ *vs* NC; $^bP < 0.01$, $^cP < 0.05$ *vs* LPS. NC: Normal control group; LPS: Crohn's disease (CD) model induced by LPS; LPS+Vector: Transfected with an empty vector based on the CD model; LPS+si-FGL1: Transfected with FGL1 siRNA based on the CD model; LPS+oe-FGL1: Transfected with the FGL1 plasmid based on the CD model; LPS: Lipopolysaccharide; FGL1: Fibrinogen-like protein 1.

Accumulating evidence has demonstrated that FGL1 plays a prominent role in the pathogenesis of various diseases, including hepatocellular carcinoma, gastric cancer, lung cancer, diabetes mellitus, and obesity[14-19]. Additionally, FGL1 is also considered a potential biomarker and drug target in certain inflammatory conditions. FGL1 may promote liver injury-induced inflammation *via* the IL-6/STAT3 signalling pathway[20]. Proteomics revealed that FGL1 is a specific biomarker for predicting the progression of rheumatoid arthritis[7]. This finding displays a fundamental role of FGL1 in regulating immune-mediated inflammation.

Pierre and colleagues have demonstrated that CD relapse is correlated with the innate immune response of the liver[21]. Given that FGL1 is a liver-derived protein and is involved in the innate immune system pathway, we hypothesize that FGL1 may influence the pathophysiology of CD based on the evidence of increased expression of FGL1 in the plasma and intestinal tissues of CD patients. Although FGL1 has been demonstrated to be a potent target for cancer immunotherapy, its precise role in CD therapy is unknown[8]. To unravel the mystery, a cell experiment was designed in the current study that focused on the FGL1-mediated regulation of signalling by NF-κB, an important proinflammatory transcription factor for inflammatory disorders.

Activation of NF-κB plays a central role in the induction and exacerbation of the intestinal inflammatory response of CD patients[22]. The NF-κB family consists of p65 (RELA), RELB, c-REL, p50/p105 (NF-κB1), and p52/p100 (NF-κB2). Activated p65 can translocate into the nucleus to upregulate the transcriptional expression of proinflammatory cytokines. In the present study, the mRNA and protein levels of FGL1 and NF-κB and the concentrations of IL-1β, IL-6, IL-17, and TNF-α were markedly upregulated

in HT-29 cells stimulated with LPS, and these effects were reversed by depleting FGL1 with specific siRNA. Correspondingly, the expression of NF-κB and the four proinflammatory cytokines was enhanced following overexpression of FGL1. These results indicate that FGL1 may promote the intestinal inflammatory response by activating NF-κB signalling. The canonical pathway of NF-κB activation involves the IKK complex, consisting of NEMO, IKKα, and IKKβ, and the IκB protein family, including IκBα, IκBβ, and IκBe. After stimulation, IKKα and IKKβ activation promotes phosphorylation of IκBα. Degradation of phosphorylated IκBα releases the p65-p50 dimer for nuclear translocation[23]. In this study, the protein expression levels of IKKα, IKKβ, p-IKKα/β, p-IκBα, and p-p65 were decreased after knockdown of FGL1 compared to those in the cell model, and the inverse effect was verified by overexpression of FGL1. Therefore, FGL1 may induce intestinal inflammation by activating the canonical NF-κB pathway.

CONCLUSION

In summary, we found for the first time that the expression of FGL1 is considerably upregulated in the plasma and intestinal mucosal and epithelial tissues of CD patients. FGL1 might induce intestinal inflammation by activating the canonical NF-κB signalling pathway to stimulate the secretion of proinflammatory cytokines, such as IL-1β, IL-6, IL-17, and TNF-α. Hence, FGL1 may be considered a potential biomarker and therapeutic target for CD. However, given the exploratory design of our study, the precise role of FGL1 in the pathogenesis of CD needs to be deeply investigated and further validated.

ACKNOWLEDGEMENT

ARTICLE HIGHLIGHTS

Research background
Currently, the etiology and pathogenesis of Crohn's disease (CD) are not completely known, which makes it incurable. It is urgent to reveal the pathophysiological mechanism of CD and investigate new therapeutic targets.

Research motivation
To explore a potential therapeutic target for CD and verify its role in the CD pathological mechanism.

Research objectives
In this study, we attempted to find a potential therapeutic target for CD and verify its role in the CD pathological mechanism *in vitro.*

Research methods
Proteomics was implemented to quantify the protein profile in the plasma of CD patients. Among the differentially expressed proteins, a hub gene that could regulate the immune response was selected for further study. The expression of the selected hub gene in the inflamed intestinal mucosa was verified by immunohistochemical staining. *In vitro*, the effects of the hub gene on the expression of proinflammatory cytokines and the NF-κB signalling pathway were evaluated by ELISA, qRT-PCR, and Western blot analysis.

Research results
Fibrinogen-like protein 1 (*FGL1*), as a hub gene of the differentially expressed proteins, was confirmed to be markedly upregulated in the plasma and intestinal mucosa of CD patients. Silencing FGL1 downregulated the levels of the proinflammatory cytokines IL-1β, IL-6, IL-17, and TNF-α. Furthermore, FGL1 knockdown repressed the mRNA

expression of *NF-κB* and the protein levels of IKKα, IKKβ, p-IKKα/β, p-IκBα, and p-p65. Overexpression of FGL1 enhanced these results.

Research conclusions

FGL1 may promote intestinal inflammation modulated by the canonical NF-κB signalling pathway and has the potential to be a therapeutic target for CD.

Research perspectives

Our findings indicate a critical role of FGL1 in the onset and progression of CD, which may serve as a potential prognostic biomarker and therapeutic target for CD.

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ORIGINAL ARTICLE

Retrospective Study

Effectiveness and safety of over-the-scope clip in closing perforations after duodenal surgery

Zhen-Zhen Wang, Xian-Bin Zhou, Yi Wang, Xin-Li Mao, Li-Ping Ye, Ling-Ling Yan, Ya-Hong Chen, Ya-Qi Song, Yue Cai, Shi-Wen Xu, Shao-Wei Li

ORCID number: Zhen-Zhen Wang 0000-0002-6274-2646; Xian-Bin Zhou 0000-0003-0048-7456; Yi Wang 0000-0002-9817-1769; Xin-Li Mao 0000-0003-4548-1867; Li-Ping Ye 0000-0001-9839-2062; Ling-Ling Yan 0000-0001-5103-9886; Ya-Hong Chen 0000-0003-0004-0187; Ya-Qi Song 0000-0001-9648-8159; Yue Cai 0000-0002-7201-6525; Shi-Wen Xu 0000-0001-6774-1979; Shao-Wei Li 0000-0002-3276-1037.

Zhen-Zhen Wang, Xian-Bin Zhou, Yi Wang, Xin-Li Mao, Li-Ping Ye, Ling-Ling Yan, Ya-Qi Song, Yue Cai, Shao-Wei Li, Key Laboratory of Minimally Invasive Techniques and Rapid Rehabilitation of Digestive System Tumor of Zhejiang Province, Taizhou Hospital of Zhejiang Province Affiliated to Wenzhou Medical University, Linhai 317000, Zhejiang Province, China

Zhen-Zhen Wang, Xian-Bin Zhou, Yi Wang, Xin-Li Mao, Li-Ping Ye, Ling-Ling Yan, Ya-Qi Song, Yue Cai, Shao-Wei Li, Department of Gastroenterology, Taizhou Hospital of Zhejiang Province Affiliated to Wenzhou Medical University, Linhai 317000, Zhejiang Province, China

Ya-Hong Chen, Health Management Center, Taizhou Hospital of Zhejiang Province Affiliated to Wenzhou Medical University, Linhai 317000, Zhejiang Province, China

Shi-Wen Xu, Taizhou Hospital of Zhejiang Province, Wenzhou Medical University, Linhai 317000, Zhejiang Province, China

Corresponding author: Shao-Wei Li, PhD, Academic Fellow, Assistant Professor, Associate Research Scientist, Instructor, Key Laboratory of Minimally Invasive Techniques and Rapid Rehabilitation of Digestive System Tumor of Zhejiang Province, Taizhou Hospital of Zhejiang Province Affiliated to Wenzhou Medical University, No. 150 Xinmen Street, Linhai 317000, Zhejiang Province, China. li_shaowei81@hotmail.com

Abstract

BACKGROUND
Endoscopic resection of duodenal subepithelial lesions (SELs) is a difficult procedure with a high risk of perforation. At present, dealing with perforation after endoscopic resection of duodenal SELs is still considered a great challenge.

AIM
To evaluate the effectiveness and safety of an over-the-scope clip (OTSC) in the treatment of perforation post-endoscopic resection of duodenal SELs.

METHODS
From May 2015 to November 2019, 18 patients with perforation following endoscopic resection of duodenal SELs were treated with OTSCs. Data comprising the rate of complete resection, closure of intraprocedural perforation, delayed bleeding, delayed perforation, and postoperative infection were extracted.

RESULTS

The rate of complete removal of duodenal SELs and successful closure of the perforation was 100%. The median perforation size was 1 cm in diameter. Seventeen patients had minor intraoperative bleeding, while the remaining 1 patient had considerable amount of bleeding during the procedure. Seven patients had postoperative abdominal infections, of which 1 patient developed an abscess in the right iliac fossa and another patient developed septic shock. All 18 patients recovered and were discharged. No delayed bleeding or perforation was reported. The mean time taken to resume normal diet after the procedure was 6.5 d. The mean postoperative hospital stay was 9.5 d. No residual or recurrent lesions were detected during the follow-up period (15-66 mo).

CONCLUSION

Closing a perforation after endoscopic resection of duodenal SELs with OTSCs seems to be an effective and reasonably safe therapeutic method.

Key Words: Over-the-scope clip; Duodenal subepithelial lesion; Endoscopic resection; Perforation; Effectiveness; Safety

Core Tip: This study presents the use of over-the-scope clip in closing duodenal perforation of 18 patients. We believe that our study makes a significant contribution to the literature because dealing with perforation after endoscopic resection of duodenal subepithelial lesions is challenging. This study aimed to evaluate the effectiveness and safety of over-the-scope clip in closing perforation after endoscopic resection of duodenal subepithelial lesions. The rate of successful closure was 100%. No delayed perforation occurred in any of the patients. Seven patients had postoperative infection, of which 1 patient developed septic shock and underwent surgery. All 18 patients recovered.

INTRODUCTION

Duodenal subepithelial lesions (SELs) include Brunner's adenomas, lipomas, heterotopic pancreas, leiomyomas, neuroendocrine tumors, and gastrointestinal stromal tumors (GISTs). Most of these are benign, while some lesions, such as neuroendocrine tumors and GISTs, are potentially malignant[1-3]. Resection of these lesions may contribute to improvement in diagnosis and treatment outcomes.

Surgery, including pancreatoduodenectomy and limited resection, is the most basic treatment for duodenal lesions. However, due to the complexity of the operation, risk of trauma, high incidence of postoperative complications, poor quality of life of patients after surgery, and other difficulties, these surgeries are not easily consented by patients, which also puts the medical staff in a difficult position. With the recent development of minimally invasive endoscopic treatment technologies, such as endoscopic submucosal dissection (ESD), endoscopic muscularis excavation, and endoscopic full-thickness resection, endoscopic treatment has become increasingly popular, which brings hope for the use of minimally invasive treatment of duodenal SELs in the future.

However, endoscopic resection of duodenal SELs is still regarded as a challenging procedure due to a high risk of perforation. The incidence of perforations in duodenal ESD has been reported to range from 6.7%-36.6% during the procedure and 0%-14.3% during the postoperative period[1,4-7]. Management of perforations after endoscopic removal of duodenal SELs is particularly challenging. However, this may be achieved

by using over-the-scope clips (OTSCs). An OTSC was developed as an endoscopic full-thickness gastrointestinal closure device and has become one of the treatment options for gastrointestinal perforation because it is less invasive compared to conventional surgical closure. At present, there are few reports on endoscopic resection of duodenal SELs and endoscopic methods for the management of perforations[1,2,8,9]. To explore further this area, this study aimed to assess the effectiveness and safety of OTSCs in the treatment of perforation after endoscopic resection of duodenal SELs.

MATERIALS AND METHODS

Patients
This was a retrospective study and was approved by the ethics committee of Taizhou Hospital of Zhejiang Province (Linhai, China). The study included 18 consecutive patients who were treated with OTSCs to close perforations that resulted after endoscopic resection of duodenal SELs, from May 2015 to November 2019. Patients were recruited if they met all of the following criteria: (1) Patients with duodenal SELs diagnosed by computed tomography and endoscopic ultrasound (EUS) with a high-frequency miniprobe (UM-2R, 12 MHz; UM-3R, 20 MHz, Olympus Optical, Tokyo, Japan); (2) Patients who underwent endoscopic resection of duodenal SELs and had intraoperative or postoperative perforations; (3) The duodenal perforation was closed using an OTSC; and (4) Patients who were able to tolerate general anesthesia and had no blood coagulation disorders prior to the procedure.

Before the endoscopic procedure, informed consent was obtained from all 18 patients. Patients were also informed that an OTSC might be used, and surgical intervention might be required in case of unsuccessful resection of the lesion or the occurrence of severe complications that cannot be successfully managed by endoscopic methods and conservative treatment.

The main outcome measurements were as follows: (1) The rate of complete closure of intraprocedural perforation; (2) Delayed perforation rate; and (3) Postoperative infection rate. All endoscopic resection procedures were performed by an experienced endoscopist in a sterile operating room while the patients were under general anesthesia with tracheal intubation.

Endoscopic procedures
The main equipment and accessories used were as follows: A single-accessory channel endoscope (Q260J; Olympus) with a transparent cap (ND-201-11802; Olympus) attached to its tip, an argon plasma coagulation unit (APC 300; ERBE, Tübingen, Germany), a high-frequency electronic cutting device (ICC 200; ERBE), a hook knife (KD-620LR; Olympus), an insulated-tip knife (KD-611L, IT2; Olympus), hot biopsy forceps (FD-410LR; Olympus), foreign body forceps (FG-B-24, Kangjin, Changzhou, China), a snare (SD-230U-20; Olympus), a carbon dioxide insufflator (Olympus), twin graspers (Ovesco Endoscopy AG, Tuebingen, Germany), an OTSC (12/6 t-type, Ovesco Endoscopy AG,), a titanium clip (HX-600-135; Olympus and M00522600), and endoloop (Leo Medical Co., Ltd, Changzhou, China).

Endoscopic resection was performed as follows (Figure 1): (1) Several marking dots were initially made around the lesion using a needle-knife to define the border; (2) A submucosal elevation was made by injection of solution (100 mL saline plus 1 mL epinephrine and 2 mL indigo carmine); (3) Subsequently, the mucosa was incised with a hook knife outside the border to reveal the lesion; (4) A circumferential excavation was made as deep as the submucosa or muscularis propria layer around the lesion using an insulated tip knife; (5) After the lesion was completely resected, it was removed using a snare or foreign body forceps; and (6) Duodenal tissues adjacent to the perforation were clamped with twin graspers and then drawn into the transparent cap of the OTSC device until they were fully inhaled into the transparent cap following which the OTSC closure system was released to close the wound. If defect closure was not complete, several clip and/or endoloops were used to close the remaining portions. The mucosa defect was closed with several clips in a 'side to center' manner, and an endoloop was placed to trap all the clips. Finally, the endoloop was slowly tightened, and all the clips were tied together with the endoloop[8].

Postoperative management and follow-up
After the operation, all patients were treated with postoperative fasting, gastrointestinal decompression, proton-pump inhibitors, and antibiotics for infection prevention. Oral intake was gradually resumed depending on the speed of recovery.
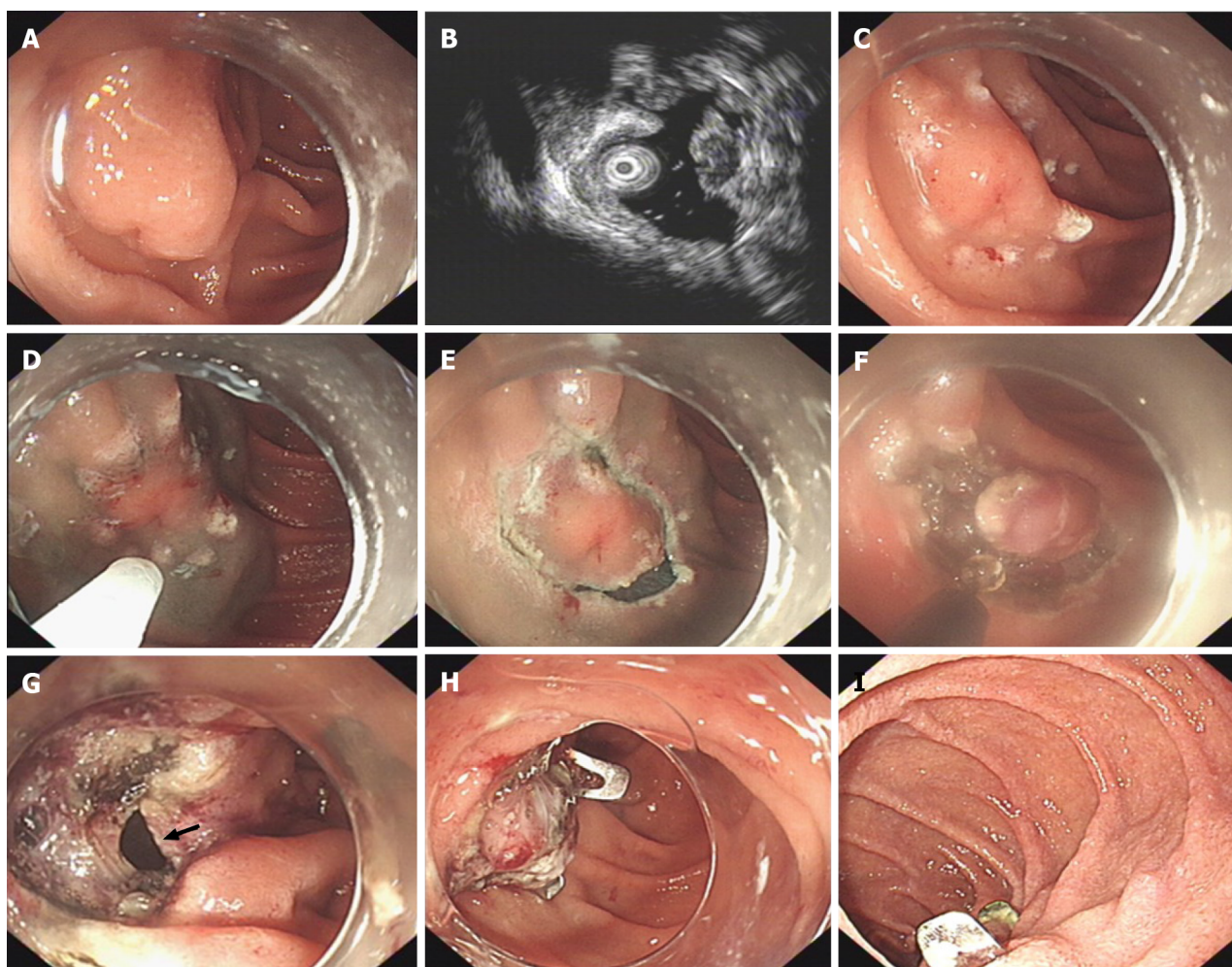
Figure 1 Endoscopic resection of a subepithelial lesion located in the descending duodenum with perforation closure using an over-the-scope clip. A: Endoscopic view of a subepithelial lesion located in the descending duodenum; B: Endoscopic ultrasound evaluation of the same lesion; C: Several marking dots are made around the lesion; D: Injection solution used to elevate the submucosa; E: The mucosa is incised outside the marking dots; F: A circumferential excavation is made as deep as the submucosa around the lesion; G: A duodenal perforation is observed (black arrow) after removal of the lesion; H: The perforation is closed with an over-the-scope clip; I: Healed wound 9 mo after the procedure.

Every patient underwent follow-up endoscopies to monitor wound healing at 3 mo and 6 mo after endoscopic resection. EUS was performed to check for residual lesions after 3 mo. Patients with potentially malignant lesions, such as neuroendocrine tumors and GISTs, were monitored by endoscopy and/or EUS to detect recurrent lesions, and abdominal US and/or computed tomography to detect distant metastasis every 12 mo.

Statistical analysis

Data were analyzed using SPSS software (version 20.0; SPSS Inc., Armonk, NY, United States). Descriptive statistics were used for this study. The median was used for variables with a skewed distribution, while the mean was used in the case of a normal distribution of variables. Enumeration data are expressed as case numbers and percentages (%).

RESULTS

Clinical characteristics and therapeutic outcome

Patient information is summarized in Table 1 and therapeutic outcomes, are described in Table 2. The rate of successful *en bloc* resection was 100%. The vertical and horizontal margins of all specimens were tumor-free. Thus, the complete resection rate was 100%.

Table 1 Clinical characteristics of the 18 patients with duodenal subepithelial lesions, *n* (%)	
Patients	
Median age, yr (range)	53.5 (29-74)
Gender	
Male	8 (44.4)
Female	10 (55.6)
Symptom	
Upper abdominal pain	3 (16.7)
Abdominal distention	4 (22.2)
Melena	5 (27.8)
Asymptomatic	6 (33.3)
Lesions	
Median size, cm (range)	2.0 (1.3-5.0)
Location of lesion	
Duodenal bulb	11 (61.1)
Descending junction of duodenal bulb	4 (22.2)
Descending duodenum	3 (16.7)
Origination of lesion	
Submucosal layer	9 (50.0)
Muscularis propria layer	9 (50.0)

All 18 patients had intraoperative perforations. The median perforation size was 1 cm in diameter (range, 0.5-3.0 cm). The wound was closed with an OTSC in 6 cases, an OTSC + a titanium clip in 1 case, and an OTSC + a titanium clip + an endoloop in 5 cases. The rate of successful intraprocedural perforation closure was 100%.

Seventeen patients had minor intraoperative bleeding. The remaining 1 patient, who had a tumor originating from the lamina propria, growing mainly out of the lumen, with rich blood supply, had considerable amount of bleeding during the procedure. All patients were treated with hot biopsy forceps to achieve hemostasis during the procedure.

None of the patients developed delayed bleeding or perforation. Seven patients had postoperative abdominal infections and were administered intensive antibiotic therapy. Among the 7 patients, 1 patient developed an abscess in the right iliac fossa that improved after puncture and drainage, while another patient developed septic shock and received peritoneal lavage and underwent distal subtotal gastrectomy with duodenal bulb resection. All 18 patients recovered and were discharged. The mean time taken to resume normal diet after the procedure was 6.5 d. The mean postoperative hospital stay was 9.5 d.

Follow-up

The median follow-up period after the procedure was 27 mo (range, 15-66 mo). No residual or recurrent lesions, duodenal stenosis, or adhesions were detected during the follow-up period in any of the patients.

DISCUSSION

Currently, endoscopic resection of duodenal SELs is a challenging procedure with a high risk of perforation. Published studies about endoscopic resection of duodenal SELs and endoscopic methods for management of perforations are limited[1,2,8,9]. In this study, we used OTSCs to close perforations in 18 patients. The rate of complete removal of duodenal SELs and successful perforation closure was 100%. No delayed bleeding or perforation occurred in any of the patients. This suggests that the use of OTSCs can effectively close perforations following endoscopic resection of duodenal

Table 2 Therapeutic outcome and adverse events of endoscopic resection for duodenal subepithelial lesions, *n* (%)

Therapeutic outcome and adverse events	
Complete resection	18 (100)
Histology diagnosis	
Brunner's adenoma	1 (5.6)
Heterotopic pancreas	7 (38.9)
GIST	7 (38.9)
Very low risk	1 (5.6)
Low risk	6 (33.3)
Neuroendocrine tumors	3 (16.6)
Complication	
Delayed perforation	0 (0)
Delayed bleeding	0 (0)
Postoperative infection	7 (38.9)
Mean time of diet recovery after the procedure, d (range)	6.5 (2-14)
Mean hospital stay after the procedure, d (range)	9.5 (4-18)
Median follow-up period, mo (range)	27 (15-66)

GIST: Gastrointestinal stromal tumor.

SELs when performed by an experienced endoscopist.

The clinical manifestations of duodenal SELs are nonspecific and related to the location, size, growth pattern, presence of mucosal ulcers, and invasion or compression of adjacent organs. Most duodenal lesions have no symptoms and are usually found incidentally during endoscopic examinations. Clinical symptoms such as gastrointestinal bleeding, abdominal pain, and abdominal distention may occur when the lesion is very large or when an ulcer develops on the surface of the lesion.

Though most duodenal SELs, such as lipomas, Brunner's adenomas, heterotopic pancreas, and cysts, are benign, some including neuroendocrine tumors and GISTs are potentially malignant[1-3]. Endoscopy and EUS are of great value in the diagnosis of duodenal SELs; however, they may be difficult to diagnose on some occasions. Patients with duodenal SELs can be monitored by endoscopy, especially for asymptomatic tumors that lack high-risk features as identified by EUS[10]. However, surveillance using only endoscopy may increase the risk of delayed diagnosis of a malignancy[11]. Furthermore, the difficulty of the operation and risk of combined evisceration will increase if the lesion is large. In such cases, removal of the lesion is inevitable.

Traditional surgical approaches for duodenal lesions, including pancreatoduoden-ectomy and limited resection, are traumatic and may result in serious complications, such as bleeding, perforation, and infection. Considering these potential risks associated with surgical therapy, endoscopic treatment is used as an alternative choice, which may be safer, more effective, and is minimally invasive. However, endoscopic resection of duodenal SELs is still considered to be a challenging procedure because the duodenal lumen is narrow and the initial part (ball to lower part) is an anti-c loop, which renders the endoscope unstable. Moreover, the abundant blood vessels and Brunner glands in the submucosa of the duodenum make it difficult to lift the mucosa after injection. In addition, compared to other parts of the gastrointestinal tract, the muscularis propria layer of the duodenum is soft and thin, and the posterior wall lacks the serosal layer; therefore, perforation can occur easily during or after the endoscopic resection of duodenal lesions, especially duodenal SELs[8]. The incidence of intrapro-cedural perforations in duodenal ESD has been reported to range from 6.7%-36.6%, and is 0%-14.3% in delayed perforations[1,4-7]. Moreover, emergency operations have been performed in 3.3%-25.0% of patients due to intraprocedural uncontrollable perforation or delayed perforation[1,4-7]. Our previous study reported that the perforation rate of endoscopic resection of duodenal SELs in our hospital was 7.4%[8].

Perioperative perforation associated with endoscopic therapy was previously considered a serious complication that usually requires surgery. With the development of endoscopic suture instruments and techniques, patients with iatrogenic gastrointestinal perforation can be successfully managed using endoscopic methods and conservative treatment without surgical intervention[12,13]. Thus, most perforations related to endoscopic treatment are no longer life-threatening complications. However, endoscopic closure of perforations after endoscopic resection of duodenal SELs remains a great challenge.

In the past, titanium clips were used for endoscopic closure of gastrointestinal perforations, especially for small acute perforations (< 5 mm). However, a titanium clip has a narrow wingspan and lacks the ability to approximate adequately the margins of the defect. Consequently, the rate of leakage after repairing a large perforation of more than 1 cm is high as the seal is confined to the surface rather than the full-thickness of the mucosa[14-16]. An OTSC has a greater holding strength[16,17]; it can clamp the entire wall of the lumen and grasp more tissue. The design can manage full-thickness perforations with diameters of up to 3 cm[14]. Moreover, the gap between the teeth of an OTSC allows blood to pass through to avoid tissue necrosis. The advantage of an OTSC lies in its ease of use, ability to close defects between 1 and 3 cm with a single clip, and safety, which allows endoscopists to deal effectively with acute perforations immediately after identification[18]. Thus, OTSCs are easy to operate and can effectively shorten operation times. Moreover, The European Society of Gastrointestinal Endoscopy recommends OTSCs for endoscopic closure of iatrogenic perforations[18]. According to a systematic review, the success rate of using OTSCs to manage perforations was 85.3%, while 9.4% of patients still required surgical intervention after an OTSC placement to achieve complete closure [19]. Voermans *et al*[14] reported 12 cases of duodenal perforation that were treated with OTSCs, nine of which were effectively closed, with an overall success rate of 75%. In our study, the rate of successful closure of intraprocedural perforations was 100%. However, we have also used a titanium clip in 1 case and a titanium clip along with an endoloop in 5 cases. It seems that if the perforation is larger than 1.5 cm, using an OTSC alone may fail to achieve complete closure. We speculate that the combination of OTSC, titanium clip, and endoloops may be more effective. Given that the duodenal lumen is narrow, caution should be exercised to avoid grasping too much tissue to avoid further narrowing of the lumen while deploying the OTSC in the duodenum. In our study, no duodenal stenosis was detected in any patient during the follow-up period.

The duodenum is exposed to pancreatic juices and bile, causing delayed perforations more likely to occur after endoscopic resection of duodenal lesions. Complete closure of the wound facilitates prevention of delayed perforation[6,7,17]. Due to its the strong tightening force and the gap between its teeth, an OTSC can manage to close full-thickness duodenal perforations and avoid tissue necrosis, which effectively reduces the occurrence of delayed perforations. A carbon dioxide pump is also recommended to use with endoscopic treatment, especially when a perforation occurs. The use of gastrointestinal decompression after endoscopic closure of perforation is helpful for the absorption of gas and liquid in the intestinal cavity. It also reduces tension in the wound, and promotes wound healing, which can reduce the incidence of delayed perforations. In this study, we placed a jejunal nutrition tube next to the wound and a gastrointestinal decompression tube to extract gas and digestive juice. Thereafter, none of the patients developed delayed perforations.

The duodenum is an interperitoneal organ, most of which is located in the retroperitoneum. After perforation or full-thickness resection, digestive fluid from the duodenum (mainly bile and pancreatic juice) flows into the peritoneal cavity or retroperitoneal cavity, which may cause serious abdominal or retroperitoneal infection. In our study, 7 patients (38.9%) had postoperative abdominal infection, including 1 who developed an abscess in the right iliac fossa and another who developed septic shock. Severe infection in the 2 cases were considered to be caused by long operation times and large amounts of digestive juice entering the abdominal cavity. Timely conversion of the endoscopic procedure to surgery or combining with laparoscopy when the resection is found to be difficult may help avoid such complications.

Due to their strong holding strength, OTSCs are more difficult to detach spontaneously from the mucosa than normal titanium clips. The OTSC is made of nitinol, which has favorable biocompatibility. Thus, this device is considered a permanent implanted material. However, OTSCs should be removed in the following circumstances: (1) Poor healing; (2) OTSC misplacement; (3) Repeat biopsy/therapy or further treatment; (4) Adverse events after OTSC implantation, such as ulcers and

stenosis of the digestive tract; (5) Removal after recovery; and (6) Patient's wishes[20]. In our study, there were no such indications for removal. During the follow-up period, OTSCs detached spontaneously in most cases.

This study has a few limitations. First, this was a single-center retrospective study and the sample size was relatively small; therefore, selection bias may have been present. Second, since this was a retrospective study, it lacked randomized and control samples. Third, our institution is a tertiary endoscopic center in Zhejiang Province where the procedures were performed by an experienced operator; thus, the results of this study may not be applicable to all other endoscopic centers.

CONCLUSION

Closing of perforations after endoscopic resection of duodenal SELs with OTSCs is an effective and reasonably safe therapeutic method. However, this procedure should be performed by an experienced endoscopic team. If the endoscopic procedure fails or the postoperative complications are difficult to manage, the patient should be planned to undergo surgery immediately.

ARTICLE HIGHLIGHTS

Research background
Currently, endoscopic resection of duodenal subepithelial lesions (SELs) is a challenging procedure with a high risk of perforation.

Research motivation
It is importance to deal with perforation after endoscopic resection of duodenal SELs. However, so far, there were few reports on endoscopic methods for management of perforations.

Research objectives
We aim to evaluate the effectiveness and safety of over-the-scope clip (OTSC) in the closing the perforation after endoscopic resection of duodenal SELs.

Research methods
This was a retrospective study. We collected data of 18 consecutive patients who were treated with OTSCs to close the perforation after endoscopic resection of duodenal SELs and analyzed the rate of complete resection, closure of intraprocedural perforation, delayed bleeding, delayed perforation, and postoperative infection.

Research results
All the perforations after endoscopic resection of duodenal SELs were successfully closed. No delayed bleeding or perforation occurred in any of the patients.

Research conclusions
OTSC can effectively and safely close the perforations after endoscopic resection of duodenal SELs by an experienced endoscopist.

Research perspectives
We need to expand the sample size to confirm further the effectiveness and safety of OTSC in closing the perforation after endoscopic resection of duodenal SELs. In addition, the long-term outcome of OTSC should be observed by extending the follow-up time.

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Retrospective Study

Hepatic perivascular epithelioid cell tumor: Clinicopathological analysis of 26 cases with emphasis on disease management and prognosis

Shan Zhang, Pan-Pan Yang, Yu-Chen Huang, Hong-Chun Chen, De-Li Chen, Wen-Tian Yan, Ning-Ning Yang, Yuan Li, Nan Li, Zhen-Zhong Feng

ORCID number: Shan Zhang 0000-0001-7891-6906; Pan-Pan Yang 0000-0003-3633-6844; Yu-Chen Huang 0000-0002-7740-5634; Hong-Chun Chen 0000-0002-7751-5390; De-Li Chen 0000-0002-4593-9295; Wen-Tian Yan 0000-0001-5260-4701; Ning-Ning Yang 0000-0003-0691-7293; Yuan Li 0000-0002-8178-5131; Nan Li 0000-0002-5522-4252; Zhen-Zhong Feng 0000-0001-9385-3157.

Shan Zhang, Department of Pathology, The Second People's Hospital of Hefei, Hefei 230011, Anhui Province, China

Pan-Pan Yang, Zhen-Zhong Feng, Department of Pathology, The Second Affiliated Hospital of Anhui Medical University, Hefei 230601, Anhui Province, China

Yu-Chen Huang, Hong-Chun Chen, Wen-Tian Yan, Ning-Ning Yang, Nan Li, Department of Pathology, The First Affiliated Hospital of Bengbu Medical College, Bengbu 233000, Anhui Province, China

De-Li Chen, Department of Gastrointestinal Surgery, The First Affiliated Hospital of Bengbu Medical College, Bengbu 233000, Anhui Province, China

Yuan Li, Department of Pathology, The First Affiliated Hospital of University of Science and Technology of China, Anhui Provincial Hospital, Hefei 230001, Anhui Province, China

Corresponding author: Zhen-Zhong Feng, PhD, Professor, Department of Pathology, The Second Affiliated Hospital of Anhui Medical University, No. 678 Furong Road, Hefei 230601, Anhui Province, China. fzz18297301626@163.com

Abstract

BACKGROUND
Perivascular epithelioid cell tumor (PEComa) is an uncommon tumor of mesenchymal origin. Cases of PEComa in the liver are extremely rare.

AIM
To analyze the clinicopathological features and treatment of hepatic PEComa and to evaluate the prognosis after different treatments.

METHODS
Clinical and pathological data of 26 patients with hepatic PEComa were collected. All cases were analyzed by immunohistochemistry and clinical follow-up.

RESULTS
This study included 17 females and 9 males, with a median age of 50 years.

Lesions were located in the left hepatic lobe in 13 cases, in the right lobe in 11, and in the caudate lobe in 2. The median tumor diameter was 6.5 cm. Light microscopy revealed that the tumor cells were mainly composed of epithelioid cells. The cytoplasm contained heterogeneous eosinophilic granules. There were thick-walled blood vessels, around which tumor cells were radially arranged. Immunohistochemical analysis of pigment-derived and myogenic markers in PEComas revealed that 25 cases were HMB45 (+), 23 were Melan-A (+), and 22 SMA (+). TFE3 and Desmin were negative in all cases. All the fluorescence *in situ* hybridization samples were negative for *TFE3* gene break-apart probe. Tumor tissues were collected by extended hepatic lobe resection or simple hepatic tumor resection as the main treatments. Median follow-up was 62.5 mo. None of the patients had metastasis or recurrence, and there were no deaths due to the disease.

CONCLUSION
Hepatic PEComa highly expresses melanin and smooth muscle markers, and generally exhibits an inert biological behavior. The prognosis after extended hepatic lobe resection and simple hepatic tumor resection is semblable.

Key Words: Hepatic tumor; Perivascular epithelioid cells; PEComa; Immuno-histochemistry; Treatment; Prognosis

Core Tip: Hepatic perivascular epithelioid cell tumor (PEComa) exhibits an inert biological behavior, and its diagnosis, treatment, and follow-up are challenging. Our study revealed that there was no difference in the prognosis between simple resection of liver tumor and extended resection of liver lobe. Optimal surgical resection currently is the best treatment option, and radiotherapy, chemotherapy, and immunotherapy may become more effective in future. The number of cases in the current retrospective study was limited by the rarity of hepatic PEComa. Therefore, further multicenter, larger-cohort studies are warranted to investigate the clinicopathological features and biological behavior of hepatic PEComa.

INTRODUCTION

Perivascular epithelioid cells were first described in 1992 by Bonetti *et al*[1]. In 2013, the World Health Organization[2] defined perivascular epithelioid cell tumor (PEComa) as "a mesenchymal tumor, which shows a local association with the vessel wall and usually expresses melanocyte markers and smooth muscle markers." Bonetti *et al*[1] were the first to propose the concept of a PEComa family, which includes angiomyolipoma, clear cell sugar tumor of the lung, lymphangioleiomyomatosis, and a group of histologically and immunophenotypically similar tumors that include primary extrapulmonary sugar tumor, clear cell myomelanocytic tumor, and abdomin-opelvic sarcoma of perivascular epithelioid cells. PEComas are mainly composed of eosinophilic and clear epithelioid cells, which are usually arranged in nests of different sizes associated with blood vessels[3,4]. The diagnosis of PEComa relies on its pathological features, including epithelioid cellular shapes with ample clear to eosino-philic cytoplasm, and in some cases, arrangement around thick-walled blood vessels and immunohistochemical phenotypes, including melanocyte and smooth muscle markers[1,4,5]. Cases of PEComa in the liver are extremely rare[6], and surgical resection currently is the most effective therapeutic strategy to cure patients or prolong

the survival period. In this study, the clinical and pathological features, immunohisto-chemical phenotypes, and information on treatment modalities of 26 cases of hepatic PEComa were collected, and the effects of different surgical methods on prognosis were evaluated to provide information for the guidance of clinical treatment.

MATERIALS AND METHODS

Patient selection

The study included 17 women and 9 men who were diagnosed with hepatic PEComa for the first time. Tumor tissue samples were collected at the time of diagnosis between January 2010 and December 2018 at the First Affiliated Hospital of Bengbu Medical College (Anhui Province, China). None of the patients received preoperative radio- or chemo-therapy. Sixteen patients underwent extended hepatic lobe resection, eight underwent simple hepatic tumor resection, and two received the oral mTOR inhibitor sirolimus. None of the 26 patients had metastasis or recurrence, and there were no deaths due to the disease. Only two patients with extended liver lobectomy had a poor prognosis (one had postoperative pain in the liver area, and the other was diagnosed with liver cancer 2 years after surgery). Informed consent was obtained from all patients. The study protocol was approved by the ethics committees of the hospitals partaking in this study.

Imageological examination

Imaging data of all patients were collected and reviewed by two experienced physicians who analyzed the imaging characteristics of the patients.

Histological observation and immunohistochemical analysis

Two experienced pathologists reviewed hematoxylin and eosin-stained sections of each tissue sample, marked the representative regions of tissue blocks, and assessed the following histological features: Tumor boundary (infiltration), tumor cell structure (trabecular and nested), tumor cell type (epithelial and fusiform), cytological features (cytoplasm and nucleus), nuclear features (atypical and pleomorphic), presence of pleomorphic tumor cells, and tumor necrosis.

Immunohistochemical staining was conducted on 4-µm-thick serial PEComa tissue sections using the standard ElivisionTM Plus/HRP detection system (Fuzhou Maixin Biotechnology, Fuzhou, China) and DAB substrate, generating a brown color. The antibodies, clones, dilutions, and pretreatment conditions used, as well as the positively stained sites, are listed in Table 1. Serial sections were incubated in parallel with rabbit IgG instead of the primary antibody as a negative control. Immunore-activity was graded according to the percentage of positive tumor cells (0, negative; 1+, 1%-5%; 2+, 6%-25%; 3+, 26%-50%; 4+, 51%-100%), and tumor cell immunore-activity was also semi-qualitatively graded: Weak, heterogeneous, or strong[7,8]. For calculation of IHC totals, a score of 1+ with weak, heterogeneous, or strong staining was considered positive for all antibodies except TFE3. A minimum of 3+ was required for TFE3 immunopositivity[8].

Fluorescence in situ hybridization

FISH was performed on paraffin-embedded tissue sections with a thickness of 4 µm and labeled with a *TFE3* gene break-apart probe (Guangzhou Anbiping Medical, Guangzhou, Guangdong Province, China). For probe preparation, *TFE3* gene was labeled with green fluorescence on the centromere side and red fluorescence on the telomere side. FISH interpretation criteria are as follows: The positive pattern for *TFE3* translocation should be 1 red, 1 green, and 1 fusion (yellow) signal in females, and 1 red, 1 green, and 1 negative signal in males; the pattern for intact *TFE3* alleles should be 2 fusion (yellow) signals in females and 1 fusion (yellow) signal in males. When the distance between the red and green signals exceeds 1 fusion signal size, it is interpreted as a red-green signal separation. A case was scored as positive if at least 10% of 100 scored nuclei showed a split signal pattern.

Table 1 Antibodies used in this study

Antigen	Clone	Dilution	Antigen retrieval	Localization
HMB-45	HMB-45	1:400	None	Cytoplasm
Melan-A	A103	1:200	Citrate buffer pressure cook	Cytoplasm
SMA	1A4	1:20000	None	Cytoplasm
Desmin	D33	1:500	None	Cytoplasm
S100 protein	Polyclonal	1:4000	Citrate buffer pressure cook	Cytoplasm/nucleus
Hepatocyte	OCH1E5	1:1000	Citrate buffer pressure cook	Cytoplasm
Vimentin	V9	1:200	Citrate buffer pressure cook	Cytoplasm
CD34	QBEnd/10	1:500	Citrate buffer pressure cook	Cell membrane
TFE-3	MRQ-0663	1:500	ETDA buffer pressure cook	Nucleus
Ki-67	MX006	1:200	Citrate buffer pressure cook	Nucleus

RESULTS

Clinical features

The clinical and pathological data for all 26 cases are summarized in Table 2. We enrolled 26 patients, including 17 females and 9 males. The median patient age was 50 years (range, 26–77 years). Of the 26 patients, 23 had liver-occupying lesions, 2 had hepatic hemangioma, and 1 had hepatic hamartoma. Six patients had a history of liver disease (cysts, hamartoma, or hemangioma). The most common site of tumors was the left hepatic lobe. Sixteen patients underwent extended hepatic lobe resection, eight underwent simple hepatic tumor resection, and two were treated only with the mTOR inhibitor sirolimus (both patients were treated for 8 mo). The clinical symptoms of hepatic PEComa were non-specific. Most patients were admitted to one of our hospitals because of space-occupying lesions in the liver during medical examination, nausea, vomiting, loss of appetite, or weight loss. During physical examination, the abdomen was soft, with no tenderness or rebound tenderness, occasional contact with the ribs at the liver margin, and no pain in the liver area. Some patients experienced compression pain under the ribs and xiphoid, or in the right abdomen when the tumor involved the caudate lobe, or in the right kidney.

Imaging findings

B-ultrasound usually revealed strong echoes in the liver, the boundary was clear, and the internal echo was uneven, suggesting that the liver had substantial space-occupying lesions (data not shown). Plain computed tomography (CT) scans commonly revealed an irregular soft tissue density (Figure 1A). Enhanced scanning in the arterial phase revealed obvious enhancement of the mass edge and of central heterogeneity (Figure 1B). Portal vein scanning revealed a low mass density (Figure 1C). Magnetic resonance imaging (MRI) revealed a solid cystic space in the liver, and tumors had clear boundaries and uneven internal signal (data not shown).

Macroscopic features

The median tumor diameter was 6.5 cm (range, 0.5-13.0 cm). PEComa tumors were located in the liver parenchyma and were round or oval. The surface was smooth and occasionally highlighted the surface of the liver. The boundary was clear and appeared to be enveloped. Tumors did not invade the surrounding tissue. The cut surface was solid and grayish yellow, had a slightly hard texture, and showed loose necrotic tissue in the center. The liver tissue surrounding the tumor was normal, and the lymph nodes in the hilar region were not swollen. Focal hemorrhage and necrosis were seen in two cases.

Microscopic features

Microscopically, the tumor cells were clearly distinct from normal liver cells, and were largely composed of proliferating epithelioid cells and spindle cells, nested in trabeculae or lamellae. In most cases, the tumor cell nest was surrounded by capillaries. Tumor cells were arranged radially around the thick-walled blood vessels (Figure 2A). Tumor cells were polygonal and cytoplasm was translucent, with hetero-

Table 2 Clinicopathological features of the 26 cases of hepatic PEComa

No.	Sex/age (yr)	Tumor location	Tumor size (cm)	First diagnosis	Treatment	Follow-up (mo) and prognosis
1	F/40	Left lobe	2.5	Left lobe occupying lesion	Left hepatic tumor simple resection	91, favorable prognosis
2	M/57	Left lobe	7.5	Left lobe occupying lesion	Left hepatic tumor simple resection	80, favorable prognosis
3	F/58	Left lobe	8.5	Left lobe occupying lesion	Left hepatic tumor simple resection	79, favorable prognosis
4	F/48	Right lobe	8.0	Right lobe occupying lesion	Right hepatic tumor simple resection	69, favorable prognosis
5	F/64	Right lobe	7.0	Right lobe occupying lesion	Right hepatic tumor simple resection	66, favorable prognosis
6	M/72	Right lobe	8.0	Right lobe occupying lesion	Right hepatic tumor simple resection	59, favorable prognosis
7	F/26	Right lobe	3.0	Right hepatic hamartoma	Extended hepatic lobe resection	55, favorable prognosis
8	M/47	Right lobe	6.5	Right lobe occupying lesion	mTOR inhibitor-sirolimus	51, favorable prognosis
9	F/47	Left lobe	5.5	Left lobe occupying lesion	Extended hepatic lobe resection	25, favorable prognosis
10	M/72	Right lobe	8.0	Right lobe occupying lesion	Extended right hepatic lobe resection	57, favorable prognosis
11	F/56	Right lobe	8.0	Right lobe occupying lesion	mTOR inhibitor-sirolimus	32, favorable prognosis
12	F/54	Right lobe	13.0	Left lobe occupying lesion	Extended left hepatic lobe resection	99, favorable prognosis
13	F/41	Caudate lobe	8.0	Caudate lobe occupying lesion	Caudate hepatic tumor simple resection	98, favorable prognosis
14	F/46	Left lobe	2.0	Left lobe occupying lesion	Extended left hepatic lobe resection	99, favorable prognosis
15	F/54	Right lobe	8.0	Right hepatic hemangioma	Extended Rright hepatic lobe resection	84, favorable prognosis
16	F/41	Caudate lobe	6.0	Caudate lobe occupying lesion	Extended caudate hepatic lobe resection	87, hepatic pain often occurs after discharge
17	M/45	Right lobe	0.5	Right hepatic hemangioma	Extended hepatic lobe resection	85, favorable prognosis
18	F/66	Right lobe	5.5	Right lobe occupying lesion	Extended hepatic lobe resection	59, favorable prognosis
19	F/43	Right lobe	2.8	Right lobe occupying lesion	Extended hepatic lobe resection	47, favorable prognosis
20	F/41	Left lobe	5.0	Left lobe occupying lesion	Extended hepatic lobe resection	49, reoperation for liver cancer in 2017
21	M/52	Left lobe	7.5	Left lobe occupying lesion	Left hepatic tumor simple resection	48, favorable prognosis
22	F/48	Right lobe	9.5	Right lobe occupying lesion	Extended right hepatic lobe resection	71, favorable prognosis
23	M/58	Left lobe	4.0	Left lobe occupying lesion	Left hepatic tumor simple resection	70, favorable prognosis
24	M/77	Left lobe	4.0	Left lobe occupying lesion	Extended left hepatic lobe resection	47, favorable prognosis
25	M/62	Left lobe	6.5	Left lobe occupying lesion	Extended left hepatic lobe resection	36, favorable prognosis
26	F/45	Left lobe	3.0	Left lobe occupying lesion	Extended left hepatic lobe resection	35, favorable prognosis

geneous eosinophilic particles; tumor nuclei were round or oval, nucleoli were
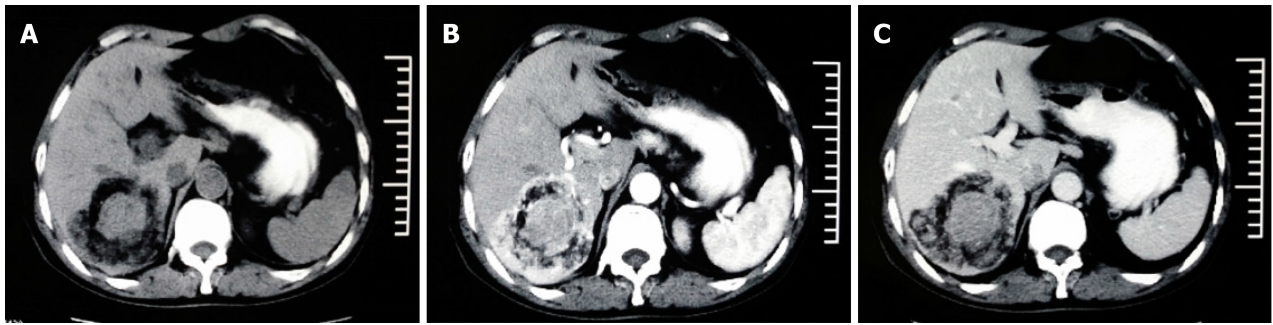
Figure 1 Computed tomography scans of the right hepatic lobe of a 72-year-old male patient with PEComa (patient 10). A: Plain computed tomography scan showing an irregular soft tissue density shadow; B: Enhanced scan showing obvious enhancement of the mass margin and of central heterogeneity in the arterial phase; C: Portal vein scan showing a low mass density.

obvious, chromatin was sparse, part of the cells were heteromorphic, and mitotic figures were not common. Collagen fibers were observed in the interstitium and were generally feathery, and a few fibers were accompanied by hemorrhage and necrosis (Figure 2B).

Immunohistochemistry findings are summarized in Table 3. Of the 26 cases, 25 were HMB45 (+), usually with multifocal or diffuse distribution and occasionally, with scattered distribution (Figure 2C), 23 were Melan-A (+) (Figure 2D), 22 were SMA (+) (Figure 2E), 20 were VIM (+), and 12 were S-100 (+). Only three cases showed focal staining (1%-5%) for TFE3. All tumors were desmin (–) (Figure 2F). The positive rate for Ki-67 was < 10%. All cases expressed at least one smooth muscle or melanocyte marker. FISH showed that no abnormal *TFE3* separation signal was found in 26 cases of hepatic PEComa (Figure 3).

Treatment and follow-up

Sixteen patients underwent extended hepatic lobe resection, eight underwent simple hepatic tumor resection, and two were treated with the mTOR inhibitor sirolimus. During a follow-up period of 25 mo to 99 mo, none of the 26 patients had metastasis or recurrence, and there were no deaths due to the disease. Only two patients with extended liver lobectomy had a poor prognosis (one had postoperative pain in the liver area, and the other was diagnosed with liver cancer 2 years after surgery). There was no difference in patient prognosis between the two surgical treatment methods, and long-term follow-up indicated that the patients went into remission.

DISCUSSION

Hepatic PEComa is a rare mesenchymal tumor derived from pericytes. Ultrasound, CT, and MRI are commonly used for preoperative diagnosis of PEComa. On contrast-enhanced CT, PEComa is characterized by vascular proliferation and arteriovenous connections[5,9,10]. MRI scans have revealed significant enhancement in PEComa in the arterial phase, but not in the portal venous and delayed phases[10]. Contrast-enhanced ultrasonography is another commonly used diagnostic method, in which the contrast agent characteristically reaches the tumor rapidly and drains the arterial blood rapidly to the vein[11]. However, due to the different proportions of smooth muscle cells, adipose tissue, blood vessels, and rare tumors, the accuracy of preoperative diagnosis is currently low. In our study, only one patient was diagnosed with hepatic PEComa before undergoing surgery.

Martignoni *et al*[12] defined PEComa as a tumor that is composed mainly of epithelioid cells and is closely associated with dilated blood vessels and contains eosinophils, but not fat cells or disordered blood vessels. The final diagnosis of PEComa currently depends on pathological features and immunohistochemical analysis. Hepatic PEComa is mainly composed of proliferating epithelioid cells and spindle cells. The tumor cells are polygonal, have translucent cytoplasm, and contain eosinophilic particles, and thick-walled blood vessels are visible in the tumors. Epithelioid cells are arranged radially around thick-walled blood vessels. Feather-like collagen fibers are visible. Nearly all PEComas have specific immunological characteristics, with melanocyte markers (*e.g.*, HMB-45 and/or melan-A) and smooth muscle markers (*e.g.*, SMA) being strongly expressed[11,13], whereas desmin, hepatocyte-

Table 3 Immunohistochemical features of the 26 cases of hepatic PEComa

Target protein	Positive cases (*n*)/total	% Positive
HMB45	25/26	96.2
Melan-A	23/26	88.5
SMA	22/26	83.6
Desmin	1/26*	3.8
S100	14/26	53.8
Hepatocyte	9/26	34.6
Vimentin	20/26	76.9
CD34	18/26	69.2
TFE3	0/26	0
Ki-67 (> 10%)	1/26	3.8

Weakly positive (1%-5%), only scattered cells.

specific antigen, and TFE3 are generally negative. In this study, 25 cases were HMB-45 (+), 17 were SMA (+), and only 3 showed focal staining (1%-5%) for TFE3.

TFE3 is a member of the MiTF family of transcription factors. A recent study[14] showed that *TFE3* gene rearrangements occur in approximately 14% of PEComas. Similar to other *TFE3* translocation-associated tumors, TFE3 (+) PEComa usually exhibits an acinar structure and epithelioid cell morphology, shows aggressive biological behavior, and has a poor prognosis. *PSF-TFE3* gene fusion has been detected in gastrointestinal tract PEComa, but fusion partners in other cases remain unknown [15]. In this study, TFE3 expression was weak and detected in only three patients with small tumors and typical morphological PEComa images, and was associated with a low malignancy and good prognosis. Moreover, no break-apart of the *TFE3* gene was detected by FISH method. Whether there is a *TFE3* fusion gene still needs to be confirmed by subsequent studies. This suggests that liver PEComa may be less malignant than PEComas in other organs.

PEComas are mainly benign tumors[16] that usually do not recur after surgical resection; however, some are malignant, and their biological behavior has not been fully elucidated. In 2005, Folpe *et al*[17] reviewed 26 cases of PEComa of soft tissue and gynecological origin, and suggested to classify PEComa into benign, uncertain malignant potential, and malignant. Further, the authors proposed seven evaluation criteria for PEComa malignancies: (1) Tumor size > 5 cm; (2) Infiltration and growth into surrounding normal tissue; (3) High nuclear grade; (4) Excessive cells; (5) Mitotic figures in > 1/50 high-power fields; (6) Coagulative necrosis of tumor; and (7) Vascular invasion. PEComas with two or more of these features are considered to be malignant, and tumors with only nuclear polymorphism, multinucleated giant cells, or tumors > 5 cm in size are considered to have malignant potential[18].

Because of the rare disease types and the scarcity of cases, treatment plans for hepatic PEComa can only be developed based on statistical analysis of a small number of cases. Surgical resection currently is the main means of treating hepatic PEComa. In clinical practice, surgical methods are usually selected based on the tumor size and on whether the tumor is benign or malignant. Larger and malignant tumors are removed by extended hepatic lobe resection, whereas simple hepatic tumor resection is used for smaller or benign tumors. In this study, the 26 cases showed clinical and biological manifestations of inertness, and no morphological criteria for malignant PEComa. Sixteen patients underwent extended hepatic lobe resection, eight underwent simple hepatic tumor resection, and two received sirolimus. The survival rate of the patients treated with the three different modalities was good, and there was no significant difference among the treatments. Hepatic pain complications were reported only in a few cases with extended lobe resection. It has been reported that when the tumor diameter is less 5 cm, resection can be suspended or regular follow-up suffices[18].

Current data do not support that chemo- or radio-therapy improves the survival time in patients with PEComa[12]; however, sirolimus is expected to improve outcomes either when used alone or in combination with other treatments[4,10,19,20]. A 31-year-old woman with hepatic PEComa showed a significant reduction in tumor
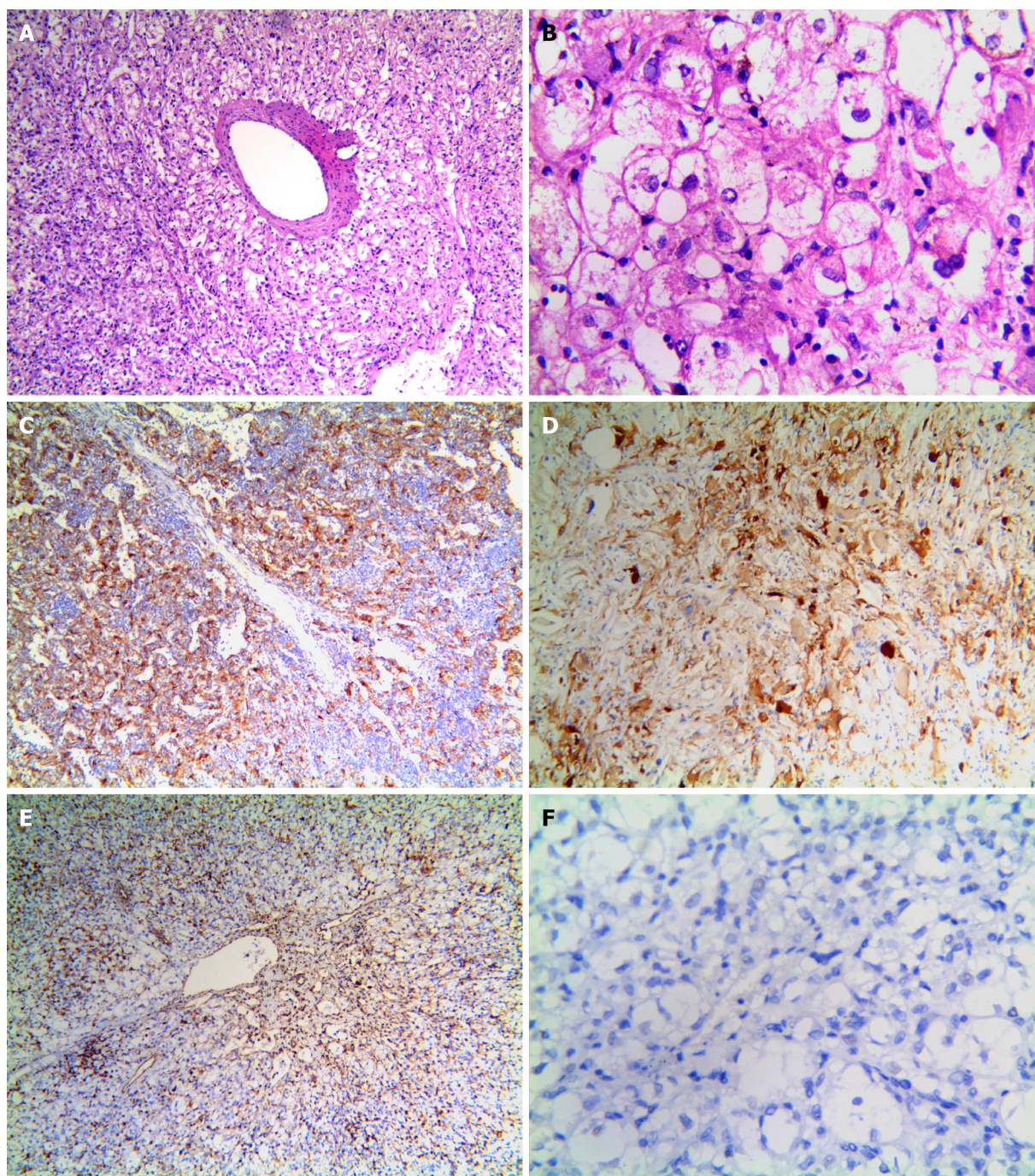
Figure 2 Morphologic appearance of hepatic PEComa. A: Tumor cells consists of proliferating epithelioid cells nested in trabeculae or lamellae and radially arranged around thick-walled vessels (HE, magnification: 100 ×); B: Tumor cells are polygonal, have translucent cytoplasm, and contain uneven eosinophilic granules. Nuclei are round or oval, with a clear nucleolus and sparse chromatin. Interstitial collagen fibers are feathery (HE, magnification, 400 ×); C: Immunoreactivity for HMB45 was detected in the cytoplasm of tumor cells in contrast to normal liver cells, which were negative for this marker (magnification, 100 ×); D: Increased expression of Melan-A was observed in both the cytoplasm and nuclei of carcinoma cells, whereas normal cells displayed lower expression of this marker (magnification, 100 ×); E: Vimentin was detected in the cytoplasm of tumor cells (magnification, 100 ×), whereas normal tissues were negative for this marker (magnification, 100 ×); F: Desmin immunoreactivity was not detected in tumor cells and normal tissues (magnification, 400 ×). Elivision[TM] Plus/HRP was used.

volume after 8 mo of treatment with sirolimus[19]. After subsequent surgical resection, there were no complications and the prognosis was favorable. This suggests that hepatic PEComa has a better prognosis when surgery is combined with chemotherapy [13,14]. In addition, Wagner *et al*[21] treated three patients with PEComa with sirolimus and found that the tumors responded to the drug, suggesting that sirolimus can be used alone or in combination to treat PEComa. Italiano *et al*[22] reported similar efficacy in a number of cases. However, large-scale clinical trials are needed. Numerous previous studies and this study showed that hepatic PEComa displays an inert biological behavior. However, due to the heterogeneous nature of PEComa, the
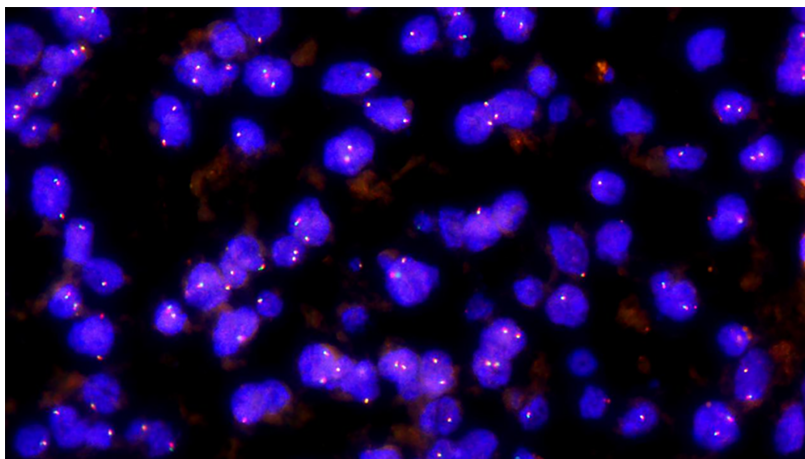
Figure 3 FISH detection of *TFE3* gene break-apart in hepatic PEComa. Most of the tumor cells show fused (yellow) signals, and the distance between the red and green signals is less than 1 fusion signal. For each sample, 100 cells were counted. Only less than 10% of tumor cells showed break-apart signals (magnification, 1000 ×).

existing diagnostic criteria cannot accurately determine the nature of this tumor, which has led to overtreatment in some cases. In addition, because the nature of hepatic PEComa is not entirely clear, there is no standard treatment, and it is difficult to develop an optimal treatment plan. Therefore, clinical observation and follow-up of more cases, and the establishment of a clinical online registration system for hepatic PEComa are needed to provide clinical data for future exploration of the differentiation and distribution of the disease and the development of more accurate diagnostic criteria.

CONCLUSION

Hepatic PEComa is a rare mesenchymal tumor that exhibits an inert biological behavior, and its diagnosis, treatment, and follow-up are challenging. Our study of 26 cases of hepatic PEComa revealed that there was no difference in the prognosis between simple resection of liver tumor and extended resection of liver lobe. Optimal surgical resection currently is the best treatment option, and radiotherapy, chemotherapy, and immunotherapy may become more effective in future[4,9]. The number of cases in the current retrospective study was limited by the rarity of hepatic PEComa. Therefore, further multicenter, larger-cohort studies are warranted to investigate the clinicopathological features and biological behavior of hepatic PEComa.

ARTICLE HIGHLIGHTS

Research background

Perivascular epithelioid cell tumor (PEComa) is an uncommon tumor of mesenchymal origin. Cases of PEComa in the liver are extremely rare.

Research motivation

Cases of PEComa in the liver are extremely rare, and surgical resection currently is the most effective therapeutic strategy to cure patients or prolong the survival period. In this study, the clinical and pathological features, immunohistochemical phenotypes, and information on treatment modalities of 26 cases of hepatic PEComa were collected, and the effects of different surgical methods on prognosis were evaluated to provide information for the guidance of clinical treatment.

Research objectives

We aimed to analyze the clinicopathological features and treatment of hepatic PEComa and to evaluate the prognosis after different treatments.

Research methods

Clinical and pathological data of 26 patients with hepatic PEComa were collected. All cases were analyzed by immunohistochemistry and clinical follow-up.

Research results

This study included 17 females and 9 males, with a median age of 50 years. Lesions were located in the left hepatic lobe in 13 cases, in the right lobe in 11, and in the caudate lobe in 2. The median tumor diameter was 6.5 cm. Light microscopy revealed that the tumor cells were mainly composed of epithelioid cells. The cytoplasm contained heterogeneous eosinophilic granules. There were thick-walled blood vessels, around which tumor cells were radially arranged. Immunohistochemical analysis of pigment-derived and myogenic markers in PEComa tumors revealed that 25 cases were HMB45 (+), 23 were Melan-A (+), and 22 SMA (+). TFE3 and Desmin were negative in all cases. All the FISH samples were negative for *TFE3* gene break-apart probe. Tumor tissues were collected by extended hepatic lobe resection or simple hepatic tumor resection as the main treatments. Median follow-up was 62.5 mo. None of the patients had metastasis or recurrence, and there were no deaths due to the disease.

Research conclusions

Hepatic PEComa is a rare mesenchymal tumor that exhibits an inert biological behavior, and its diagnosis, treatment, and follow-up are challenging. Our study of 26 cases of hepatic PEComa revealed that there was no difference in the prognosis between simple resection of liver tumor and extended resection of liver lobe. Optimal surgical resection currently is the best treatment option, and radiotherapy, chemotherapy, and immunotherapy may become more effective in future.

Research perspectives

The number of cases in the current retrospective study was limited by the rarity of hepatic PEComa. Therefore, further multicenter, larger-cohort studies are warranted to investigate the clinicopathological features and biological behavior of hepatic PEComa.

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ORIGINAL ARTICLE

Retrospective Study

Diagnosis of focal liver lesions with deep learning-based multi-channel analysis of hepatocyte-specific contrast-enhanced magnetic resonance imaging

Róbert Stollmayer, Bettina K Budai, Ambrus Tóth, Ildikó Kalina, Erika Hartmann, Péter Szoldán, Viktor Bérczi, Pál Maurovich-Horvat, Pál N Kaposi

ORCID number: Róbert Stollmayer 0000-0003-4673-7588; Bettina K Budai 0000-0002-3982-7887; Ambrus Tóth 0000-0002-1150-957X; Ildikó Kalina 0000-0002-2647-9123; Erika Hartmann 0000-0001-6073-9286; Péter Szoldán 0000-0002-3808-8541; Viktor Bérczi 0000-0003-4386-2527; Pál Maurovich-Horvat 0000-0003-0885-736X; Pál N Kaposi 0000-0002-7150-3495.

Róbert Stollmayer, Bettina K Budai, Ambrus Tóth, Ildikó Kalina, Viktor Bérczi, Pál Maurovich-Horvat, Pál N Kaposi, Department of Radiology, Medical Imaging Centre, Faculty of Medicine, Semmelweis University, Budapest 1083, Hungary

Erika Hartmann, Department of Transplantation and Surgery, Faculty of Medicine, Semmelweis University, Budapest 1082, Hungary

Péter Szoldán, MedInnoScan Research and Development Ltd., Budapest 1112, Hungary

Corresponding author: Bettina K Budai, MD, Department of Radiology, Medical Imaging Centre, Faculty of Medicine, Semmelweis University, Korányi Sándor st. 2., Budapest 1083, Hungary. budai.bettina@med.semmelweis-univ.hu

Abstract

BACKGROUND

The nature of input data is an essential factor when training neural networks. Research concerning magnetic resonance imaging (MRI)-based diagnosis of liver tumors using deep learning has been rapidly advancing. Still, evidence to support the utilization of multi-dimensional and multi-parametric image data is lacking. Due to higher information content, three-dimensional input should presumably result in higher classification precision. Also, the differentiation between focal liver lesions (FLLs) can only be plausible with simultaneous analysis of multi-sequence MRI images.

AIM

To compare diagnostic efficiency of two-dimensional (2D) and three-dimensional (3D)-densely connected convolutional neural networks (DenseNet) for FLLs on multi-sequence MRI.

METHODS

We retrospectively collected T2-weighted, gadoxetate disodium-enhanced arterial phase, portal venous phase, and hepatobiliary phase MRI scans from patients with focal nodular hyperplasia (FNH), hepatocellular carcinomas (HCC) or liver metastases (MET). Our search identified 71 FNH, 69 HCC and 76 MET. After volume registration, the same three most representative axial slices from all

sequences were combined into four-channel images to train the 2D-DenseNet264 network. Identical bounding boxes were selected on all scans and stacked into 4D volumes to train the 3D-DenseNet264 model. The test set consisted of 10-10-10 tumors. The performance of the models was compared using area under the receiver operating characteristic curve (AUROC), specificity, sensitivity, positive predictive values (PPV), negative predictive values (NPV), and f1 scores.

RESULTS
The average AUC value of the 2D model (0.98) was slightly higher than that of the 3D model (0.94). Mean PPV, sensitivity, NPV, specificity and f1 scores (0.94, 0.93, 0.97, 0.97, and 0.93) of the 2D model were also superior to metrics of the 3D model (0.84, 0.83, 0.92, 0.92, and 0.83). The classification metrics of FNH were 0.91, 1.00, 1.00, 0.95, and 0.95 using the 2D and 0.90, 0.90, 0.95, 0.95, and 0.90 using the 3D models. The 2D and 3D networks' performance in the diagnosis of HCC were 1.00, 0.80, 0.91, 1.00, and 0.89 and 0.88, 0.70, 0.86, 0.95, and 0.78, respectively; while the evaluation of MET lesions resulted in 0.91, 1.00, 1.00, 0.95, and 0.95 and 0.75, 0.90, 0.94, 0.85, and 0.82 using the 2D and 3D networks, respectively.

CONCLUSION
Both 2D and 3D-DenseNets can differentiate FNH, HCC and MET with good accuracy when trained on hepatocyte-specific contrast-enhanced multi-sequence MRI volumes.

Key Words: Artificial intelligence; Multi-parametric magnetic resonance imaging; Hepatocyte-specific contrast; Densely connected convolutional network; Hepatocellular carcinoma; Focal nodular hyperplasia

Core Tip: Our study aimed to assess the performance of two-dimensional (2D) and three-dimensional (3D) densely connected convolutional neural networks (DenseNets) in the classification of focal liver lesions (FLLs) based on multi-parametric magnetic resonance imaging (MRI) with hepatocyte-specific contrast. We used multi-channel data input to train our networks and found that both 2D and 3D-DenseNets can differentiate between focal nodular hyperplasias, hepatocellular carcinomas or liver metastases with excellent accuracy. We conclude that DensNets can reliably classify FLLs based on multi-parametric and hepatocyte-specific post-contrast MRI. Meanwhile, multi-channel input is advantageous when the number of clinical cases available for model training is limited.

INTRODUCTION

Artificial intelligence (AI)-based analysis is one of the fastest evolving fields in medical imaging, thanks to the rapid development of medical physics, electronic engineering, and computer science. The need for computer-aided diagnostics has been further amplified by the continuously increasing demand for imaging studies and the arrival of new modalities that put extra pressure on radiologists while also increasing the probability of diagnostic errors[1]. Meanwhile, deep learning (DL)-based algorithms have started to gain attention among medical researchers, since they provide excellent reproducibility and the ability to quantify aspects of imaging data unobservable to the human eye, resulting in automatically generated statistical reports and predictions, such as the potential of malignancy or metastatic spread and automated volume

assessment, among other uses. Nowadays, AI has become compatible with the full spectrum of imaging modalities and has evolved the capacity to diagnose lesions in various organ systems with greater accuracy than a human reader[2]. The processed data often include two-dimensional (2D) slices or three-dimensional (3D) image volumes; moreover, in the case of magnetic resonance imaging (MRI) studies, the different sequences are condensed into a multi-channel input. Due to their efficiency, convolutional neural networks (CNNs) have replaced other machine learning (ML) approaches in most image classification and segmentation tasks[3,4]. Recently, densely connected CNNs (DenseNets) have become more popular than plain CNN architectures. DenseNets use shortcut connections between the convolutional layers to facilitate gradient flow and optimize the number of trainable parameters. In return, these networks yield improved accuracy and efficiency in medical image classification tasks[5].

Focal liver lesions (FLLs) are common incidental findings during imaging studies, and the work-up often requires further diagnostic procedures, such as dynamic contrast-enhanced ultrasound, computed tomography and liver biopsy. Meanwhile, the excellent soft-tissue contrast, volumetric image acquisition and avoidance of ionizing radiation make multi-phase dynamic post-contrast MRI the primary tool for detection and characterization of liver lesions. The use of hepatocyte-specific contrast agents (HSAs), such as gadoxetic acid and gadobenate dimeglumine, further improves the sensitivity and specificity of the diagnosis of FLLs, as the enhancement characteristics of these lesions in the hepatobiliary phase (HBP) correlates with hepatocyte uptake[6,7]. Additionally, HSA-enhanced MRI is capable of detecting lesions smaller than 10 mm, making it an optimal modality for the early detection of liver metastases (METs)[8].

In the present study, we compared the performance of 2D and 3D-DenseNets in the classification of three types of FLLs, including focal nodular hyperplasia (FNH), hepatocellular carcinoma (HCC) and MET. To guarantee the highest possible prediction rate, we used HSA-enhanced multi-phase dynamic post-contrast MRI scans for the classification task. According to our knowledge, this is the first study to evaluate 2D and 3D-DenseNets for the diagnosis of FLLs and using multi-channel images combining four different MRI sequences. The reporting of this study follows the STROBE Statement checklist of items[9].

MATERIALS AND METHODS

Patient and MRI study selection
In our single-center study, we retrospectively collected multi-phasic MRI studies of patients with FNHs, HCCs or METs, that were acquired using Primovist (gadoxetate disodium), an HSA, from the picture archiving and communication system of the Medical Imaging Centre of our university. As this is a retrospective study, the need for written patient consent was waived by the Institutional Research Ethics Committee. The collected images were acquired between November 2017 and October 2020 using a Philips Ingenia 1.5 T scanner (Cambridge, MA, United States). T2-weighted (T2w) spectral-attenuated inversion recovery (commonly referred to as SPAIR), arterial phase (HAP), portal venous phase (PVP), and HBP scans were collected from each eligible patient for further analysis. Included lesions were either histologically confirmed or exhibited typical characteristics of the given lesion type with MRI. Patients younger than 18 years of age at the time of imaging were excluded from the study. Table 1 contains details of patient demographics, properties of each lesion class, and metastatic lesion origin.

Data preparation and dataset creation
MRI scans were exported as DICOM files, that were then anonymized to remove the patients' social security numbers, birth date, sex, age, body weight, and date of the imaging study. Anonymized PVP and HBP files were resampled and spatially aligned to the corresponding T2w volume using BSpline as a non-rigid registration method *via* an open-source visualization and medical image computing software, called 3D Slicer (www.slicer.org). 3D Slicer was also used for annotation cropping and file conversion [10,11]. Lesions were annotated by cubic regions of interest (referred to as ROIs). The lesions were then cropped from the aligned HAP, PVP, HBP, and T2w volumes using the same ROI. The cropped volumes were saved as NIfTI files, which were then combined into one four-dimensional (4D) file for each lesion (Figure 1). Cropped lesions were randomly sorted into datasets. After 10-10 lesions were added to the test

Table 1 Patient demographics, imaging properties of each lesion class, and details of metastatic lesion origin

Patent properties	FNH	HCC	MET	Total
Number of patients	42	13	14	69
Age in years at imaging, mean ± SD	45 ± 12	66 ± 5	57 ± 10	54 ± 14
Sex				
Male	11	8	8	27
Female	31	5	6	42
Lesion properties				
Number	71	69	76	216
Primary type				
CRC			21	
Leiomyosarcoma			18	
GI adenocc. or cholangiocc.			15	
Breast cc.			11	
Pancreas cc.			7	
Neuroendocrine ileum cc.			3	
Papillary thyroid cc.			1	

cc.: Carcinoma; CRC: Colorectal cancer; FNH: Focal nodular hyperplasia; GI: Gastrointestinal; HCC: Hepatocellular carcinoma; MET: Metastasis; SD: Standard deviation; T: Tesla.
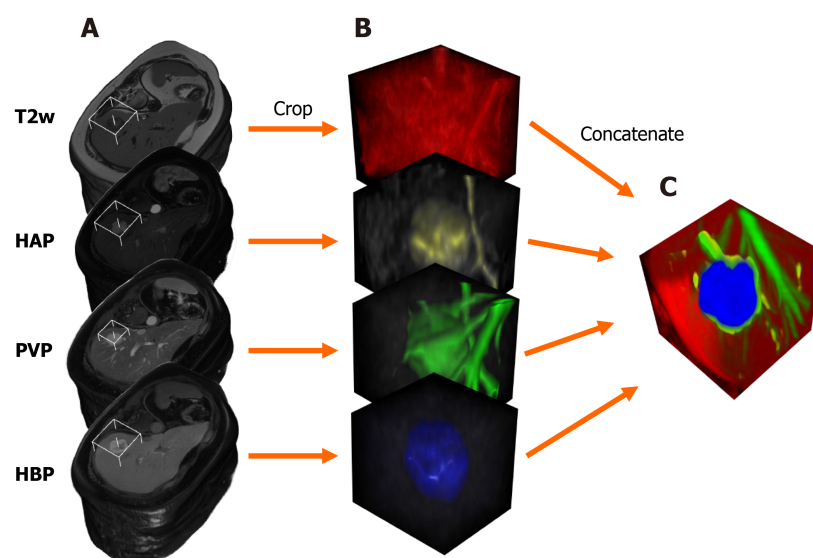

Figure 1 Steps of input data preparation for the three-dimensional densely connected convolutional neural network. A: Three-dimensionally rendered whole volumes at the level of the lesion (indicated by the white frame); B: Cropped cubic volumes containing the lesion; C: The four cubic volumes are concatenated into one four-dimensional file; each volume is represented by a different color. T2w: T2-weighted; HAP: Hepatic arterial phase; PVP: Portal venous phase; HBP: Hepatobiliary phase.

and validation dataset from each class, the remaining tumors were added to the training dataset. NIfTI files were sliced up into axial PNG images. The resulting T2w, HAP, PVP, and HBP PNG files were concatenated (Figure 2) using a custom-written computer program in Python. The training and validation datasets contained three axial slices of each lesion (*i.e.* three most representative axial slices of the NIfTI files), while the test set consisted of only one slice from each lesion.
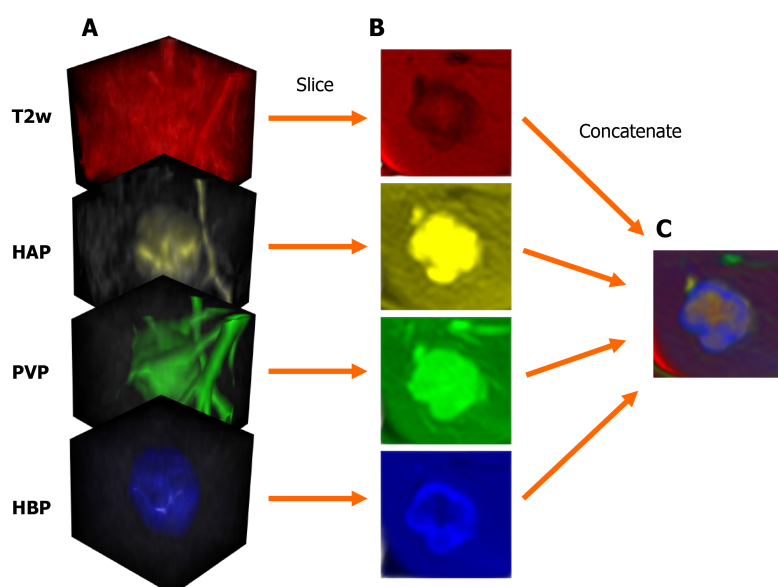
Figure 2 Steps of input data preparation for the two-dimensional densely connected convolutional neural network. A: Cubic magnetic resonance image volumes containing the lesion; B: Axial slices acquired from the cropped volumes; C: The four axial slices are concatenated into one three-dimensional image; each slice is represented by a different color. T2w: T2-weighted; HAP: Hepatic arterial phase; PVP: Portal venous phase; HBP: Hepatobiliary phase.

Data processing, training, and testing

Parameters of concatenated files were modified *via* transform functions. Image pixel intensity was scaled between -1.0 minimum and 1.0 maximum values. Data augmentation transforms were applied to the training samples, including random rotation (70° range along two axes) and zoom (0.7–1.4 scaling) to enrich training data. PNGs were resized to 64 × 64 resolution. Transformed images were converted to tensors (2D images were converted into 3D tensors, with the additional dimension equaling the number of network input channels), which were then fed to DenseNet264 that used 2D convolutional layers[5].

In the case of the 3D-DenseNet264 network, NIfTI voxels were resampled to isovolumetric shape, voxel intensities were rescaled between -1.0 minimum and 1.0 maximum value and NIfTI files were resized to 64 × 64 × 64 spatial resolution. The four NIfTI files were concatenated (T2w, HAP, PVP, HBP) to be used as multi-channel input for the 3D CNN. We used random 90° rotation (along two spatial axes), random 60° rotation (along x and y axes), random zoom (between 0.8 and 1.35), and random flipping on the training samples. MR volumes were converted to 4D tensors (number of channels, x-, y- and z-dimensions) that were used as network input. We used DenseNet264 models through the Pytorch-based open-source Medical Open Network For Artificial Intelligence (*i.e.* MONAI) framework[12]. We used categorical cross-entropy loss to measure the prediction error of the network during training and an Adam optimizer to update model parameters[13]. Networks were trained for 70 epochs. Using a Tesla T4 graphical processing unit, the 2D network was trained for 18 min, while the 3D CNN was trained for 41 min. Validation set area under the receiver operating characteristic curve (AUROC) values were calculated after each epoch, and the model with the highest average AUC value was saved as the final model.

The trained models were used to make predictions on an independent test dataset consisting of 10 lesions from each class. The tumor type with the highest probability, according to the last softmax layer of the convolutional networks, was chosen as the predicted lesion type *via* an argmax function, encoding the predicted diagnosis as 1, while the predicted incorrect classes as 0. Specificity, sensitivity, f1 score, positive predictive value (PPV), negative predictive value (NPV) were calculated for each class based on these outputs.

Classification performance was also measured using AUC values of each class, calculated from the softmax layer probability outputs. DeLong's test was used to determine the statistical significance between the test performance of the 2D and 3D classifiers[14].

RESULTS

The 2D model achieved the highest average validation set AUC after the 46th epoch, while the best average AUC value of the 3D network was reached after the 62nd epoch. These models were saved and then used to make test set predictions (Figure 3).

The finalized 2D and 3D networks were evaluated on the same independent test set, consisting of 10 lesions from each tumor type. On the independent test set, the finalized 2D model achieved 0.9900 [95% confidence interval (CI): 0.9664–1.0000], 0.9600 (95%CI: 0.8786–1.0000) and 0.9950 (95%CI: 0.9811–1.0000) AUC values for FNH, HCC and MET respectively, with an average AUC of 0.9783 (95%CI: 0.9492–1.0000). The finalized 3D model achieved 0.9700 (95%CI: 0.9077–1.0000), 0.9050 (95%CI: 0.7889–1.0000) and 0.9550 (95%CI: 0.8890–1.0000) AUC values for FNH, HCC and MET diagnosis, and an average AUC value of 0.9433 (95%CI: 0.8942–0.9924) on the test dataset (Figure 4). No statistically significant difference was found between the diagnostic performance of the 2D and 3D classifiers based on the ROC curve comparison for the three classes (Z = 0.7007, P = 0.4835 for FNH; Z = 0.7812, P = 0.4347 for HCC; Z = 1.3069, P = 0.1913 for MET). The 2D input data achieved excellent results in the distinction between all three lesion classes, similar to the 3D network (Table 2). Both networks achieved excellent PPV, sensitivity, f1 score, NPV, and specificity values for all three classes. The highest diagnostic accuracy was achieved by both networks for FNH and MET, while both networks demonstrated lower AUC values for HCC (Table 2). PPV, sensitivity, f1 score, specificity and an NPV of 0.9091, 1.0000, 0.9524, 0.9500, 1.000 values were achieved by the 2D model for FNH diagnosis. The 3D network performed FNH classification with similar PPV (0.9000), sensitivity (0.9000), f1 score (0.9000), specificity (0.9500) and NPV (0.9500) values as the 2D network. During HCC classification both the 2D and 3D models reached acceptable metrics with PPVs of 1.000 and 0.8750, sensitivities of 0.8000 and 0.7000, f1 scores of 0.8889 and 0.7778, specificities of 1.000 and 0.9500, lastly NPVs of 0.9091 and 0.8636. For the differentiation of METs from FNHs and HCCs the use of the 2D DenseNet resulted in a PPV of 0.9091, sensitivity of 1.000, f1 score of 0.9524, specificity of 0.9500 and NPV of 1.000, while the 3D DenseNet achieved values of 0.7500, 0.9000, 0.8182, 0.8500 and 0.9444 for PPV, sensitivity, f1 score, specificity and NPV respectively. On average, both the 2D and 3D trained models could distinguish FNHs, HCCs and METs reliably with PPVs of 0.9394 and 0.8417, sensitivities of 0.9333 and 0.8333, f1 scores of 0.9312 and 0.8320, specificities of 0.9667 and 0.9167, NPVs of 0.9697 and 0.9194.

In addition, these results are supported by the extraction of attention maps from the trained models using test set images. We implemented an open-source software (M3d-CAM) to visualize the most important regions for diagnosis-making[15]. The extracted attention maps may correlate with the certainty with which a model classifies FLLs. By marking the areas within images, based on which the model makes a decision, attention maps form optimal bases of training dataset tailoring for certain radiological or other medical computer vision tasks by focusing on image regions that are difficult to analyze for the trained neural network (Figure 5).

DISCUSSION

FLLs are common findings during liver imaging, and the differentiation of benign and malignant types of FLLs is a significant diagnostic challenge, as imaging signs may overlap between different pathologies which can substantially alter the therapeutic decision. Therefore, precise and reproducible differential diagnosis of FLLs is critical for optimal patient management.

Today, the most accurate imaging modality to diagnose FLLs is multi-phase dynamic contrast-enhanced MRI. Extracellular contrast agents (ECAs) are commonly used to perform multi-phase dynamic post-contrast MRI studies to differentiate between lesions based on their distinct contrast enhancement patterns, such as HAP hyper-enhancement or washout in the PVP[16]. In comparison to ECAs, HSAs are taken up by hepatocytes and (in part) excreted through the biliary tract; thus, they can better differentiate between those lesions that consist of functionally active and impaired hepatocytes or those that are extrahepatic in origin[7]. This behavior of HSAs is utilized for making a distinction between FNH and hepatocellular adenoma, or to detect small foci of HCC and MET within the surrounding liver parenchyma[17,18].

In the current study, we evaluated different AI models on liver MRI images for the prediction of 216 FLLs compiled from three different types of lesions, namely FNHs, HCCs and METs. To ensure that the models could achieve the highest possible

Table 2 Evaluation metrics of the two-dimensional and three-dimensional densely connected convolutional neural networks

Input data	PPV	Sensitivity	F1 score	Specificity	NPV
FNH 2D	0.9091	1.0000	0.9524	0.9500	1.0000
3D	0.9000	0.9000	0.9000	0.9500	0.9500
HCC 2D	1.0000	0.8000	0.8889	1.0000	0.9091
3D	0.8750	0.7000	0.7778	0.9500	0.8636
MET 2D	0.9091	1.0000	0.9524	0.9500	1.0000
3D	0.7500	0.9000	0.8182	0.8500	0.9444
Mean 2D	0.9394	0.9333	0.9312	0.9667	0.9697
3D	0.8417	0.8333	0.8320	0.9167	0.9194

2D: Two-dimensional; 3D: Three-dimensional; FNH: Focal nodular hyperplasia; HCC: Hepatocellular carcinoma; MET: Metastasis; NPV: Negative predictive value; PPV: Positive predictive value.
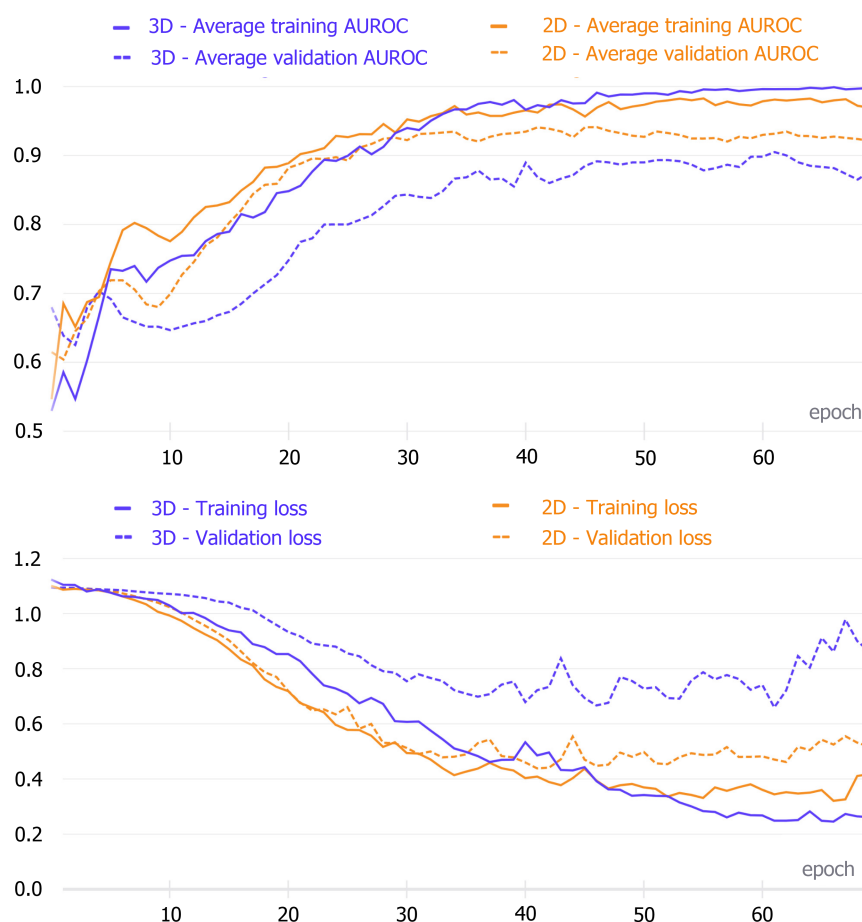

Figure 3 Comparison of the training evaluation metric curves and loss curves. The upper figure shows the area under the receiver operating characteristic curve (AUROC) values after each training epoch of the two-dimensional (2D) and three-dimensional (3D) densely connected convolutional neural networks (DenseNets). The best average AUC was obtained after the 46th (2D network) and 62nd (3D network) epochs. The lower figure indicates the loss values for each training epoch of the two networks. 2D: Two-dimensional; 3D: Three-dimensional; AUROC: Area under the receiver operating characteristic curve.

prediction rate, we narrowed down our data collection to only those four MRI sequences that provided the highest tissue contrast compared to the neighboring parenchyma or depicted distinctive imaging features of the lesion types. For the same reason, we used only HSA-enhanced scans for the analysis. We collected post-contrast images from HAP, PVP and HBP, and a T2w SPAIR image in the case of each lesion. A similar image analysis strategy was used by Hamm *et al*[19], who predicted 494 FLLs from six categories, including simple cyst, cavernous hemangioma, FNH, HCC,
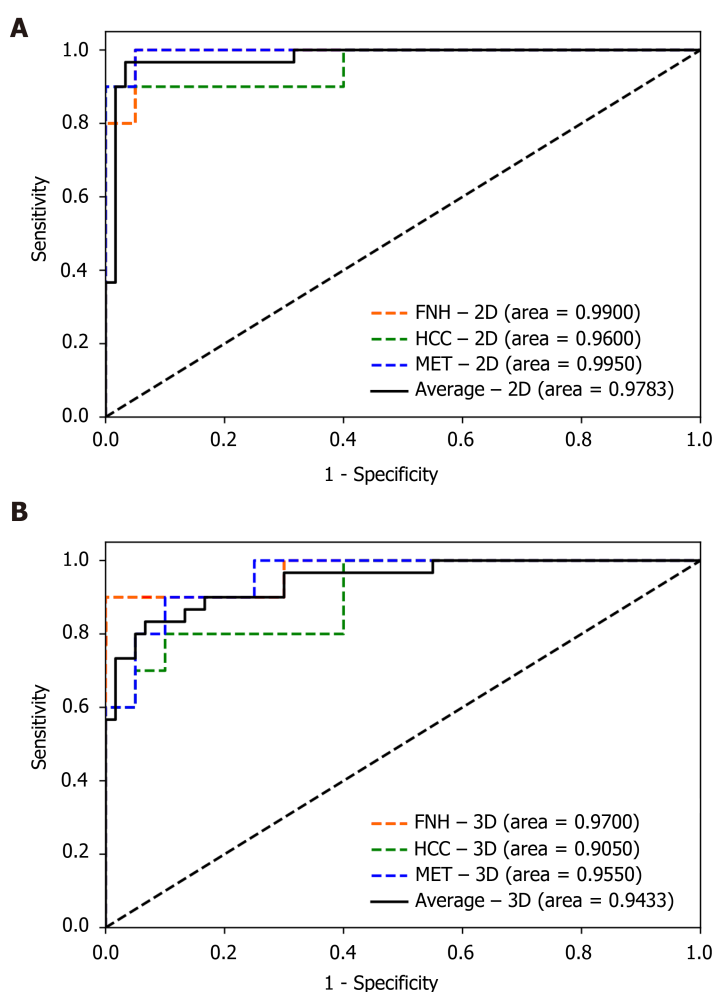
Figure 4 Receiver operating characteristic curves of the two-dimensional and three-dimensional densely connected convolutional neural network 264 models' performance on the test set. A: Two-dimensional; B: Three-dimensional. 2D: Two-dimensional; 3D: Three-dimensional; FNH: Focal nodular hyperplasia; HCC: Hepatocellular carcinoma; MET: Metastasis.

intrahepatic cholangiocarcinoma, and colorectal cancer METs using a 3D CNN model. The authors used HAP, PVP and delayed venous phase MRI images for the classification of the FLLs. They reported that the CNN model demonstrated 0.92 accuracy, 0.92 sensitivity and 0.98 specificity. The disadvantage of this study compared to ours was that it did not include HBP images, with only ECA images used for the MRI scans.

There are a handful of studies that included conventional ML methods and achieved reasonably good results. Wu *et al*[20], for example, extracted radiomics features from non-enhanced multi-parametric MRI images of FLLs and used them in ML models to differentiate between hepatic haemangioma and HCC. The final classifier achieved an AUC of 0.89, a sensitivity of 0.822 and a specificity of 0.714. Jansen *et al*[21], in their 2019 paper, used traditional ML methods for the same problem achieving an average accuracy of 0.77 for five major FLL types.

Our models' performance in the test set was comparable to or even surpassed those from previous publications, as the AUC, sensitivity and specificity were excellent for both the 2D (0.9783, 0.9333 and 0.9667 respectively) and 3D (0.9433, 0.8333 and 0.9167 respectively) architectures, which demonstrates the robustness of our data collection and analysis.

The quality and quantity of input data are pivotal when training neural networks. MRI liver tumor analysis using DL methods has steeply increased, but there is evidence lacking to support the use of 2D or 3D data. The additional dimension in 3D network inputs makes them computationally more demanding and the different data augmentation methods and hyperparameters must be well chosen to avoid artifacts. The 2D neural networks have the advantage of pretraining, which may improve classification accuracy[16,22,23]. Our study supports the results of Wang *et al*[24] and Hamm *et al*[19], emphasizing the need for multi-channel input volumes in order to achieve better accuracy. In contrast to these approaches, we have also utilized HBP
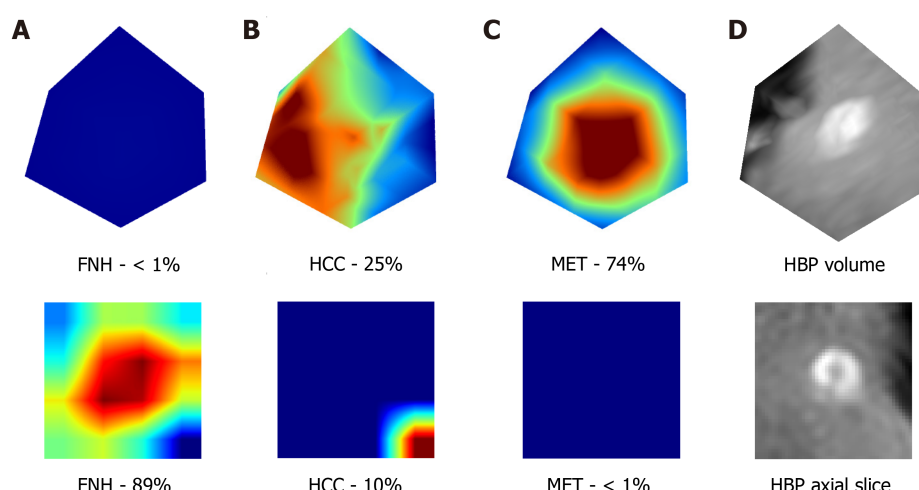
Figure 5 Visualization of the attention maps extracted from the two-dimensional and three-dimensional densely connected convolutional neural networks compared to the hepatobiliary phase input images. Two-dimensional (lower row) and three-dimensional (upper row) attention maps (column A-C) and hepatobiliary phase (column D) images were extracted from the 3rd dense block of the trained network. A-C: Two-dimensional (lower row) and three-dimensional (upper row) attention maps; D: Hepatobiliary phase images. Column A contains the attention maps for focal nodular hyperplasia (FNH), column B for hepatocellular carcinoma, and column C for metastasis diagnosis. The correct diagnosis is FNH in this case. Probabilities for different lesion classes are annotated below each attention map. The red areas are more important for the classification than other image regions. FNH: Focal nodular hyperplasia; HCC: Hepatocellular carcinoma; MET: Metastasis; HBP: Hepatobiliary phase.

images, thereby increasing the number of input channels to four in order to improve accuracy and additionally trained 2D CNNs, proving them to be just as effective classifiers as 3D models.

The selected architecture of the DL model can substantially alter classification accuracy. It is a novelty of our analysis that compared to previous examinations we utilized a DenseNet architecture. DenseNets contain multiple dense blocks, where each layer is connected with the residuals from previous layers. DenseNets require fewer trainable parameters at the same depth than conventional CNNs, as newly learned features are shared through all layers[5]. Our results are among the first to indicate that this highly efficient network design can enhance the performance of AI models for the classification of multi-parametric MRI images of FLLs.

Our study's limitations are the low number of patients involved, the retrospective nature of the study, and that it was conducted within a single institute. Further improvement may be achieved by additional data collection (including additional lesion classes) and the use of more MRI volumes and different data augmentation methods as well as the use of pre-trained networks.

CONCLUSION

Based on our study, we can say that routinely acquired radiological image materials can be used for analysis with AI methods, such as CNNs. According to our results, densely connected CNNs trained on multi-sequence MRI scans can be promising new alternatives to single-phase approaches; furthermore, the use of multi-dimensional input volumes can help the AI-based diagnosis of FLLs. According to our results, 3D and 2D DenseNets can reach similar performance in the differentiation of FLLs based on a small dataset of MRI images. The use of gadoxetate disodium-enhanced MRI scans can also enhance the diagnostic performance of MRI-based hepatic lesion classification.

ARTICLE HIGHLIGHTS

Research background

Interest in medical applications of artificial intelligence (AI) has steeply risen in the last few years. As one of the most obvious beneficiaries of the advances in computer vision, radiology research has also put AI in a prominent position. Convolutional

neural networks are the state-of-the-art methods used in computer vision. Focal liver lesions (FLLs) are common findings during imaging, which can best be evaluated *via* hepatocyte-specific contrast-enhanced magnetic resonance imaging (MRI).

Research motivation

Though convolutional neural networks are widely used for medical image research purposes, the effect of input, such as data dimensionality and the effect of multiple input channels, has not yet been widely examined in this area. MRI volumes presumably hold more complex information about each lesion; as such, three-dimensional inputs may be more difficult to process and properly use for classification tasks in comparison to two-dimensional axial slices. The combination of multiple MRI sequences in addition to the use of hepatocyte-specific contrast agents (HSAs) may also affect diagnostic accuracy.

Research objectives

Our research aimed to compare two- and three-dimensional DenseNets264 networks for the multi-phasic hepatocyte-specific contrast-enhanced MRI-based classification of FLLs.

Research methods

T2-weighted, arterial phase, portal venous phase, and hepatobiliary phase volumes of focal nodular hyperplasias, hepatocellular carcinomas and liver metastases were used to train the two models. Diagnostic performance was evaluated on an independent test set, based on area under the curve, positive and negative predictive values (NPVs), sensitivity, specificity and f1 score.

Research results

The study found that *via* the use of either two- or three-dimensional convolutional neural networks and the combination of multiple MRI sequences, the average area under the curve, sensitivity, specificity, NPV, positive predictive value and f1 scores of comparable level can be achieved.

Research conclusions

According to our findings, two- and three-dimensional networks can both be used for highly accurate differentiation of multiple classes of FLLs by combining multiple MRI phases and using HSAs.

Research perspectives

This study's findings can help to clarify the potential applicability of two- and three-dimensional multi-channel MRI images for the convolutional neural network-based classification of FLLs using HSAs.

ACKNOWLEDGEMENTS

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