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REVIEW

Recent advances in the diagnosis and treatment of primary biliary cholangitis

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Abstract

Primary biliary cholangitis (PBC), formerly referred to as primary biliary cirrhosis, is an infrequent progressive intrahepatic cholestatic autoimmune illness that can evolve into hepatic fibrosis, hepatic cirrhosis, hepatic failure, and, in some cases, hepatocellular carcinoma. The disease itself is characterized by T-lymphocyte-mediated chronic non-suppurative destructive cholangitis and elevated serum levels of extremely specific anti-mitochondrial autoantibodies (AMAs). In this article, we will not only review epidemiology, risk factors, natural history, predictive scores, radiologic approaches (*e.g.*, acoustic radiation force impulse imaging, vibration controlled transient elastography, and magnetic resonance elastography), clinical features, serological characteristics covering biochemical markers, immunoglobulins, infections markers, biomarkers, predictive fibrosis marker, specific antibodies (including AMAs such as AMA-M2), anti-nuclear autoantibodies [such as anti-multiple nuclear dot autoantibodies (anti-sp100, PML, NDP52, anti-sp140), anti-rim-like/membranous anti-nuclear autoantibodies (anti-gp210, anti-p62), anti-centromere autoantibodies, and some of the novel autoantibodies], histopathological characteristics of PBC, diagnostic advances, and anti-diastole of PBC. Furthermore, this review emphasizes the recent advances in research of PBC in terms of therapies, including ursodeoxycholic acid, budesonide, methotrexate, obeticholic acid, cyclosporine A, fibrates such as bezafibrate and fenofibrate, rituximab, mesenchymal stem cells transplant, and hepatic transplant. Currently, hepatic transplant remains the only optimal choice with acknowledged treatment efficiency for end-stage PBC patients.

Key words: Autoimmune liver diseases; Primary biliary cholangitis; Primary biliary cirrhosis; Diagnosis; Therapy

Core tip: Primary biliary cholangitis (PBC), previously called primary biliary cirrhosis, is an autoimmune non-suppurative inflammatory disease of the bile duct

that is usually complicated by intrahepatic cholestasis and intrahepatic bile ductule damage, and eventually leads to liver fibrosis and cirrhosis. This review will focus on the clinical, serological and histopathological characteristics of PBC, as well as the advances in the diagnosis and treatment of the disease.

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INTRODUCTION

Primary biliary cholangitis (PBC)[1-8] is a relatively rare chronic intrahepatic cholestatic illness characterized by a T-lymphocyte-mediated attack on small intralobular biliary ducts and the presence of elevated plasma concentrations of specific anti-mitochondrial antibodies (AMAs), resulting in hepatic fibrosis and, ultimately, hepatic cirrhosis or hepatic failure, with the potential for hepatic cellular carcinoma *via* complications[9-11]. PBC predominantly affects women, at a ratio of approximately 12:1 of women to men, who are normally diagnosed at middle-age, primarily in an initial symptomless early stage[9-12]. There is positive association between the national incidence of PBC and socioeconomic status, as estimated by the Human Development Index (HDI)[13]. Moreover, in less-developed countries, the incidence of PBC might be less common[13]. Fatigue and pruritus are incipient clinical manifestations that appear in approximately 20% of PBC patients[14]. Although the clinical presentation and natural disease history of PBC patients have progressively improved over the years due to the recognition of earlier widespread use of ursodeoxycholic acid (UDCA), about 1/3 of PBC patients display suboptimal biochemical responses to UDCA and a poor prognosis[9-12]. At present, hepatic transplant remains the most beneficial therapeutic modality for patients with end-stage PBC[9-12]. This article will focus on the epidemiology, risk factors, clinicopathologic characteristics, serological features, histopathological characteristics, radiologic evaluation approaches, diagnosis, and differential diagnosis, as well as recent advances in the therapy of PBC.

ALTERED TERMINOLOGY FOR PBC: FROM "PRIMARY BILIARY CIRRHOSIS" TO "PRIMARY BILIARY CHOLANGITIS"

The disorder generally referred to currently as "primary biliary cirrhosis" was primitively depicted in 1851, but not formally named until 1950[1-8]. However, it was later rightly recognized that the application of the terminology "primary biliary cirrhosis" is for a catachresis in patients in the presence of early-stage disease and histopathological characteristics of non-suppurative destructive cholangitis that are usually complicated with intrahepatic cholestasis and intrahepatic bile ductule damage. In recent decades, the prognosis of PBC patients has been observably ameliorated since the disease entity was first described more than 150 years ago due to the application of UDCA. Since a great number of PBC patients do not suffer from hepatic cirrhosis, this tag has perceptibly disrupted many PBC patients, who strive for more accurate nomenclature[1-8]. At the second European Association for the Study of the Liver (EASL) monothematic conference on "primary biliary cirrhosis" in 2014, representatives of multitudinous patient cohorts from a variety of countries worldwide requested altering the eponym "cirrhosis" to another that would more precisely represent the characteristics of the disorder[1-8]. From the point of view of the patient, the eponym "cirrhosis" is misdirecting in some ways, and may result in stigmatization and confusion with alcoholic cirrhosis, as well as a shortage of transparency with regards to the stage and prognosis of the disease. From the physician's perspective, misapplication of the terminology "cirrhosis" is counter-productive to their job. In order to assist and cure patients both within and without the hospital setting who are trying to balance their private lives with their medical demands, it is vital that the term "cirrhosis" be changed[1-8]. The suggested change of "cirrhosis" to "cholangitis" was ratified by the EASL in November 2014, by the American Association for the Study of Liver Diseases in April 2015, and by the AGA in July 2015, respectively[1-8]. In order to inform more people worldwide regarding this change, an article was published in 2015 titled "Changing nomenclature for PBC: From "cirrhosis" to "cholangitis" " in various well-known international medical journals, such as *Gastroenterology*, *Am J Gastroenterol*, *Gut*, *Hepatology*, *J Hepatol*, *Dig Liver Dis*, *Clin Res Hepatol Gastroenterol*, and *Clin Gastroenterol Hepatol*[1-8]. Adopting the terminology "primary biliary cholangitis" for the illness known by the acronym PBC is therefore long overdue, so as to bring it into correspondence with a very recent global consensus.

Epidemiology

Epidemiology provides significant clues towards our comprehension of the unsearchable etiopathogenesis of PBC. In the past two decades, there have been a chain of epidemiological retrospective investigations concerning patients with PBC[15-22]. The epidemiology of PBC has not only changed significantly over the past twenty years, with a trend towards increasing prevalence in many places around the world[15-22], but is also positively correlated with the national HDI[13]. There is a positive, but not significant, correlation between PBC incidence and HDI on a global level (r = 0.348, P = 0.082)[13]. However, in Europe, a significantly positive correlation exists between PBC incidence and HDI (r = 0.455, P = 0.044)[13]. Moreover, the PBC incidence

is positively related to the health index (r = 0.422, P = 0.036), but negatively related to the education index (r = -0.650, P < 0.01)[13]. The prevalence and incidence rates of PBC patients have been reportedly augmenting annually worldwide, making changing the name "cirrhosis" vital[15-22]. A study in the United States showed that, during the period of 1975-1995, the overall age and sex-adjusted incidence rate of PBC was 27/1000000 per year, with the incidence in female and male populations being 45/1000000 and 7/1000000 per year, respectively[15]. In 1995, the age- and sex-adjusted prevalence was 654/1000000 for women, 121/1000000 for men, and 402/1000000 overall[15]. A study in Canada revealed that, from 1996 to 2002, the overall age and sex-adjusted incidence rate of PBC was 30.3/1000000 per year (female: 48.4/1000000, male: 10.4/1000000); the prevalence was 100/1000000 in 1996 and 227/1000000 in 2002[16]. A study in Lombardy, Italy and in Denmark suggested that during 2000-2009, the overall age and sex-adjusted incidence rate of PBC in Lombardy was 16.7/1000000 per year (female-to-male ratio 2.3:1), the point prevalence in Lombardy was 160/1000000 in 2009, the incidence of PBC in Denmark was 11.4/1000000 per year (female-to-male ratio 4.2:1), and the point prevalence in Denmark was 115/1000000 in 2009[17]. A study in Crete, Greece showed that, from 1990 to 2010, the incidence of PBC was 20.88/1000000, and the prevalence was 365/1000000[18]. A study in the Netherlands demonstrated that, between 2000 and 2008, the incidence of PBC was 11/1000000 (3/1000000 in men and 19/1000000 in women) and the point prevalence in 2008 was 132/1000000[19]. A study in Iceland indicated that the point prevalence in 2010 was 383/1000000, while the age-standardized rate of incidence for female patients in the 1st (1991-2000) and 2nd phases (2001-2010) were 34/1000000 and 41/1000000, respectively[20]. Overall incidence rates in the 1st and 2nd phases were 20/1000000 and 25/1000000, respectively[20]. Although the prevalence of PBC was higher in some regions of North America and northern Europe, it was rarely seen in Australia. A study in Australia demonstrated that the age-adjusted prevalence rate of PBC was 51/1000000[21]. A study in South Korea revealed that, between 2009 and 2013, the age and sex-adjusted incidence of PBC from 2011 to 2013 was 8.57/1000000 per year (ratio of female to male was 6.2:1), while the age and sex-adjusted prevalence rate from 2009 to 2013 was 47.50/1000000[22]. At the time of writing, there is still a notable lack of large, nationwide population-based solid epidemiological information concerning PBC in China. One study in China indicated that the point prevalence rate of adult PBC patients who received a health examination in southern China was 492/1000000, with the prevalence in women over 40 years old being up to 1558/1000000 (ELISA method)[23]. The overall prevalence of PBC reported by Liu *et al*[23] was much higher than previously reported in the literature, which was likely due to the methodology used in the study. In general, the different prevalence and incidence rates of PBC reported in the aforementioned literature were mainly due to differences in assay methods, gender and age distributions of population groups, and geographic regions. There is presently no precise epidemiologic data on the prevalence of PBC in Africa, but it is speculated to be one of the lowest in the world.

RISK FACTORS

To date, the pathogenesis of PBC remains largely unknown, although geographical distributions, genetic susceptibility, and environmental factors may be some potential risk factors for the disease[9-10]. Both familial clustering and monozygotic twins with an identical DNA sequence provided good evidence for its genetic susceptibility and high degree of consistency[11]. Environmental factors, such as smoking, drug abuse, and microbiome complexities, may play a vital role in breaking the immune tolerance of individuals with genetic susceptibility[9-11]. Furthermore, recent novel hypotheses on latent environmental triggers, such as chemical xenobiotics, which result in the breaking of self-tolerance within the unparalleled immunological environment of the liver, have also been suggested[11,12]. As PBC overwhelmingly affects females, factors such as major defects in sex chromosomes, abnormal genetic architecture, and epigenetic abnormalities strongly suggest an effect of genetic and epigenetic factors in the triggering and perpetuation of autoimmune aggression in PBC[9-12]. Several human leukocyte antigen (HLA) risk loci that provide prognostic information and a few non-HLA risk loci associated with the development of PBC have recently been confirmed by means of genome-wide association studies (GWAS)[24-31]. GWAS showed that *HLA-DQB1* (*0402), *HLA-DRB1* genes (*08,*14), and *HLA-DPB1* gene (*03:01) were predisposing risk alleles for PBC susceptibility[24,25]. Aside from the HLA locus, a number of non-*HLA* genes including *IL-12A* (*rs6441286*, *rs574808*), *IL-12RB2* (*rs3790567*), *STAT4*, *CD80*, *DENND1B*, *CXCR5*, *IL-7R*, *TNFRSF1A*, *NFKB1* and *CLEC16A* were also closely related to PBC susceptibility[24,26]. These data demonstrate not only that there are extraordinary associations between PBC and the usual heritable aberrance at HLA class Ⅱ, IL12A, and IL12RB2 loci, but also that the IL-12 immunoregulatory signaling axis plays an outstanding role in the physiopathology of PBC. Several recent GWAS have shown that some non-*HLA* genes, such as *STAT4 SNPs* (*rs10168266*, *rs11889341*, *rs7574865*, *rs8179673*, *rs10181656*)[27], *ESR2 rs1256030 T* allele[28], *CLEC16A, SOCS1, SPIB* and *SIAE* genes[29], may also be significant risk factors for the progression of PBC. One study demonstrated that the downregulated expression of IL-12A in lymphoblastoid cell lines obtained from Han Chinese were markedly associated with the risk alleles of rs4679868 and rs6441286 (P = 0.0031 and 0.0073, respectively)[30]. Furthermore, the risk alleles of the 2 SNPs were observably related to a decreased expression

of *SCHIP1* gene that is 91.5 kb, located upstream of IL-12A and associated with susceptibility to celiac disease[30]. These data have disclosed the IL-12/JAK-STAT signaling pathway as a pivotal etiologic factor for PBC. In addition, the allele of rs79267778 was observably relevant to PBC[31]. The amino acid at position 1904 (NM_001037335) from threonine (ACG) had been changed to methionine (ATG)[31]. This gene locus was exceedingly conservative in mammals and estimated to have the potential risk score of 0.469 by PolyPhen-2 (bioinformatics tools)[31]. PBC and gene expression were related to allele-specific transcription factor binding to usual and infrequent geno-variation[32]. DNA methylation analysis of the X chromosome exposes abnormal demethylation on CXCR3 promoter in PBC[33]. Furthermore, other associated risk factors include concurrent autoimmune disease, lifestyle factors (*e.g.*, cigarette smoking), urinary tract infection, vaginal infection, and environmental influences (*e.g.*, toxic and chemical exposure to such substances as nail polish and hair dye)[34-36]. In general, the close link between environment factors and genetic susceptibility may play a vital part in the epigenetic mechanisms of PBC.

Clinical characteristics

Common clinical symptoms of PBC include fatigue, pruritus, weakness, daytime sleepiness, loss of weight, xanthelasma palpebrarum, jaundice, skin hyperpigmentation, upper abdominal discomfort, hepatosplenomegaly, osteodystrophy, osteoporosis, cholelithiasis, malabsorption syndrome, and extrahepatic manifestations of an autoimmune nature, although roughly 50% of PBC patients are asymptomatic at diagnosis[9-12]. Patients with PBC normally suffer from itching and fatigue, regardless of disease severity[14,37,38]. Serum fat-soluble vitamin D deficiency may be detected, particularly in advanced PBC patients[39,40]. Metabolic bone disease includes osteoporosis and, more rarely, osteomalacia, which have been considered important complications of PBC[41,42]. The extremely infrequent PBC complication of tubulointerstitial nephritis with Fanconi syndrome should be highly suspected in adult PBC patients in the presence of agnogenic halisteresis, even without the presence of abnormal liver function[41]. The serum levels of sclerostin were found to be observably increased in PBC patients in comparison with controls ($P < 0.001$), while the hepatic mRNA overexpression of sclerostin and elevated serum levels of sclerostin were inversely related to osteogenesis and reabsorption biological markers[42]. In addition, liver sclerostin was mainly distributed in the bile ducts, was relevant to the seriousness of cholangitis ($P = 0.02$), and was indirectly related to the extent of inflammation in the hepatic lobule ($P = 0.03$)[42]. These results indicated that sclerostin overexpression in the bile duct of patients with PBC in the presence of chronic intrahepatic cholestasis may affect metabolic osteopathia in PBC[42]. In general, although the main target organ is the liver, multiple systems may also be involved, such as interstitial lung disease (ILD)-related pulmonary hypertension (PH) and esophageal dysfunction. PBC is also often accompanied by nephritis, connective tissue diseases (CTDs), hepatocellular carcinoma, and other rare diseases. The concurrence of these rare diseases often augments the difficulty in establishing an exact diagnosis. Specific clinical features are as follows.

Fatigue

Fatigue is not an uncommon complaint of PBC patients, and is related to a lower quality of life[9-11]. In recent years, PBC is mainly diagnosed in the majority of patients who are asymptomatic[9-11]. However, fatigue is a significant problem in approximately 50% of PBC patients, with 20% of all PBC patients experiencing significant or life-altering fatigue[9-11]. The pathogenesis of fatigue in PBC has not been fully elucidated, although it isn't relevant to the seriousness of the underlying illness and is unresponsive to UDCA[9-11]. As the symptom of fatigue is non-specific, multifactorial, and potentially incapacitating, conditions such as anemia, diabetes, hypothyroidism, and depression should be considered and excluded[9-11]. Fatigue is typically identified with a subset of PBC patients who are predominantly young women who have particularly active illness, a suboptimal response to UDCA therapy, and are more likely to develop hepatic cirrhosis and its complications[37]. At present, there is no special drug therapy for the management of PBC-related fatigue and no significant improvement following liver transplantation[9-11]. The clinical efficacy of modafinil in the treatment of PBC-related fatigue for 12 wk has been proved to be secure and reasonably well-tolerated in randomized, placebo-controlled, phase Ⅱ clinical trials[38]. Nevertheless, it did not give rise to an advantageous impact on fatigue when compared to a placebo-treated group[38].

Pruritus

Pruritus is a pre-eminent symptom in PBC patients with chronic cholestasis and is variably reported in PBC characterized by cholestasis[9-11,14]. More than two-thirds of PBC patients experience pruritus during the process of the illness[9-11]. Compared to asymptomatic PBC patients without pruritus, symptomatic PBC patients with pruritus more frequently suffer from hepatic cirrhosis and its related complications ($P = 0.004$)[37], and are less likely to respond to UDCA treatment ($P = 0.006$). The pathogenesis of cholestatic pruritus remains largely elusive[9-11]; its natural history, related pathogenesis, and molecular mechanisms are under continued investigation[9-11]. The autotaxin (ATX)-lysophosphatidic acid signaling axis may play a vital part in the nosogenesis of pruritus, and has lately has been connected with pruritus in PBC[14]. Several pieces of evidence have showed that a circulating pruritogen will take responsibility for it, but identification of the small molecule has yet to be ultimately identified[14]. In comparison, plasma ATX activity is observably associated with pruritus in PBC,

suggesting a new molecular targeting therapy[14].

FAT-SOLUBLE VITAMIN DEFICIENCY

Malabsorption, steatorrhea, and fat-soluble vitamin D deficiency are uncommon, except in cases of advanced liver disease and long-standing, severe cholestasis[9,10,39,40,43]. In addition to vitamin D deficiency, as luminal bile acid levels in severe cholestasis are below the critical concentration required for micelle formation and subsequent lipid absorption, clinically-relevant fat-soluble vitamin (vitamin A, E and K) deficiencies may also exist in PBC[43]. Deficiencies in fat-soluble vitamins A, D, E and K have been reported in 33.5%, 13.2%, 1.9% and 7.8% of PBC patients, respectively[43]. Vitamin A deficiency appears to be markedly associated with advanced PBC stage, decreased cholesterol, and increased Mayo risk score[43]. High Mayo risk score, low serum albumin level, and elevated total bilirubin have been shown to be independently related to vitamin D deficiency[43]. Baseline vitamin D deficiency was associated with severity of disease and response to UDCA treatment[39]. Serum 25(OH)D concentrations reduced with elevating histological grading of stage (P = 0.029) and were inversely associated with serum bilirubin and alkaline phosphatase (ALP) concentrations in PBC[39]. Serum 25(OH)D concentration at baseline was observably reduced in non-responders to UDCA (P = 0.005)[39]. Baseline vitamin D deficiency was related to an elevated risk of an inappropriate response with no relationship to advanced histological stages (P = 0.047)[39]. Mean serum concentrations of vitamin D were observably reduced among PBC patients compared to the control group (P = 0.029) and vitamin D deficiency ($\leqslant$ 10 ng/mL) was observed in 33% of PBC patients *vs* 7% of the control group (P < 0.0001)[40]. Vitamin D concentrations were negatively associated with advanced hepatic injury, as well as the existence of accompanying autoimmune disorders[40]. The potential role of vitamin D in PBC may involve genetic and cell signaling mechanisms. Relatively few PBC patients have vitamin E or K deficiencies[43].

PBC complicated with portal-venous hypertension

Portal-venous hypertension is not an uncommon aftermath of PBC, and may even occur before cirrhosis develops in PBC patients[9-11]; approximately 10% of PBC patients presented with characteristics of portal hypertension as an initial clinical symptom[9-11]. Gastroesophageal varices may occur in any of the different histological phases of PBC[9-11]. Signs of portal hypertension should therefore be carefully observed for in PBC patients at the moment of diagnosis, as well as during the observation period[9-11]. The pathogenesis of portal hypertension in PBC is still unclear[9,10]. A recent study suggested sinusoidal blockage as a potential physiopathology mechanism during the early phases of PBC, which was verified by the obvious intrahepatic portal vein in 3 non-cirrhotic PBC patients, with intrahepatic portal vein hepatica interflow being responsible for relieving the hepatic venous pressure gradient[44]. Another study indicates that angiogenetic and fibrotic responses are presumably induced by aquaporin-1 (AQP-1), resulting in the enhanced perfusion of arterial blood flow to the sinusoids[45]. The result demonstrates that AQP-1 is related to arterial capillary wall proliferation and hepatic sinusoidal transformation facilitating portal-venous hypertension in PBC[45]. Esophageal varicosities (EV) can be found in PBC patients with early histological stages[46,47]. A study revealed that 6% (8/127) of early histological stage PBC patients suffered from EV and 95% of PBC patients in the presence of varices were required to meet at least one of the following criteria: Male sex, hypoalbuminemia (< 3.5 g/dL), hyperbilirubinemia ($\geqslant$ 1.2 mg/dL), and/or prolonged prothrombin time (PT) ($\geqslant$ 12.9 s)[46]. Therefore, these parameters that include male sex, hypoalbuminemia, hyperbilirubinemia, and/or PT can be used as a tool for non-invasive prediction of EV[46]. A study demonstrated that among 256 cases of PBC with early histological stage, 22 cases suffered from EV at the time of diagnosis, with elevated serum ALP levels and decreasing platelet counts being markedly related to the presence of EV in early histological stage PBC[47]. The prominent relationship between these two factors with the development of EV was also disclosed, and PBC with early-stage and elevated ALP ratios $\geqslant$ 1.9 had an observably high risk of progressing EV[47]. In addition, another study has shown that quantitative parameters in the diagnosis of hepatic fibrosis in portal, septal and fibrillar areas may accurately predict gastroesophageal varices in PBC; the diagnostic specificity and sensitivity in PBC was 75% and 100%, respectively[48].

PBC complicated with PH

PH is generally complicated by heart or lung disorders, but it is also known to be related to PBC[49]. PH that suggests poor prognosis as a complication of PBC is not only common, but is closely related to portal-venous hypertension and immunological dysregulation[49]. PH is significantly more frequent than was previously assessed in PBC patients with portal hypertension[49]. The risk of progressing PH could be enhanced with the persistent time of portal hypertension without any explicit relationship with the degree of portal hypertension, liver failure, or amount of blood shunted[49]. The prevalence rate of PH in PBC patients with portal hypertension has been reported by McDonnell *et al*[50] as 0.61% in a clinical series of 2459 PBC patients with biopsy-proved cirrhosis of the liver. PH associated with PBC without portal hypertension is very infrequent; among 178 PBC patients, 21 (11.8%) suffered from PH[49]. Four cases (19.0%) suffered from medium to severe PH and one died of right ventricular dysfunction rather than hepatic dysfunction[49].

PBC complicated with esophageal dysfunction

Esophageal dysmotility can exist in some PBC patients[51], particularly in those with scleroderma or Sjögren's syndrome in the absence of scleroderma[51]. As a result, some

esophageal motor disturbances could be considered associated with Sjögren's syndrome[51]. Esophageal motor dysfunction is by no means uncommon in Sjögren's syndrome or scleroderma; however, whether any esophageal dysmotility also exists in PBC without Sjögren's syndrome or scleroderma is still controversial. A recent study showed that, among 37 PBC patients, 17 (45.9%) had esophageal dysmotility (10 cases of non-specific esophageal motor disorder, 5 cases of esophageal hypomotility, 1 case of nutcracker esophagus, and 1 case of hypertensive lower esophageal sphincter)[51]. These results demonstrate that sub-clinical esophageal motor dysfunction is common in PBC patients[51].

PBC complicated with ILD

ILD is a frequent and major complication of PBC[52,53]. PBC patients who suffer from Raynaud's phenomenon and other CTDs were considered to have the greater possibility of developing ILD[52,53]. PBC with concomitant Sjögren's syndrome was considered to have a higher risk of developing ILD and presenting a poor prognosis[53]. A study showed that, among 178 PBC patients, 28 (15.7%) suffered from ILD, with 53.6% said patients suffering from difficult breathing and tussis, and 88.2% demonstrating restrictive and diffusing ventilation impairment by means of pulmonary function test[52]. Patients with PBC in the presence of ILD were older in age and displayed higher serum levels of sedimentation rate of erythrocyte compared to those without ILD (P < 0.05)[52]. Raynaud's phenomenon, as well as the coexistence of PBC and CTDs, were considered to be risk factors for PBC patients developing ILD (P = 0.04, OR = 3.12 and P = 0.01, OR = 3.18, respectively), although 42.9% of patients with PBC in the presence of ILD had not suffered from other CTDs[52]. There was much higher incidence rate of ILD in PBC patients with concomitant Sjögren's syndrome compared to those without the syndrome (P = 0.005)[53]. In some instances, ILD can even appear to precede PBC[54].

PBC complicated with nephritis

Symptomless distal renal tubular acidosis should be considered the main feature of PBC-related kidney damage, and can appear in approximately 1/3 of PBC patients[55]. However, various rarer methods of PBC-associated kidney damage have also been described in the literature, including: Fanconi syndrome, microscopic polyangiitis, membranous nephropathy, membranous glomerulonephritis, tubulointerstitial nephritis, fibrillary glomerulonephritis, interstitial nephritis, Goodpasture syndrome, anti-neutrophil cytoplasmic autoantibody (ANCA)-associated rapidly progressive glomerulonephritis, and focal segmental glomerulosclerosis[41,56-64].

PBC complicated by CTDs

PBC can be complicated by CTDs, more specifically systemic lupus erythematosus (SLE), systemic sclerosis (SSc), rheumatoid arthritis (RA), Sjögren's syndrome (SS), polymyositis (PM), and dermatomyositis[65]. Moreover, combined PBC and CTDs often enhance the difficulty in making an exact diagnosis and treatment of PBC[65]. One study showed that, among 322 patients with PBC, 150 cases (46.6%) suffered from CTDs, of which 11 cases (3.4%) suffered from two or more CTDs[65]. SS should be considered the most common CTD (122 cases, 36.2%)[65]. Other CTDs in this group of patients, in order of rarity from high to low, were as follow: 12 cases of SLE (3.7%), 10 cases of PM (3.1%), 9 cases of SSc (2.8%), and 9 cases of RA (2.8%)[65].

PBC complicated by hepatocellular carcinoma

It is by no means uncommon for PBC patients to suffer from hepatocellular carcinoma (HCC)[66-69]. Of two retrospective studies in China, one demonstrated an incidence of HCC in PBC patients of 4.13% (52/1255)[66], while the other found it to be 3.75% (70/1865)[67]; this incidence was observably higher in men (9.52%) than in women (3.31%)[66]. Risk factors for PBC-related HCC in China for the two studies were found to be body mass index (BMI) $\geqslant$ 25, male sex and a history of drinking alcohol for the first study[66] and age > 54 years, male sex, co-existence of diabetes, and previous hepatitis B virus (HBV) infection for the second study[67]. A retrospective Japanese study found the incidence of HCC in PBC patients to be 5.2% (11/210), with the only risk factor for PBC-associated HCC being associated with advanced histological stage[68]. Recently, a multicenter international study demonstrated that incidence rates of HCC in PBC patients were 2.69% (123/4565), with markedly higher rates in male PBC patients compared to female patients (P < 0.0001). Univariate analysis of potential risk factors in establishing diagnosis of PBC related to HCC progression were: Male sex (P < 0.0001), increased aspartate aminotransferase (AST) (P < 0.0001), progressing liver illness (P = 0.022), platelet decline (P < 0.0001), and decompensated hepatic function (P < 0.0001)[69]. According to the Paris-I criteria, one year stratification by inappropriate biochemical response with UDCA therapy was markedly related to risk factors of progressing HCC (P < 0.0001)[69]. Biochemical non-response to UDCA therapy predicted future trends of HCC in early stage PBC (stages Ⅰ-Ⅱ) (P = 0.005) and advanced stage PBC (stages Ⅲ-Ⅳ) (P = 0.02)[69]. The international multicenter study clearly demonstrates that one year biochemical non-response to UDCA is related to incremental future risk factors of progressing HCC in PBC[69]. In addition, another study showed that repeat liver resection for recurrent HCC complicating PBC is an option and may provide and improved outcome[70]. In general, PBC with hepatic cirrhosis or non-response to one year of UDCA therapy are at incremental risk of HCC.

PBC with concurrent viral hepatitides

The difficulty in identifying hepatitides C virus (HCV) and/or HBV infections in PBC patients is such that an accurate diagnosis of PBC is usually observably delayed in this particular patient cohort[71]. In PBC patients with

accompanying HCV infection, impact therapy might be approved in consideration of the relevant and more serious cirrhosis[72]. A retrospective Greek study showed that, among 1493 HBV and 526 HCV patients, 17 were confirmed as having a coexistence of viral hepatitides and PBC (8 cases of HCV and 9 cases of HBV)[71]. It is very difficult to make an exact diagnosis of PBC in HBV or HCV-infected patients, meaning that a precise diagnosis is usually delayed[71]. Cholestasis should therefore be an important indication of PBC for physicians[71]. A study in Taiwan showed that, among 76 patients with PBC, 9 cases were confirmed as having a coexistence of HCV infection and PBC, and suffered from more serious hepatic cirrhosis on the basis of Child-Pugh (P = 0.019) and the Model for End-Stage Liver Disease (MELD) (P = 0.01) scores[72]. One case report showed that a patient with chronic HBV infection was later found to have active, asymptomatic PBC due to a persistently elevated ALP level after optimal HBV DNA suppression on antiviral therapy[73]. This report emphasizes the significance of keeping a high clinical index of suspicion for potential PBC, even after a patient with HBV has been successfully treated for a primary liver condition[73]. Clinical vigilance, particularly in atypical clinical manifestations, can result in earlier accurate diagnosis and prompt treatment of PBC[73].

PBC with concurrent rare diseases

Although uncommon, the coexistence of PBC and some rare diseases are frequently believed to enhance the difficulty in making an exact diagnosis of PBC, as well as its treatment, due to the very complicated clinical manifestation of diseases with coexistence conditions. PBC is occasionally associated with some rare diseases, including Guillain-Barré syndrome, warm autoimmune hemolytic anemia, primary hepatic mucosa-associated lymphoid tissue lymphoma, ANCA-associated vasculitis, pseudolymphoma, hereditary hemorrhagic telangiectasia, generalized morphea, myasthenia gravis, hepatic inflammatory pseudotumor, idiopathic retroperitoneal fibrosis, celiac disease, Wilson's disease, bullous pemphigoid, idiopathic granulomatous hepatitis, CREST syndrome, Crohn's disease, hepatic sarcoidosis, Evans syndrome, and Hürthle cell adenoma[74-92].

SEROLOGICAL FEATURES

Serum antibody specific for PBC

AMAs: AMAs, including AMAs-M2, are a specific and sensitive marker for the diagnosis of PBC[9,10,93,94]. The existing evidence shows that AMAs and AMAs-M2 have excellent diagnostic value, with high specificity and sensitivity for PBC[93]. Compared with AMAs-M2, AMAs is a faster and more comprehensive diagnostic marker[93]. AMAs consist of nine subtypes, four of which are associated with PBC: AMA-M2, AMA-M4, AMA-M8, and AMA-M9[9-11]. Although these four AMA subtypes have comparatively specific diagnostic value for PBC, AMA-M2 remains the foremost subtype applied as a routine diagnostic marker for PBC[9-11]. AMAs are present in 95% of PBC patients; however, 5% of patients with PBC are still AMA-negative[94]. AMA-negative PBC patients had an observably worse prognosis in comparison with AMA-positive PBC patients[94]; however, an obvious distinction between positive and negative PBC AMAs should not have been found on the basis of clinical manifestation, serum biochemical features, histopathological characteristics, disease process, or response to UDCA treatment[94]. Notably, AMA-negative PBC patients had an observably decreased free survival of liver-associated complications covering liver transplant and death in comparison with AMA-positive PBC patients (P = 0.0182)[94].

Anti-nuclear antibodies: Besides AMAs, PBC patient serum is able to demonstrate other PBC-related autoantibodies, especially anti-nuclear antibodies (ANAs) covering anti-multiple nuclear dot autoantibodies (anti-sp100, PML, NDP52, anti-sp140), anti-nuclear envelope protein autoantibodies (lamin, lamin B receptor), and anti-rim-like/membranous anti-nuclear autoantibodies (anti-gp210, anti-p62)[95-103]. Determination of AMAs and PBC-specific ANAs identified them as being associated with PBC severity[9,10]. Elevated serum concentrations of ANAs should be found in approximately 50% of PBC patients and 85% of AMA-negative PBC patients[96]. In short, 44% of PBC patients had anti-sp100, 15.1% had PML, 25% had anti-gp210 and 25% had ACAs[97-100]. AMAs and ANAs (anti-gp210, anti-sp100, ACAs) are particularly prevalent in PBC[101]. Although changes in most autoantibodies that occur naturally with the passage of time do not appear to associate with clinical results in PBC, changes in serum anti-sp100 antibody levels can be used as an evaluation of prognostic factors with regard to the progress of liver fibrosis diagnosed *via* hepatic biopsy[102]. Sp140L is the phylogenetically nearest family member to anti-sp100 protein, and serves as an autologous antigen in PBC patients[103]. The polymerization of anti-p62 is significantly augmented in the impaired biliary ducts of PBC and may reflect the inappropriate autophagy and subsequent senescence of biliary ducts cells in the etiopathogenesis of biliary duct injury in PBC[104]. In clinical practice, it is vital to detect these autoantibodies in order to establish PBC diagnosis, assess disease severity, determine the PBC clinical phenotype, and calculate the long-term outcome[101]. Positive anti-gp210 antibody and elevated vanishing biliary duct score were observable risk factors for elevated ALP predicted worsened response[105]. Positive anti-gp210 antibody and elevated hepatitides score were observable risk factors for elevated IgM predicted worsened response[105]. Elevation of ALP and IgM worsened response were observable risk factors for development to end-stage liver illness in the absence of jaundice[105]. Therefore, in the classical or typical form of PBC, characterized by the chronic progressive disappearance of small intrahepatic biliary ducts with a simultaneous augment in hepatic fibrosis, anti-gp210 autoantibodies are a powerful risk factor for development

to icterus and liver failure[101,105]. Age, positive anti-gp210 antibody, and positive ACAs were observable risk factors for elevated of alanine aminotransferase (ALT) worsened response[105]. Elevation of ALT worsened response was an observable risk factor for development to end-stage hepatic illness with persistent icterus[105]. Of PBC patients with ACAs positivity, 30% had serious bile duct damage and portal hypertension[106]. Therefore, the presence of ACAs is a risk factor for development to hepatic cirrhosis and portal-venous hypertension[101,105,106]. Biochemical response to UDCA therapy at two years, which is affected by the serum autoantibody status of ACAs, anti-gp210, and histological and morphometric variables at baseline, may predict long-term clinical results in PBC patients[105]. By contrast, another study showed that continuous variations of anti-sp100 titers, rather than anti-gp210 titers, might be effective for the surveillance of disease procession and UDCA treatment outcome[107]. The study revealed a reduced rate of eGFR, an elevated possibility of chronic kidney disease (CKD), and an elevated rate of annual eGFR decline in PBC patients with ACAs compared to those without ACAs ($P < 0.05$, separately)[108]. ACAs may serve as an independent predictor for CKD in patients with PBC[108]; therefore, it is important to assess ACAs and renal function in order to deter CKD evolution in PBC[108].

New autoantibodies: The recognition of novel auto-antibodies as a non-invasive serum hallmark is still an important area of PBC research[109]. Hu *et al*[109] created a PBC-focused microarray with 21 of these recently affirmed alternatives, as well as 9 supererogatory familiar PBC autoantigens[109]. By screening the PBC-focused microarrays with PBC patients, 6 proteins were identified as new PBC autoantigens in the presence of high specificities and sensitivities, covering hexokinase-1 (HK 1, and isoforms Ⅰ and Ⅱ), Kelch-like protein 7 (KLHL7), KLHL12, zinc finger, BTB domain-containing protein 2, and eukaryotic translation initiation factor 2C, subunit 1[109]. In addition, both anti-KLHL12 and anti-HK1 antibodies with higher specificity and sensitivity were more likely to be detected in PBC in comparison with controls without PBC ($P < 0.001$)[110]. Anti-HK1 in combination with anti-KLHL12 in the presence of usable signs (*i.e.*, MIT3, gp210 and sp100), improved the sensitivity of PBC diagnosis[110]. Importantly, both anti-KLHL12 and anti-HK1 autoantibodies had been detected in 10% to 35% of AMA-negative patients with PBC, and increasing both biomarkers in routine PBC tests significantly increased the sensitivity in AMA-negative patients with PBC from 55% to 75% by means of immunoblot and from 48.3% to 68.5% with the ELISA method[110]. Supplementing both anti-KLHL12 and anti-HK1 autoantibodies with highly specific assays for AMAs and ANA serological tests observably enhanced the serological surveillance effect and PBC diagnosis, particularly for AMA-negative patients[110].

Serum biochemical sign in PBC: The enhanced serum activity of ALP, gamma-glutamyltransferase (γ-GT), ALT, AST, total bilirubin (TBIL), and bile acids can be detected in most patients with PBC[9,10,47,111-113]. Evidence from several studies has shown that the presence of elevated serum activity of ALP is not only an obvious guidepost of intrahepatic cholestasis, but also a pronounced succedaneous hallmark PBC severity[111-113]. A Japanese study showed that elevated serum ALP levels were not only markedly related to the presence of esophageal varicosities in PBC patients with early histological stage, but also associated with the progression of esophageal varicosities during the follow-up period[47]. In addition, a meta-analysis of individual patient information from 4845 PBC cases covering 15 European and North American countries demonstrated that serum levels of ALP and TBIL detected at research enrollment and every year for 5 years were significantly related to clinical results[111]. The study result showed that serum levels of ALP and TBIL may predict clinical results (*i.e.*, hepatic transplant or dying) of PBC patients, and could serve as alternative terminal points in treatment tests[111]. In addition, a study in China showed that, among serum biolabeling in PBC patients, the serum concentrations of bile acids were augmented with the development of PBC, while the concentrations of carnitines were reduced with the development of PBC[113]; these factors, high serum levels of ALP, TBIL, and bile acids, are markedly associated with progressive PBC and worsened outcomes.

Serum immunoglobulins in PBC: PBC patients characteristically show elevated serum levels of IgM[9,10]. Environmental factors, but not genetic ones, are considered to play an important role in the pathogenesis of high serum IgM in PBC[114]. In addition, serum IgG2 and IgG3 levels were most prominently increased in PBC[115]. However, evidence of decreased serum levels of IgA, IgM, and IgG in a PBC patient seems to demonstrate that immunoglobulin-mediated etiopathogenesis may be unessential for the development of PBC[116].

Serum markers of infection in PBC: As a screening test, serum from 69 PBC patients were detected for IgG-antibodies against *Toxoplasma gondii* (anti-*T. gondii*), *Helicobacter pylori* (anti-*H. pylori*), Epstein-Barr virus (anti-EBV), cytomegalovirus (anti-CMV), anti-HBV and anti-HCV[117]. The results demonstrated that the prevalence rates of 4 anti-infectious agent antibodies: Scilicet anti-*T. gondii* ($P < 0.0001$), anti-*H. pylori* ($P < 0.01$), EBV early antigen ($P < 0.0001$), and anti-CMV ($P < 0.05$) in PBC patients was observably higher than in the controls[117]. The coexistence of the 4 anti-infectious agent antibodies was comparatively ordinary in PBC, but the infection burden was infrequent in normal controls ($P < 0.0001$)[117]. In addition, peculiar contagion reciprocities that potentially accelerate PBC patient risk were also pointed out. Seropositivity of ammodytoxin A was negatively related to hepatic cirrhosis among patients with PBC ($P < 0.05$)[117].

Serum biomarkers in PBC: Serum microRNAs (miRNAs), which are sufficiently steady and control RNase-mediated

degeneration in body fluids, have been used as novel potential biomarkers for many illnesses. However, the expression spectrum of serum miRNAs in patients with PBC is poorly understood. Recently, a miRNA panel (hsa-miR-122-5p, hsa-miR-141-3p, and hsa-miR-26b-5p) was confirmed to have prominent diagnostic accuracy for PBC (sensitivity = 80.5%, specificity = 88.3%)[118]. There was a remarkable difference between expression profiles of the miRNA panel, those of serum ALP (P < 0.001), and those of serum ANAs (P = 0.0282)[118]. Seventeen miRNAs were confirmed to be distinctively expressed in peripheral blood mononuclear cells from PBC patients[119]. In addition, the downregulated expression of hsa-miR-505-3p and miR-197-3p can be used as biological markers of PBC[120]. Functional bioinformatics analysis showed prediction of microRNA target genes involved in multiple signaling passageways and biological processes[119]. In general, serum biological markers for inchoate diagnosis of PBC are a new subject of ongoing research.

Serum predictive marker in PBC: Non-invasive predictive markers of hepatic fibrosis in PBC patients should be used for predicting illness development. The Wisteria floribunda agglutinin-positive Mac-2-binding protein [WFA (+)-M2BP] could serve as an effortless and dependable non-invasive succedaneous serum glycol-biomarker for the diagnosis of hepatic fibrosis in PBC[121]. Serum WFA (+)-M2BP was not only considered to be better than the other non-invasive markers in determining the important and serious fibrosis stages of PBC, but was also forcefully and separately related to clinical result[121]. Serum FGF19 is related to hepatic illness severity, and can also be used as a potential predictive marker of chronic cholestatic hepatic lesion in PBC[122]. Serum ANAs, total cholesterol, and bile acids are predictors of liver failure in PBC[123]. Elevated serum levels of fractalkine in patients with PBC could serve as predictive markers of cholangitis activity at early stages[124]. Comparative proteomics analysis demonstrated not only obvious elevated serum levels of vitronectin in AMA-negative PBC patients compared to those of AMA-positive PBC (P < 0.01), but also a potential association with the more serious bile duct destruction found in this group[125]. Serum hyaluronan is considered a hopeful hallmark for the estimation of hepatic fibrosis in PBC[126]. In addition, serum cartilage oligomeric matrix protein might be a novel non-invasive biomarker for estimating PBC and the risk of HCC[127].

PREDICTIVE SCORES IN PBC

A retrospective analysis showed that PBC predictive scores, covering the European and Yale model, MELD score, and Child-Pugh score, should be interpreted prudently, with the Mayo Risk Score being deemed beneficial in predicting a helpful result[128]. As current approaches for risk stratification of PBC patients are limited and single-center-based, as well as often dichotomous, the novel prognostic tool of GLOBE score was recently proposed by the Global PBC Study Group on the basis of an international meta-analysis of 4119 PBC patients receiving UDCA[129]. A GLOBE score to forecast transplant-free survival of PBC patients receiving UDCA therapy within 1 year was formulated and confirmed by means of clinical and biochemical variables, and the prognostic capacity of the GLOBE score was assessed along with those of the Paris-1, Barcelona, Toronto, Rotterdam, and Paris-2 criteria[129]. Serum levels of ALP, albumin, and hematoidin, as well as blood platelet counts and age, were all independently related to patient mortality or hepatic transplant[129]. There were significantly reduced times of transplant-free survival in patients with risk scores > 0.30 compared to matched normal subjects (P < 0.0001)[129]. The 5-year and 10-year survival rates for patients with positive predictive values verified by the GLOBE score were 98% and 88%, separately[129]. The GLOBE score can therefore not only be considered predictive of the transplant-free survival of PBC patients treated with UDCA, but may also be used to choose the therapy and nursing scheme[129].

RADIOLOGIC APPROACHES TO ASSESSING FIBROSIS IN PBC

At present, there are three proposed important radiologic prediction approaches for assessing hepatic fibrosis: Acoustic radiation force impulse (ARFI), vibration controlled transient elastography (VCTE), and magnetic resonance elastography (MRE)[130-132]. The diagnostic value of the degree of liver fibrosis by means of ARFI together with the 4 serum prediction markers of hepatic fibrosis covering laminin, hyaluronan (HA), type Ⅲ collagen, and type Ⅳ collagen is of a satisfying effect and has significant practical value[130]. ARFI elastography correlated observably with hepatic histological stage (r = 0.74, P < 0.001) in PBC patients[131]. The area under the receiver operating curve of ARFI elastography for predicting histological stage equal to or higher than Ⅱ or Ⅲ and equal to Ⅳ were 0.83, 0.93 and 0.91, respectively[131]. The optimal cut-off values of ARFI elastography were 1.51 m/s, 1.79 m/s, and 2.01 m/s for PBC stage equal to or higher than Ⅱ or Ⅲ and equal to Ⅳ, respectively[131]. ARFI elastography is therefore an acceptable and powerful technique for the quantitative assessment of PBC stage[131]. Dependable VCTE consequences can exclude advanced hepatic fibrosis and avoid the need for biopsy in the lowest 45% of patients[132]. A recent prospective study in the United States has demonstrated that three-dimensional (3D)-MRE at 40 Hz has supreme diagnostic precision in diagnosing advanced hepatic fibrosis[133]. Both 2D-MRE and 3D-MRE at 60 Hz, the standard shear-wave frequency, are also reasonably precise in diagnosing advanced hepatic fibrosis[133]. MRE has obvious diagnostic precision in advanced hepatic fibrosis and cirrhosis in hepatic transplantation receivers, independent of the extent of inflammation

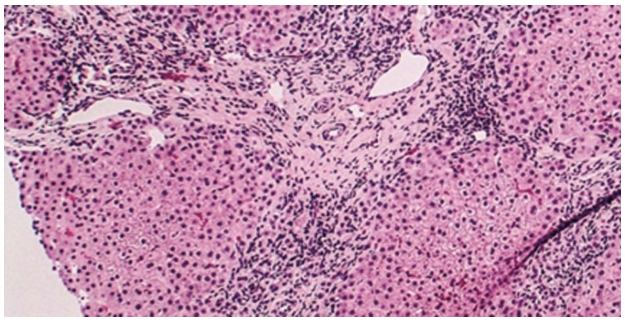

Figure 1 Histology of primary biliary cholangitis (hematoxylin and eosin staining; × 200 liver biopsy). An absence/paucity of bile ducts is seen with focal chronic inflammation in a portal area consistent with late-stage primary biliary cholangitis[9].

and BMI[134]. Magnetic resonance imaging (MRI) has potential diagnostic value for PBC, and the periportal halo sign and signal strength contribute to assessing the extent of hepatic fibrosis[135]. In addition, Gd-EOB-DTPA-enhanced MRI might offer beneficial detection approaches for hepatopathy in PBC patients[136]. In general, none of the radiologic approaches have perfect accuracy in any published study to date; however, VCTE outperformed all other non-invasive current surrogate markers of hepatic fibrosis in PBC. Due to its high acceptability and ability to predict hepatic decompensation, VCTE could be a useful tool in allocating PBC patients into different categories of risk.

HISTOPATHOLOGICAL FEATURES

Histopathologically, PBC is not only characterized by predominantly different stages of hepatic fibrosis that eventually result in cirrhosis of the liver or hepatic failure, but also as a granulomatous lymphocytic cholangitis that consequently leads to such small bile duct loss such as vanishing bile duct syndrome and cholestasis (Figures 1 and 2)[9,137]. The typical dendritic-cellular CD11c marker has markedly effective expression and significant sensitivity compared with classical hematoxylin and eosin (H and E) staining in discovering hepatic granulomatous lesions related to PBC and other illnesses[137]. There are significantly elevated serum concentrations of IgM and earlier stages of illness in PBC patients in the presence of CD11c-positive expression hepatic granulomas[137]. There are hallmarks of immature dendritic cells, namely CD11b, decreased expression of MHC Ⅱ, IL23, CD83 and CCR7, and increased expression of C1q in granulomatous lesions from PBC and other illnesses[137]. PBC-related granulomatous lesions largely represented by B lymphocytes and IgM-positive plasmocytes together with macrophagocytes[137]. Put simply, dendritic cells play a pivotal role in the etiopathogenesis of granulomas, regardless of their origin[137]. More specifically, hepatic granulomas may be caused by the reciprocities between IgM and immature dendritic cells in PBC[137]. In addition, spleen tissue samples from PBC demonstrated accumulation of IgM-positive cells, along with CXCL13-positive cells, in CD21-positive lymph follicles[138]. CXCL13-positive follicular dendritic cells might be conducive to the production of excess IgM from the spleen[138]. The deviant expression of mitochondrial autoantigens and subsequent autoimmune mechanism in PBC may be closely associated with deregulated autophagy and the following cellular senescence in biliary epithelial cells (BECs)[139]. Activated NKT cells may prompt BEC death, leading to the development of PBC[140]. A novel hepatic histological grading system for PBC, proposed by Japanese scholars, includes the degree of chronic cholangitis activity (CA 0-3), which is associated with clinic-laboratory characteristics of cholangitis, and hepatitides activity (HA 0-3), which is related to the progression of cirrhosis-related conditions[141]. French scholars proposed another novel histological scoring system for PBC, which covers assessment of hepatic fibrosis, leukomonocyte interface hepatitides, and absence of biliary ducts[142]. Abnormal expression of K-7 in hepatic cells may serve as an accessional hallmark for predicting rapid development to hepatic failure in diagnosed asymptomatic patients with PBC[143]. In other words, the histological features of PBC, in addition to typical non-suppurative destructive cholangitis and hepatic granulomatous lesions, include portal inflammation, chronic cholestasis, hepatic changes (interface hepatitides or lobular hepatitides), and bile duct loss. The two histological classifications by Ludwig's and Scheuer's stages have been used globally for PBC staging since the 1960s, and are based on the histopathological findings of PBC. In addition, two novel histological scoring systems for PBC have been proposed by Japanese and French scholars, respectively.

DIAGNOSIS

Cholestasis, which is a general clinical manifestation in hepatic illnesses that gives rise to reactive hyperplasia of the bile ducts, is the main complication in PBC patients. PBC diagnosis can be made in a patient *via* high serum AMAs in the presence of significantly elevated serum ALP, after ruling out other common or rare causes of cholestasis, such as viral hepatitides, drug-induced hepatic damage, alcoholic liver disease, intrahepatic cholestasis of pregnancy, progressive familial intrahepatic cholestasis, autoimmune hepatitides (AIH), primary sclerosing cholangitis (PSC), immunoglobulin G4-associated sclerosing cholangitis (IgG4-SC), and autoimmune hepatic illnesses overlap syndrome (PBC/AIH, PSC/AIH, PBC/PSC, PBC/IgG4-SC), as well as biliary obstructions such as biliary calculi, biliary ascariasis, biliary tract inflammation, postoperative bile duct benign stricture, pancreatic pseudocyst, cholangiocarcinoma, and pancreatic head carcinoma. PBC diagnosis requires two of the three following objective criteria: (1) biochemical proof of intrahepatic cholestasis based primarily on elevated levels of serum ALP greater than or equal to 1.5 times the upper limit of normal (ULN) for more than

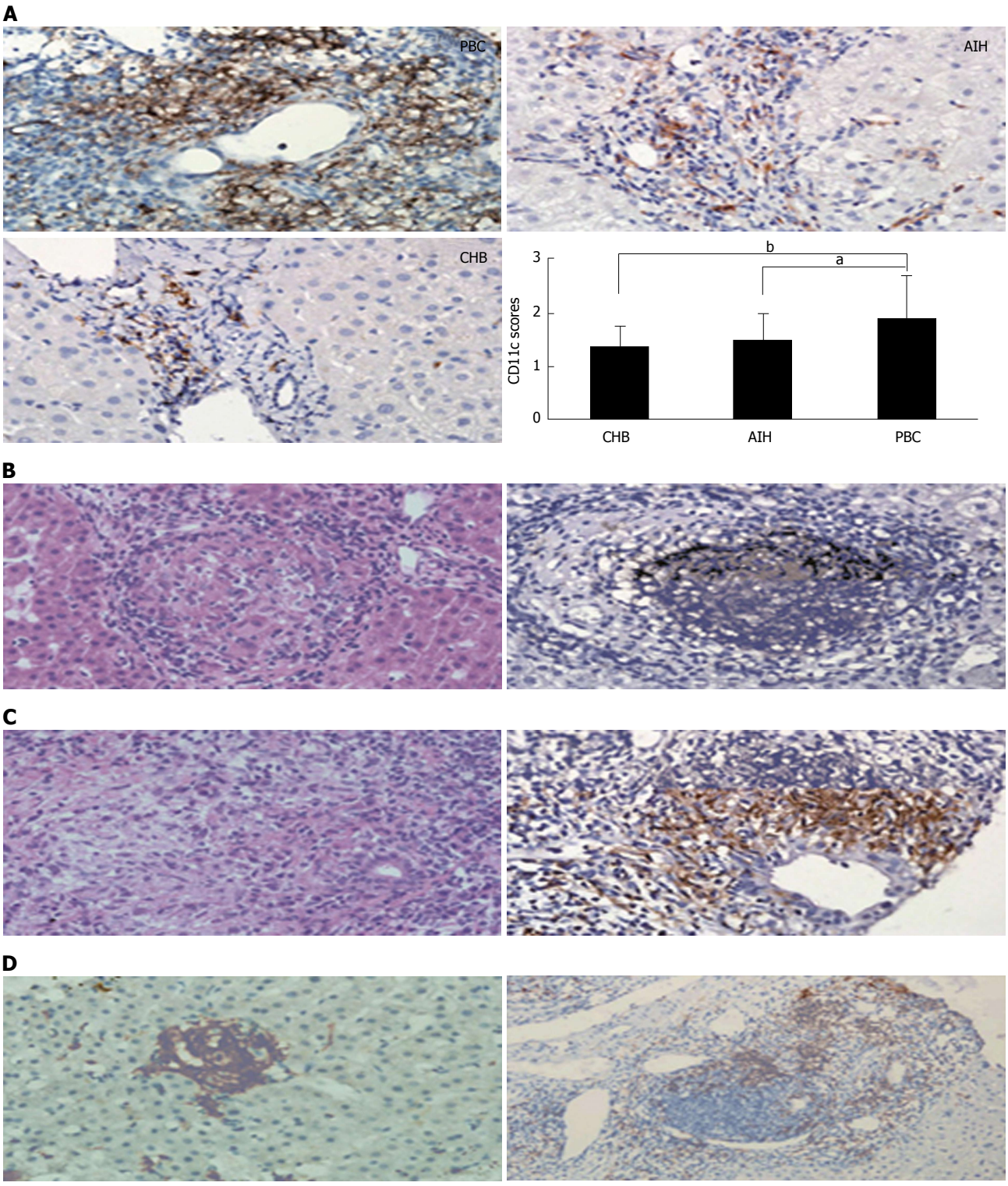

Figure 2 Histology (hematoxylin and eosin staining) and immunochemical staining of primary biliary cholangitis. A: Magnification × 400; B: Magnification × 400; C, D: Magnification × 400 (left); Magnification × 200 (right). PBC livers demonstrated observably stronger portal area immunostain for CD11c, the position of CD11c sedimentation scored on a 0-4 scale to be compared among PBC, AIH and CHB patients ($^{a}P < 0.05$, $^{b}P < 0.01$) (A); PBC hepatic granulomatous lesions were classically situated within portal areas, generally near or around the impaired bile duct (B); Hepatic granulomatous lesions were also occasionally detected in the liver lobule or close to the germinal center (C)[137]. PBC: Primary biliary cholangitis; AIH: Autoimmune hepatitides; CHB: Chronic hepatitis B.

24 wk; (2) presence of serum titers of AMAs greater than or equal to 1:40; and (3) liver histology characterized by non-suppurative cholangitis and granulomatous destruction of interlobular bile ducts[9,10,144]. Furthermore, patients with PBC frequently have elevated serum levels of ALT, AST and IgM[144].

DIFFERENTIAL DIAGNOSIS OF PBC

Autoimmune liver diseases (AILDs) cover PBC, PSC,

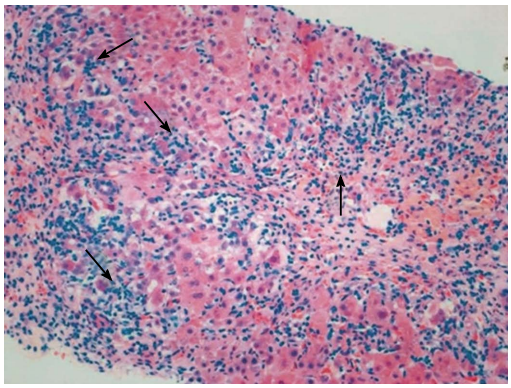

Figure 3 Histology characteristic of autoimmune hepatitis (hematoxylin and eosin staining). The inflammatory cell infiltration feature of autoimmune hepatitides is composed of leukomonocytes, monocytes/macrophagocytes, and plasmocytes (interface hepatitis, arrows) in the portal and periportal areas[145].

IgG4-SC and AIH; PBC should therefore be distinguished from AMA-positive AIH, PSC, or IgG4-SC. In addition, some AILDs patients present with features of PBC or PSC and AIH or IgG4-SC, either simultaneously or consecutively. They are traditionally deemed as obvious entities, although shared modes in so-called "overlap syndrome" have been recognized across the spectrum. The diagnosis of such overlap syndromes as PBC/AIH, PSC/AIH, PBC/PSC, and PBC/IgG4-SC is still challenging, but it is indispensable to diagnosis due to its rapid progression to cirrhosis and liver failure. Overlap syndromes should be considered in AIH patients with cholestatic findings, concurrent inflammatory bowel disease (IBD), or steroid-refractory disease. Clinical, biochemical, immunological, histological, and bile duct imaging characteristics contribute to the diagnosis of AILD overlap syndrome.

AMA-positive AIH

AIH is an immune-mediated severe hepatopathy characterized by elevated serum levels of ALT, AST and IgG, a high percentage of circulating non-organ-specific autoantibodies, and histologically with interface hepatitis (Figure 3)[145]. The incidence rates of AIH display a positive correlation with the national HDI (r = 0.638, P = 0.014) and the income index (r = 0.649, P = 0.012)[13]. AIH is divided into type 1, which is defined according to the seropositivity of smooth muscle autoantibody (SMA) and/or ANAs, and type 2, which is defined according to the seropositivity of liver-kidney microsome type 1 and/or liver cytosol type 1[145]. The non-classical clinical phenotypes of AIH, particularly AMA-positive AIH, should be distinguished from PBC. In general, AMAs-M2 antibody is specific to PBC patients, but may also be occasionally discovered in certain AIH patients[146]. Efficient means of discriminating between AIH and PBC are required, due to the fact that their clinical process and treatment are disparate[146]. One recent study has shown that antibodies to filamentous-actin (anti-F-actin) protein can not only be considered the serological marker of type 1 AIH, but may also predict AIH recurrence[147]. Furthermore, the application of repetition hepatic biopsy is an efficient method for AIH diagnosing comorbid liver conditions[148]. Although certain AIH patients were detected to be AMAs-M2 (+), the titers were markedly reduced compared to PBC patients[146]. During the follow-up period, the serum titers of AMAs-M2 were reduced in AIH patients[146].

PSC

PSC is an immune-mediated chronic idiopathic cholestatic hepatobiliary illness characterized by progressive fibrosis and the stricturing of medium and large-sized extrahepatic and/or intrahepatic bile ducts (Figure 4)[9]. PSC is related to an elevated risk of cholangiocarcinoma and when IBD is present for colorectal carcinoma. Approximately 75% of PSC patients suffer from IBD; primarily ulcerative colitis (UC)[9]. Although no statistical correlation between PSC incidence and HDI was discovered (r = 0.116, P = 0.706), the income index was positively related to PSC incidence (r = 0.599, P = 0.031)[13]. PSC classically evolves slowly over 10 to 15 years, eventually leading to biliary cirrhosis and premature death due to decompensated hepatic illness in the majority of patients[149]. Additional complications of PSC include hepatic osteodystrophy, dominant bile duct stenosis, recurrent cholangitis, and such disease-related malignancies as hepatobiliary (especially cholangiocarcinoma), pancreatic, and colorectal (especially with IBD) carcinoma[149]. In one recent study, 65% of patients with long-term IBD had subclinical PSC related to progressive IBD, with no biochemical anomalousness and mild illness, based on magnetic resonance cholangiography findings[150]. There is currently no specific biomarker for PSC, although the prevalence of p-ANCA has been reported to range from 33% to 85% in patients with PSC[149]. Other non-specific autoantibodies in PSC cover ANAs and SMA[149].

IgG4-SC

IgG4-SC is an immune-mediated peculiar sclerosing cholangitis of unknown etiopathogenesis that is frequently related to autoimmune pancreatitis (AIP)[151]. The diagnosis of IgG4-SC is performed on the basis of a combination of the following four standards: (1) distinctive cholangiography features; (2) elevated serum levels of IgG4; (3) concurrence of IgG4-associated illnesses, excluding those of the bile duct; and (4) typical histopathological characteristics[151,152]. Moreover, the efficiency of corticosteroid treatment is a selectable additional diagnostic standard to affirm a precise diagnosis of IgG4-SC[151,152]. Typical characteristics of IgG4-SC may be divided into four types on the basis of the stenosis regions disclosed by endoscopic retrograde cholangiography and anti-diastole (Figure 5)[152].

PBC/AIH overlap syndromes

PBC/AIH overlap syndrome patients, including both characteristics of PBC and AIH, were diagnosed based on the Paris diagnostic criteria proposed by Chazouillères

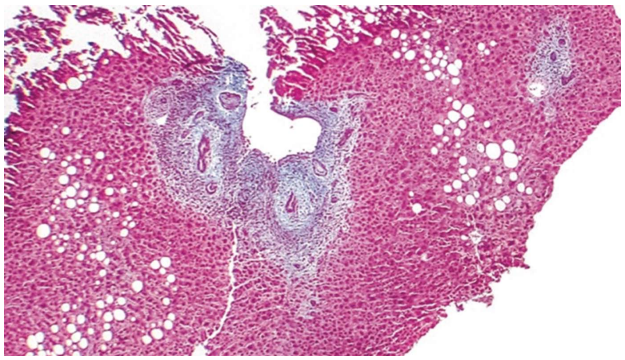

Figure 4 Histology of primary sclerosing cholangitis. Trichrome × 40, liver biopsy. Low power view demonstrating a focal lesion typical for primary sclerosing cholangitis. Periductular layered fibrosis (featuring "onion skin" pattern) is found with edema and inflammation around the interlobular bile ducts in the center of the field[9].

et al[153]. Characteristics of PBC were the following: (1) serum ALP levels more than twice the ULN value and/or γ-GT five or more times the ULN value; (2) serum AMA positivity; and (3) histopathological evidence of bile duct damage[153]. Characteristics of AIH were: (1) ALT elevation at a minimum of five times the ULN value; (2) levels of IgG at a minimum of twice the ULN valve and/or SMA positivity; and (3) hepatic biopsy revealed interface hepatitides in the presence of moderate to serious periportal lymphocyte infiltration[153]. Diagnosis of PBC/AIH overlap syndrome was considered with the presence of 2/3 of the criteria[153]. According to the Paris diagnostic criteria: (1) PBC/AIH overlap syndromes are uncommon; (2) flares of AIH can appear either voluntarily or under UDCA; and (3) a combination of corticosteroids and UDCA is requested in the majority of patients in order to achieve the best efficient biochemical response[153]. In addition, the revised and simplified diagnostic criteria for AIH were established by the International Autoimmune Hepatitis Group (IAIHG) in 1999[154] and 2008, respectively[155]. The latter is constructed on the basis of four clinical components that appear to be more peculiar in PBC/AIH patients[156]. The simplified diagnostic criteria seem to be more effective in comparison with the Paris diagnostic criteria and revised diagnostic criteria for patients with PBC/AIH overlap syndrome[157]. However, the IAIHG's position statement on this controversial issue suggests that patients with AILDs should be classified on the basis of their dominant characteristics as PBC, AIH or PSC/small duct PSC, and that those with overlapping characteristics should not be referred to as unique diagnostic entities[158]. Combination treatment with budesonide and UDCA was more efficacious than UDCA monotherapy for PBC/AIH overlap syndrome[159]. Furthermore, combination treatment with immunosuppression and UDCA offered better short-term responses in PBC/AIH overlap syndromes[160].

PSC/AIH overlap syndrome

PSC/AIH overlap syndrome is a comparatively infrequent variant of PSC[161]. There were remarkable distinctions in the below listed arguments, such as mean age ($P < 0.01$), serum levels of AST ($P < 0.005$), ALT ($P < 0.005$), and IgG ($P < 0.0001$) in PSC/AIH overlap syndromes compared with "typical" PSC patients[161]; the former seemingly profits from combination treatment with UDCA and immunosuppression, while survival is distinctly superior in the latter[161]. In addition, the clinical course of PSC/AIH overlap syndrome appears to be superior to typical PSC, suggesting that immunosuppression likely has an active efficacy on the development of PSC composition[162].

PBC/PSC overlap syndromes

PBC/PSC overlap syndromes demonstrating the clinical manifestations of both PBC and PSC are an exceedingly uncommon condition that has been reported in a mere eight published cases, including the previously mentioned two cases[163].

PBC/IgG4-SC overlap syndromes

IgG4-SC and PBC are two distinct autoimmune liver diseases. Approximately 90% of IgG4-SC patients have AIP, so therefore the presence of AIP may contribute to the diagnosis of IgG4-SC[151,152]. Nevertheless, PBC/IgG4-SC overlap syndrome is an extremely rare condition that has been reported in very few published cases to date, with the diagnosis of PBC/IgG4-SC overlap syndromes without the coexistence of AIP being particularly difficult[164]. Serum IgG4 concentrations may be worthwhile detecting in patients with PBC intractable to routine therapy[164].

NATURAL HISTORY OF PBC

The natural history pattern of PBC has observably changed over the past 20 years due to earlier diagnosis and the introduction of UDCA treatment. However, little is known about the natural history of PBC patients without efficient therapy. Hence, an epidemiological survey of the natural history of PBC patients in the absence of treatment might contribute to a greater understanding of the natural history of patients with UDCA-resistant PBC and in developing criteria for estimating UDCA response. A recent study demonstrated greatly reduced serum levels of ALP and very slight fluctuations in the other biochemical parameters of PBC patients treated with placebo at the 2 year follow-up period[165]. There was histological development in 39.4% of patients treated with placebo and a mild worsening of histological grade after 2 years of research[165]. In the meantime, histological progression was observed in 39.4% of the placebo-treated patients, with a moderate deterioration in histological scores noted after 2 years. Furthermore, the pooled 2 year rates of death, transplant, and progression of varicosities were 11.4%, 8.7% and 10.6%, respectively, in patients treated with placebo[165]. The natural history of PBC patients with AIH characteristics significantly differs from those without AIH characteristics[160]. In addition, although considered to possess a higher prevalence

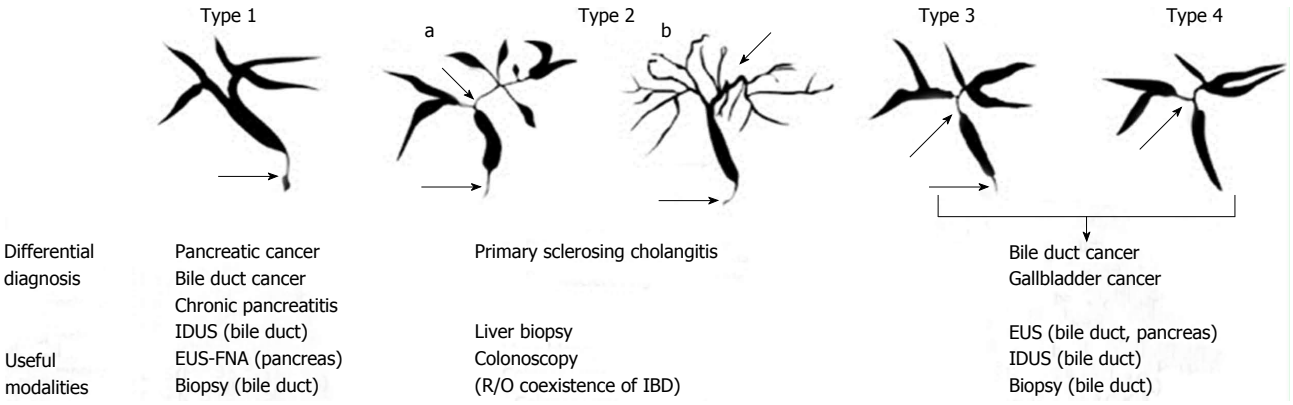

Figure 5 Endoscopic retrograde cholangiography classification of IgG4-SC and anti-diastole. Stricture is distributed only in the distal choledoch in type 1; stricture is widely spread throughout in the intrahepatic and extrahepatic biliary ducts in type 2. Type 2 is once more divided into two. Expanded stenosis of the intrahepatic biliary ducts in the presence of pre-stenotic expansion is diffusely spread all over in type 2a. Stenosis of the intrahepatic biliary ducts in the absence of pre-stenotic expansion and decreased biliary tree are diffusely spread throughout in type 2b; stricture is found in both porta hepatis damage and the distal choledoch in type 3; stenosis of the biliary ducts are found only in the porta hepatis damage in type 4. IDUS: Intraductal ultra-sonography; EUS-FNA: Endoscopic ultrasonography-guided fine-needle aspiration[152]; IBD: Inflammatory bowel disease.

rate of AMAs, first-degree relatives of PBC patients have a lower risk of developing PBC over time, especially in those without baseline biochemical test evidence of intrahepatic cholestasis[166].

THERAPY OF PBC

UDCA

The optimal dosage for UDCA of 13-15 mg/kg per day is the standardized treatment for PBC[9,10], as it can postpone its development, improve long-term clinical outcomes, and is extremely safe and well-tolerated. Therefore, reliable identification of so-called treatment non-response to UDCA is very important, not only for selecting PBC patients who could benefit from new therapeutic approaches, but also for discerning those who are at low risk of developing end-stage PBC. The biochemical response to UDCA after 1 year of treatment in PBC has been deemed to be a powerful predictive indicator of long-term clinical outcomes and thus facilitate the rapid recognition of patients requiring novel treatment methods. However, another study demonstrated that, in comparison with biochemical responses assessed after 12 mo of UDCA treatment, biochemical responses at the 6 mo mark showed higher positive predictive value and negative predictive value, as well as lower negative likelihood ratio according to all criteria used in the Paris, Toronto, Barcelona, and Ehime definitions[167]. Therefore, the biochemical responses at the 6th month may be served as a new standard of prediction substitute for those assessed after 12 mo of UDCA treatment[167]. In addition, the UK-PBC risk scores (composed of baseline albumin, bilirubin, platelet count, ALT, AST and ALP) after 1 year of UDCA treatment might not only be available for identification in higher risks patients for rigorous surveillance and 2nd-line treatments, as well as lower risks patients who could possibly be tracked after observation during initial treatment, but the 5-, 12- and 15-year risk scores might also be considered extremely precise[168].

Budesonide

Budesonide is a corticosteroids receptor/pregnane X receptor (PXR) agonist[9,10]. Treble treatment with budesonide (6 mg/d), UDCA (13-15 mg/kg per day), and mycophenolate mofetil (1.5 g/d) may afford an advantage in non-cirrhotic PBC patients with characteristics of serious illness without biochemical response to UDCA[169]. Combination therapy of budesonide (6 mg/d) and UDCA (15 mg/kg per day) was able to ameliorate the plasma biochemical index of hepatic function and hepatic histology, particularly in PBC patients with hepatic fibrosis (grade Ⅰ-Ⅲ), whereas the treatment effectiveness of UDCA alone was principally on lab results[170]. Although larger studies are still required, the preparatory results of agents targeting PXR, such as budesonide, have been encouraging, particularly in subsets of patients with PBC, and may mark a new therapeutic era[169,170].

Methotrexate

The immunosuppressive agent methotrexate (MTX) has a long history in the treatment of PBC, however little is known about its action mechanisms and roles, if any[9,10]. MTX was assessed for PBC treatment, which is currently recommended only in patients for whom PBC failed to respond adequately to UDCA and in AIH/PBC overlap syndromes[171,172]. PBC patients with characteristics of AIH should be considered for immunosuppressive therapy[158], with the therapeutic goal being to attain normal serum aminotransferase levels and histological improvement[158]. In patients who responded improperly to UDCA, MTX observably improved hepatic enzyme tests and hepatic histology[171]. Combination therapy of UDCA and MTX brought about continuous clinical anesis in a subgroup of PBC patients, and the response to a combination of MTX and UDCA appears to be more time-proof[172].

FXR agonist

FXR is the receptor for primary bile acids expressed in enterohepatic tissues, where it regulates bile acid uptake, metabolism, and disposal, and has been considered

a significant target for intrahepatic cholestatic illness therapy[173-176]. Obeticholic acid (OCA) is a semi-synthetic bile acid analogue for 6α-ethyl-chenodeoxycholic acid that is nearly 100-fold more potent than chenodeoxycholic acid (CDCA) and is a powerful, first class alternative FXR agonist derived from primary human bile acid CDCA, the natural endogenous FXR agonist[173]. OCA is being developed by Intercept Pharmaceuticals for the treatment of a variety of intrahepatic cholestatic illnesses, and has lately been permitted expedited approval in the United States for the treatment of PBC in combination with UDCA in adults with inappropriate response to UDCA or as monotherapy in adults unable to tolerate UDCA[174]; OCA (Ocaliva™) is in preregistration for this function in the European Union[174]. A randomized controlled clinical trial showed that treatment with OCA (10-50 mg/d) observably decreased the serum concentrations of γ-GT, ALP and ALT in PBC patients with inappropriate response to UDCA, in comparison with placebo[175]. Furthermore, PBC patients treated with OCA (10 mg/d) had the lowest incidence rates and seriousness of itching[175]. Clinical trials demonstrated the treatment effectiveness of OCA in PBC without biochemical response to UDCA, as evidenced by changes in laboratory parameters substituted for long-term clinical outcomes[176]. Dose-dependent itching is a usual side-effect of OCA, but can be overcome *via* dose-titration[176]. Furthermore, INT-767, which is another steroidal semi-synthetic bile acid analogue, has been testified to be able to modulate the activity of monocytes and macrophages, decrease inflammation though the inactivation of NF-κB *via* a protein kinase A dependent pattern, and decrease hepatic damage by promoting biliary bicarbonate excretion as a dual FXR and TGR5 agonist[173].

Cyclosporine A

Recurrence of PBC after hepatic transplant has been proven to adversely influence transplant and patient survival. Protective potencies of cyclosporine A (CyA) against PBC recurrence after hepatic transplant have been reported[177]. Changing from tacrolimus to CyA was possible without sequelae, with no patients demonstrating recurrence of PBC[177]. Therefore, CyA might be serviceable for the prevention of PBC recurrence after living-donor hepatic transplant[177]. However, a retrospective multicenter study in Japan showed that although there was no influence on patient survival, original immunosuppression with CyA was considered to be major risk for PBC recurrence after hepatic transplant[178]. However, in subset analysis, switching from tacrolimus to CyA within 12 mo reduced recurrence[178].

Fibrates

Fibrates, including bezafibrate and fenofibrate, may be useful for treating asymptomatic patients with PBC who exhibit inappropriate response to UDCA[179-183]. A nationwide retrospective survey in Japan demonstrated that normalizing serum ALT concentrations with accessional bezafibrate therapy observably reduced the occurrence of hepatic illness-associated clinical signs in symptomless patients, with PBC responding incompletely to UDCA[179]. Moreover, long-term combined bezafibrate and UDCA treatment in PBC not only observably ameliorated the Mayo risk score and serum concentrations of ALP, but also observably elevated the serum concentrations of creatinine[180]. Hence, it is very important to consider adverse drug reaction associated with long-term combination treatment[180]. Although the treatment effectiveness of fenofibrate has been confirmed to be related to obvious improvement in ALP, decompensation amelioration, and hepatic transplantation-free survival in patients with PBC who reveal inappropriate responses to UDCA, fenofibrate should be more prudently applied in PBC, with frequent supervision for biochemical/clinical maladjustment[181]. Long-term fenofibrate therapy as a second-line auxiliary drug in PBC patients without appropriate response to UDCA was considered to be safe and efficient in ameliorating ALP, but did not markedly decrease the evaluated possibility of hepatic-associated death or demand for hepatic transplant[182]. In addition, the optimal dosage for fenofibrate (100-200 mg/d) seems to be efficient for assistant treatment in PBC patients without optimal biochemical response to UDCA[183].

Rituximab

Rituximab, an anti-CD20 monoclonal antibody that selectively depletes B cells, which are precursors of the autoantibody-producing plasmocytes, may be successfully used in autoimmune-mediated hepatic illnesses[184]. Selective depletion of B-cells with rituximab was safe and related to an obvious reduction in autoantibody product, but had limited biochemical effect in PBC patients without optimal biochemical response to UDCA[184]. The efficacy of B-cell depletion with rituximab therapy and the significant improvement in both biochemical and immunologic markers that it provides has been found in PBC patients with inappropriate biochemical response to UDCA[185]. The results of these studies demonstrate that depletion of B-lymphocyte affects the inductiveness, maintenance, and activation of both B- and T-lymphocytes, and offers an underlying principle for the treatment of PBC patients with incomplete response to UDCA[184,185].

Mesenchymal stem cells

Mesenchymal stem cell (MSC) transplantation is considered to be safe, and has been diffusely tested in autoimmune hepatic disease clinical trials with encouraging results[186,187]. MSC transplantation could modulate the systemic immune response and promote recovery in hepatic inflammation of PBC[186,187]. One single-arm clinical trial has shown that umbilical cord-derived MSC (UC-MSC) transplantation is viable and well-tolerance in patients with PBC, who response only partly to UDCA therapy, hence the need for a new treatment method for PBC patients in this subset[186]. However, the exact effect of UC-MSC transplantation in patients with PBC

still requires confirmation by a larger placebo-controlled randomized clinical trial[186]. In addition, allogeneic bone marrow MSC transplantation has been confirmed to safely improve histologic fibrosis and hepatic function in UDCA-resistant PBC patients[187].

Liver transplantation

At present, liver transplantation (LT) is still a lifesaving approach with outstanding results for end-stage PBC patients[177,178]. Although the 15 year survival of PBC patients was confirmed as 52.6% after LT, regrettably, the recurrent rates of PBC were 21%-37% and 43% at 10 and 15 years after LT, respectively[178,188]. Though there is still no specific treatment for recurrent PBC (rPBC), cyclosporine A and UDCA may be useful for the prevention of rPBC after LT[177,188,189]. Furthermore, the expression of mitochondrial proteins in small biliary ducts may be a beneficial diagnostic hallmark for both end-stage PBC and rPBC after LT[190].

CONCLUSION

In the past decade, recent advances in PBC have attempted to improve the accuracy of the disease's diagnosis and prognosis, as well as affording the chance to refine therapeutic methods. Promising novel therapies, including budesonide, fibrates, and rituximab, are being tested in PBC patients on the basis of understanding thoroughly the cellular and molecular mechanisms touched upon in all histological stages of PBC, from the early autoimmune-mediated bile duct epithelial cell damage to the destructive and illness-persistent influences of intrahepatic cholestasis, and finally giving rise to hepatic fibrosis and hepatic cirrhosis progression. Although much progress has been seen in the last 5 to 10 years, including in the diagnosis and treatment of PBC, the ultimate challenge for physicians is reducing UDCA non-responders and recurrent PBC after liver transplantation. Some novel therapeutic agents, including FXR agonists like OCA, FXR/TGR5 agonists like as INT-767, and PPAR-alpha have been identified as novel targets for drug development, with further investigation in PBC-related clinical trials still being implemented. Results of ongoing clinical trials and burgeoning therapeutic paradigms for PBC patients will likely further improve medical management and stride toward accurate treatment in the near foreseeable future.

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ORIGINAL ARTICLE

Basic Study

Performance of cold-preserved rat liver Microorgans as the biological component of a simplified prototype model of bioartificial liver

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Abstract

AIM

To develop a simplified bioartificial liver (BAL) device prototype, suitable to use freshly and preserved liver Microorgans (LMOs) as biological component.

METHODS

The system consists of 140 capillary fibers through which goat blood is pumped. The evolution of hema-

tocrit, plasma and extra-fiber fluid osmolality was evaluated without any biological component, to characterize the prototype. LMOs were cut and cold stored 48 h in BG35 and ViaSpan® solutions. Fresh LMOs were used as controls. After preservation, LMOs were loaded into the BAL and an ammonia overload was added. To assess LMOs viability and functionality, samples were taken to determine lactate dehydrogenase (LDH) release and ammonia detoxification capacity.

RESULTS

The concentrations of ammonia and glucose, and the fluids osmolalities were matched after the first hour of perfusion, showing a proper exchange between blood and the biological compartment in the minibioreactor. After 120 min of perfusion, LMOs cold preserved in BG35 and ViaSpan® were able to detoxify 52.9% ± 6.5% and 53.6% ± 6.0%, respectively, of the initial ammonia overload. No significant differences were found with Controls (49.3% ± 8.8%, $P < 0.05$). LDH release was 6.0% ± 2.3% for control LMOs, and 6.2% ± 1.7% and 14.3% ± 1.1% for BG35 and ViaSpan® cold preserved LMOs, respectively ($n = 6$, $P < 0.05$).

CONCLUSION

This prototype relied on a simple design and excellent performance. It's a practical tool to evaluate the detoxification ability of LMOs subjected to different preservation protocols.

Key words: Rat liver Microorgans; Cold preservation; BG35 preservation solution; Bioartificial liver device; Acute liver failure

Core tip: This work describes the development of a simplified bioartiticial liver prototype (BAL, suitable to use rat liver Microorgans (LMOs) as biological component, and the evaluation of these tissue slices performance in this new model. We demonstrate that the minibioreactor constructed allows a good performance of fresh and cold preserved LMOs, showing the importance of architecture and model configuration on these devices design. Besides its application as BAL, this minibioreactor could serve as a suitable laboratory tool to evaluate the behavior and functionality of LMOs subjected to different preservation protocols due to its simple design and the utilization of standard materials.

Pizarro MD, Mediavilla MG, Quintana AB, Scandizzi ÁL, Rodriguez JV, Mamprin ME. Performance of cold-preserved rat liver Microorgans as the biological component of a simplified prototype model of bioartificial liver. *World J Hepatol* 2016; 8(33): 1442-1451 Available from: URL: http://www.wjgnet.com/1948-5182/full/v8/i33/1442.htm DOI: http://dx.doi.org/10.4254/wjh.v8.i33.1442

INTRODUCTION

To date, acute liver failure continues to be a defeating syndrome in the clinical practice due to its rapid development and its high risk of mortality. Patients always require a multidisciplinary approach for adequate management and subsequent organ transplantation. Unfortunately, the scarcity of donor organs often limits liver transplantation in time. Among the different approaches that have been tested to maintain the patients until transplantation and/or to facilitate self-regeneration of the damaged liver is the bioartificial liver (BAL)[1]. In BAL devices, the plasma of the patient is treated by its circulation through a bioreactor that accommodates a biologically active component which performs the diminished or lacking hepatic metabolic functions. Ammonia detoxification is one key task this biological component must carry out because increased blood levels of this metabolite are toxic to the central nervous system[2].

Investigations concerning the development of BAL devices containing normal hepatocytes are still being conducted[3,4]. Some researchers have chosen to employ immortalized hepatocytes[5] while others have focused their efforts in preparing bioreactors housing isolated hepatocyte with or without extra-cellular matrix and structural components[6,7].

Our group has already reported the construction of a minibioreactor (MBR) consisting in a hollow fiber based cartridge with blood flowing through the fiber lumens. Rat isolated hepatocytes were used as the biological component, showing an effective ammonia depuration rate[8]. Since it is thought that the "ideal" biological component for a BAL should contain all the constituents present in a liver lobule in order to obtain maximal function, we became interested in evaluating the performance of rat liver Microorgans (LMOs). These are thin fragments of tissue that retain the basic microarchitecture of the liver lobe, including cell to cell contact and cell to cell communication[9,10].

On the other hand, in order to become a useful clinical tool, any BAL device must be ready to use when a patient needs it. This means the biological component should be not only available but viable and functional. In a previous work we have presented BG35 [Bes-Gluconate-Polyetyleneglycol (PEG) 35 kDa], a novel preservation solution, that exhibited an efficacy similar to that of the ViaSpan® to give protection to LMOs against injury produced by the ischemia followed by reoxygenation suffered as a consequence of cold preservation[11].

The objectives of this work were to develop a simplified prototype BAL suitable to use LMOs as biological component, and to evaluate the performance of fresh and cold preserved rat LMOs in this model.

MATERIALS AND METHODS

MBR

The MBR (Figure 1) was constructed using a 25 cm^2

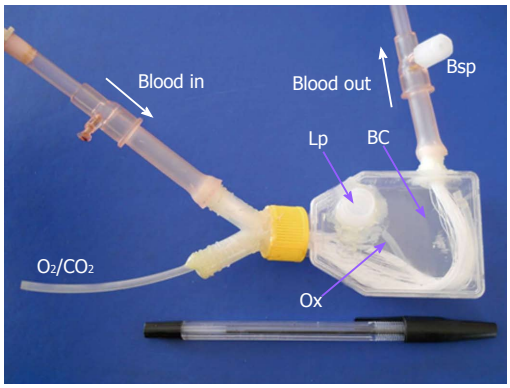

Figure 1 Minibioreactor device. BC: Biological compartment; Lp: Loading port; O_2/CO_2: Carbogen supply line; Ox: Silicone tube oxygenator; Bsp: Blood sample port.

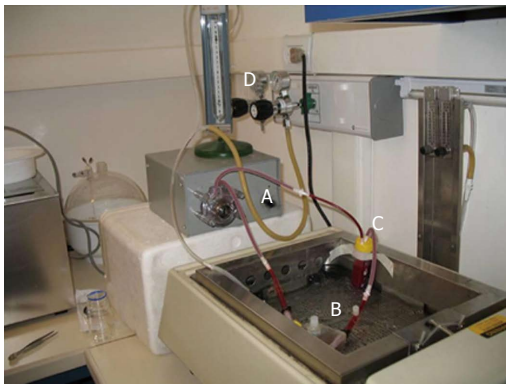

Figure 2 *In vitro* perfusion system. A: Peristaltic pump; B: Minibioreactor; C: Blood reservoir; D: External oxygen supply.

culture flask, adding a loading port (Lp) on top, a Y-polypropylene connector (Nalgene cat. 6152-0375) onto its lid and a simple connector at one side. One hundred and forty Polyamix™ hollow fibers (Gambro, Hechingen, Germany) are assembled to these two connectors and sealed with epoxy glue. The diverse parts that made up the MBR can be appreciated in Figure 1.

In the MBR, two main compartments can be distinguished: The hollow fibers internal lumen constitutes the blood compartment, while the biological compartment (BC) comprises the space outside the hollow fibers (total volume of 50 cm^3). A silicone tube (Ox, oxygenator) enters to the BC through the Y connector and allows the oxygenation of the BC fluid. The LMOs were placed in the biological compartment through the Lp port and released on the flat surface of the device. This allows a homogeneous distribution of LMOs and a better oxygenation and exchange of solutes.

Components of the perfusion system and its manipulation

The components of the perfusion system used are detailed in Figure 2. The blood reservoir, that contains a clot filter, and the MBR are immersed in a water bath at 37 ℃. The peristaltic pump (model 7554-60, Cole Parmer, United States) allows the recirculation of heparinized goat blood (total volume: 35 mL) through all the system at a constant flow of 9 mL/min.

In all the experiments performed, we first filled the system with goat blood *via* the inlet tube and then inoculated 1 g of LMOs into the BC (or Krebs-Henseleit Reoxygenation media (KHR) alone, in the experiments done to characterize system operation, which composition is shown in page 11). The silicone tube was used to oxygenate the BC compartment with carbogen gas (95% O_2/5% CO_2) at a stable pressure of 85 mmHg. The blood pH was kept at 7.40 ± 0.50 adding 8.4% sodium bicarbonate if necessary.

To test ammonia detoxification capability of the rat LMOs, we added an aliquot of an ammonium chloride solution (approximate concentration: 350 mmol/L) to the blood in order to achieve an initial ammonia plasma concentration of 1.06 ± 0.12 mmol/L, n = 6 (blood sample t = 0). Then, we initiated blood perfusion and took blood and BC fluid samples after 60 and 120 min of operation to perform the different assays detailed below.

Characterization of the MBR perfusion system

In order to characterize the operation of the system "*in vitro*", *i.e.*, without LMOs, different MBR were perfused for 120 min with only KHR solution inside the BC compartment and the following parameters were evaluated:

Hematocrit, to determine the probable rupture of some fibers with the concomitant passage of blood to the BC, and to study the possible hemolytic action of the peristaltic pump. Blood samples were taken from blood sample port at different perfusion times and were centrifuged (1000 x g - 3 min, Rolco CH24 centrifuge). The hematocrit was calculated using the next equation:

Hematocrit (%) = red blood cells volume/blood total volume.

Plasma and extra-fiber fluid osmolality, were measured in order to monitor the correct transfer of fluids between blood and the BC, using a freezing point osmometer (Osmomat 030, Gonotec, GmbH, Berlin, Germany).

Protein analysis using fast protein liquid chromatography (FPLC), in order to study the diffusive properties of the hollow fibers used in the construction of the MBR and to determine the possible passage of plasmatic proteins towards the BC, especially those belonging to the immune system that could damage the biological component (described below).

Metabolite concentration in both compartments, such as glucose and ammonia, to determine their correct distribution in the MBR (described below).

Hemolysis determination

Samples of plasma were taken after 0, 60 and 120 min of perfusion and hemoglobin concentration was determined using the oxyhemoglobin method[12].

Table 1 Composition of the preservation solutions ViaSpan® and BG35

	ViaSpan®	BG35
Impermeants (mmol/L)		
Lactobionate	100	
Gluconate		100
Raffinose	30	
Buffers (mmol/L)		
KH_2PO_4	25	2.5
BES		50
Substrates (mmol/L)		
Allopurinol	1	1
Glutathione	3	3
Adenosine	5	5
Glycine		15
$MgSO_4$	5	5
Colloids (g/L)		
HES	50	
PEG 35000		40
pH	7.40	7.40
Osm (mOsm/kg water)	320 ± 4	339 ± 4

Dexamethasone 16 mg/L, insulin 40 UI/L and penicillin G 200000 UI/L were added to ViaSpan® before use. Streptomycin 0.25 mg/mL and penicillin G 10 UI/mL were added to BG35 before use. All the solutions were bubbled with 100 % N2 for 45 min at 0 ℃ before use. BES: N, N-bis (2-hydroxyethyl)-2-aminoethanesulfonic acid; HES: Hydroxyethyl starch; PEG: Polyethyleneglycol.

To calculate the percentage of hemolysis, we used the following equation, described by Arnaud[13]:

Hemolysis (%) = 100 × {[Hb_S × (1 - Ht)] ÷ Hb_T}

where Hb_S is the hemoglobin content, expressed in g/100 mL, of the different samples; Hb_T is the total hemoglobin content (in whole blood), and Ht is the hematocrit value measured after 0, 60 or 120 min of perfusion.

FPLC analysis

Samples of basal plasma and BC fluid were taken after 60 and 120 min of perfusion and analyzed by Gel Filtration Chromatography. They were centrifuged (12100 × *g* - 5 min), filtered and 100 μL were seeded in a Tricorn Superdex-200 column (30 × 1 cm, GE Healthcare, Sweden), equilibrated with 50 mmol/L Tris, 150 mmol/L NaCl buffer, pH 7.00, previously degassed by vacuum filtration. The column was manipulated using an ÄKTA-Prime equipment (GE Healthcare, Sweden), at a constant flow of 0.5 mL/min. Each sample was analyzed in duplicate. Chromatograms were registered measuring absorbance at 280 nm and, to determine the protein molecular weight, a standard calibration curve was made using a "Molecular Weights 29000-700000" kit, following the supplier's instructions (Sigma-Aldrich, St Louis, Missouri, United States).

Animals

The livers were obtained from male Wistar rats weighing 250-300 g. Animals had access to regular laboratory food for rodents and water *ad libitum*. Animals were cared in conformity with the principles and recommendations for the care and utilization of laboratory animals, suggested by the National Academy of Sciences. The rats were adapted to experimental laboratory environment for fourteen days before to experimentation. All experimental procedures were authorized by the School of Biochemical and Pharmaceutical Sciences Institutional Animal Care and Use Committee (Res No. 139/2011).

Preparation of rat LMOs

LMOs were manually cut from rat livers into slices of 338 ± 27 μm thickness, n = 25. They were cut using a microtome blade attached to a plastic handle. We performed all the manipulations on ice (at 0 ℃) to decrease tissue injury, and on top of a paper filter to avoid the pieces of livers from sliding what could impede the correct cutting of the tissue[14].

Subsequently, LMOs were allocated in various solutions. Control group (non-preserved or fresh) LMOs were suspended in KHR and directly put in the MBR perfusion. KHR buffer was composed as follows: 114 mmol/L NaCl, 25 mmol/L $NaHCO_3$, 1.2 mmol/L KH_2PO_4, 1.2 mmol/L $MgSO_4$, 4.8 mmol/L KCl, 1.5 mmol/L $CaCl_2$, 10 mmol/L HEPES, 25 mmol/L glucose, 5 mmol/L fructose, 1 mmol/L allopurinol, 3 mmol/L glycine, 10 μmol/L adenosine, 6 mmol/L ornithine, 10 mmol/L sodium lactate; pH 7.40, 328 ± 7 mOsm/kg water (n = 6)[15]. Preserved LMOs were stored 48 h in BG35 and ViaSpan® solutions (Table 1) before MBR perfusion as explained in the next section.

Preservation of LMOs

As it was stated, in the case of the preserved groups, LMOs were stored in two different preservation solutions. Fifty LMOs were preserved during 48 h at 0 ℃[11] in a crystal flask immerse in 50 mL of one of these preservation solutions: (1) ViaSpan® (Bristol-Myers Squibb Pharmaceutical Limited; ViaSpan® group); and (2) BG35 (Bes-Gluconate plus 4% PEG 35 kDa; BG35 group).

The composition of the preservation solutions used are shown in Table 1. A period of 48 h of preservation was selected since in initial investigations (data not exposed) the viability evaluated by lactate dehydrogenase (LDH) leakage was modestly changed by 1 d of cold ischemia, but a pronounced increase was observed after 2 d.

After 48 h of cold preservation, LMOs were completely rinsed with a flush solution earlier reported by our group[16] to fully eliminate residual cold preservation solution. After that, LMOs were placed into the MBR.

LDH release

Viability of LMOs was tested by LDH release. LDH activity was determined in the BC fluid and the slices as earlier explained[17]. Data are shown as the percentage of the total enzyme activity released into the incubation medium.

Measurement of plasma and BC fluid ammonia concentrations

Samples of blood and BC fluid were taken at different

Table 2 Time course evolution of minibioreactor functional parameters during 120 min of perfusion

Perfusion time	$(Osm)_B/(Osm)_{BC}$	Hto (%)	Hemolysis (%)	$(Glucose)_B/(Glucose)_{BC}$	$(NH_4^+)_B/(NH_4^+)_{BC}$	QNH_4^+ (μmol)
0 min	0.94 ± 0.02	47 ± 3	0.27 ± 0.09	0.09 ± 0.04	52.8 ± 4.0	36.3 ± 1.6
60 min	1.00 ± 0.02	44 ± 5	0.59 ± 0.10	0.77 ± 0.07	1.1 ± 0.1	36.1 ± 1.6
120 min	1.00 ± 0.01	45 ± 3	0.79 ± 0.12	0.90 ± 0.06	1.2 ± 0.3	36.1 ± 1.5

B: Blood; BC: Biological compartment.

periods of time (0, 60 and 120 min), blood samples were centrifuged (12000 × *g*, 3 min) and all samples were conserved in liquid nitrogen until the determinations were performed. Ammonia was measured using the van Anken enzymatic determination in a volume of 0.8 mL consisting of 66.7 mmol/L phosphate buffer, pH 8.30, 0.14 mmol/L, NADPH, 6.5 mmol/L sodium-ketoglutarate, 2.5 mmol/L ADP, 120 UI/mL glutamate dehydrogenase (cat. #G2626, Sigma Aldrich St. Louis, MO, United States)[18].

The following equations were then used to calculate ammonia mass balance:

$Q_{B,t} = [(A)_{B,t} \times V_{B,t}] - [(A)_{B,Bas} \times V_{B,t}]$

$Q_{BC,t} = [(A)_{BC,t} \times V_{BC,t}] - [(A)_{BC,Bas} \times V_{BC,t}]$

$Q_{T,t} = Q_{B,t} + Q_{BC,t}$

Where: $Q_{B,t}$ and $Q_{BC,t}$ represent the ammonia mass at time t in blood and the BC fluid respectively; $(A)_{B,t}$ and $(A)_{BC,t}$ are the ammonia concentrations in blood and BC fluid at different times; $(A)_{B,Bas}$ is basal blood ammonia concentration; $V_{B,t}$ and $V_{BC,t}$ are the blood and BC fluid volumes, and $Q_{T,t}$ is the total ammonia mass at different times.

The ammonia detoxification capacity is expressed as the % of the initial dose detoxified at different times and was calculated using the following equation.

$\%\ Dose = 100 - [(Q_{T,t} \times 100)/Q_{T,0}]$

Where $Q_{T,0}$ is total ammonia mass at time 0.

Determination of plasma and BC fluid glucose concentrations

Glucose was determined using a commercial kit ("Glicemia Enzimática AA", Wiener Laboratories, Rosario, Argentina) and following the manufacturer's instructions.

Histology

Samples of livers from all experimental groups were fixed in 10% formaldehyde, dehydrated, embedded in paraffin, sectioned with a micrometer, stained with hematoxylin-eosin and mounted. Sections were microscopically analyzed and some aspects of the hepatic parenchyma were taken into consideration: Hepatic cell plate organization, the form of endothelial cells and hepatocytes, presence of necrotic areas and blebs in the plasmatic membrane of the hepatocytes. To perform the analyses, we used a light field microscope (Olympus Co, LTD. Model U-MDOB), equipped with a digital camera (Olympus model D-360 Zoom-3.2 megapixels of resolution).

Materials

Chemicals were purchased from Sigma (St. Louis, Missouri, United States) and were analytical grade pure.

Statistical analysis

Results are presented as mean ± SD. We performed a one-way or multifactor analysis of variance with Scheffe's multiple range test as post-test to establish the statistical significance of the differences between means. *P* values smaller than 0.05 were taken as statistically significant. The statistical review of the study was performed by a biomedical statistician.

RESULTS

Time course evolution of MBR functional parameters during 120 min of perfusion

In order to characterize the "*in vitro* system" operation, different MBR were perfused for 120 min, without any biological component. The mean data of six individual runs are shown in Table 2. The plasma/BC relationship did not change during the experiments. Plasma and KHR solution osmolalities were arrived to equilibrium after the first hour of perfusion, demonstrating a proper exchange of solutes between the two compartments. No significant variation of the hematocrits was observed during the function of the system, but a minimum breakup of the erythrocytes was generated after 120 min of perfusion by the activity of the peristaltic pump. Ammonia concentration became equal in both compartments after the first hour of perfusion and the total mass (Q) of this metabolite remained constant during the whole experiment, indicating that no loss or interactions with any system component occurred. Similar behavior was observed for glucose distribution.

FPLC analysis

The protein analysis by gel filtration chromatography is shown in Figure 3. In the chromatogram obtained for a sample of basal plasma (Figure 3B) we can observe the presence of two main peaks. Based on the calibration curve obtained (Figure 3A), they can be assigned to the major plasma proteins: Albumin [elution volume (Ve) = 14.15 mL] and immunoglobulins (mainly IgG, Ve = 12.52 mL). Two minor peaks are also appreciated (Ve < 9 mL) that correspond to proteins of high molecular weight (MW > 700 kDa). These could be α2-macroglobulin (MW = 725 kDa, Ve = 8.92 mL) and the pentameric form of IgM (MW = 950 kDa, Ve = 8.22 mL). Figure 3C shows the chromatogram obtained for a sample of the BC fluid after 120 min of blood perfusion (the same result was obtained after 60 min). It can be noticed that none of the

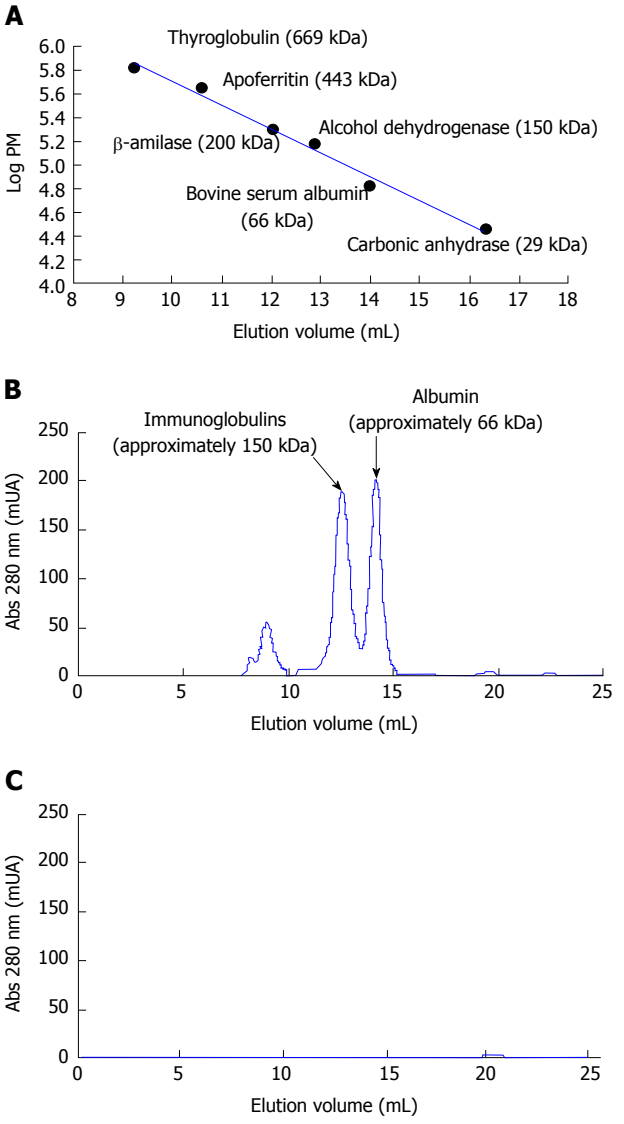

Figure 3 Protein analysis by gel filtration chromatography. A: Calibration curve carried out with molecular weight markers; B: Chromatogram obtained for a sample of basal plasma; C: Chromatogram obtained for a sample of biological compartment, taken after 120 min of perfusion $n = 5$.

plasma proteins was capable of crossing the membrane of the hollow fibers used in the construction of the MBR.

Evolution of the amount of LDH released by fresh LMOs and LMOs cold preserved in BG35 and ViaSpan® solutions after two hours of MBR perfusion

Figure 4 exposes the time changes in LMOs viability (determined by LDH release) throughout the two hours of the experiments performed. One gram of fresh LMOs (controls) or LMOs cold preserved in BG35 and ViaSpan® solutions was loaded into the BC and the MBR was then perfused during 120 min. The amount of enzyme released by fresh LMOs and LMOs cold preserved in BG35 showed a minor increase after two hours of perfusion. However, LMOs preserved in ViaSpan® solution showed a statistically significant raise in this parameter as perfusion time increased. The values of LDH release reached after 120 min in the MBR were: 6.0% ± 2.3% for controls;

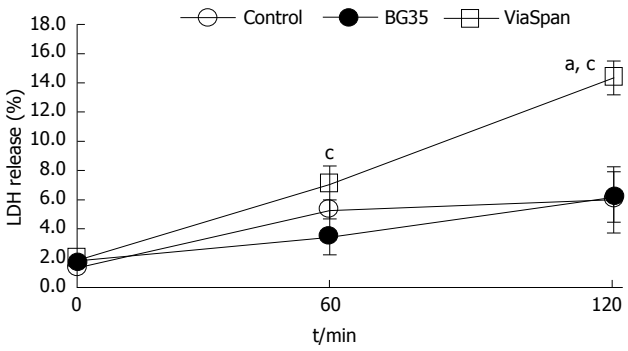

Figure 4 Time course of lactate dehydrogenase release during 120 min of minibioreactor perfusion determined for fresh and cold preserved liver microorgans in BG35 and ViaSpan® solutions. Data are expressed as mean ± SD for 6 liver microorgans (LMOs) preparations. Different from control, $^{a}P < 0.05$; Different from all the other reoxygenation times, $^{c}P < 0.05$, $n = 6$ LMOs independent preparations for each condition. LDH: Lactate dehydrogenase.

6.2% ± 1.7% for LMOs cold preserved in BG35 and 14.3% ± 1.1% for the group cold preserved in ViaSpan®, ($P < 0.05$, $n = 6$).

Evolution of ammonia detoxification for fresh LMOs and LMOs cold preserved in BG35 and ViaSpan® solutions

The ammonia detoxification capability of the device was evaluated by measuring the time course evolution of plasma ammonia concentration: An ammonia overload was added to the blood to obtain an ammonia plasma concentration of 1.06 ± 0.12 mmol/L, $n = 6$. We determined the ammonia content in blood and BC fluid samples obtained before initiating the perfusion (time 0) and after the first and second hour of operation (time 60 and 120 min, respectively). In Figure 5A it can be appreciated the LMOs ammonia detoxification capacity during two hours of MBR functioning. It can be observed that both preserved groups were able to detoxify a percentage of ammonium initial doses similar to control group, during the whole experiment. After two hours, the percentage of the initial dose detoxified (Figure 5A) was 49.3% ± 8.8% for controls LMOs; 52.9 ± 6.5 for BG35 and 53.6 ± 6.0 for ViaSpan® preserved LMOs ($n = 6$). To get a better knowledge about the amount of ammonia that LMOs were able to metabolize in the MBR, Figure 5B shows the μmols of this compound detoxified per gram of wet tissue. The values reached at the end of the perfusion period were: Control: 13.2 ± 2.2; BG35: 14.2 ± 3.8, and ViaSpan® 16.0 ± 1.1 μmol of NH_4^+ detoxified/g wet tissue ($n = 6$).

Histology

Control and cold preserved LMOs (48 h in BG35 and ViaSpan® solutions) were morphologically analyzed to assess hepatic tissue integrity, at the beginning and after 2 h of perfusion in the MBR.

Control LMOs showed normal hepatocyte cords with fusiform endothelial cells attached to the extracellular matrix of perisinusioidal space (EMPS), both at 0 min and at 120 min of perfusion period in the MBR (Figure

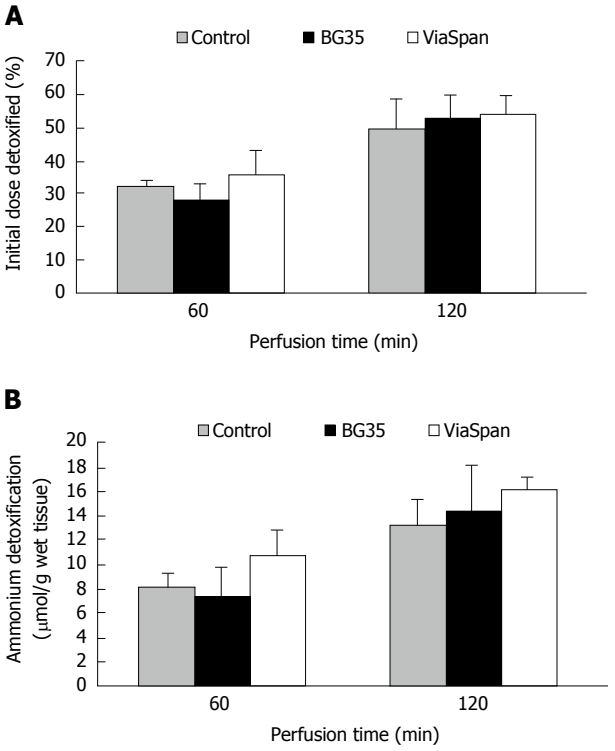

Figure 5 Evolution of (A) initial dose of ammonium detoxified (%) (B) detoxification of ammonia (μmol/g wet tissue) for fresh cold preserved liver Microorgans in BG35 and ViaSpan® solutions used as a biological component in the minibioreactor. Data are expressed as mean ± SD, *n* = 6 liver Microorgans independent preparations for each condition.

6A and B).

LMOs preserved in BG35 had organized hepatocyte cords with sinusoids slightly dilated and endothelial cells with two different morphology patterns: Fusiform or rounded, both attached to EMPS (Figure 6D) at 0 min. After 120 min, morphological features changed. Heptocyte cords continued to be organized but sinusoids were dilated with abundant rounded endothelial cells either attached to EMPS or seen inside sinusoidal lumen (Figure 6E).

At 0 min, LMOs preserved in ViaSpan® solution showed balonized hepatocytes and abundant rounded endothelial cells. Endothelial cells were attached to EMPS and sinusoidal lumen was dilated (Figure 6D). At 120 min LMOs had abundant blebs and areas of disrupted hepatocyte cords (Figure 6E).

DISCUSSION

The goal of this study was the development of a simplified BAL prototype suitable to use LMOs as biological component, and the evaluation of fresh and cold preserved rat LMOs performance in this model.

Our simple hollow fiber MBR was constructed to enable the control of LMOs performance (*i.e.,* viability and detoxification, but also suitable for the measurement of other parameters such as synthesis functions specific of liver) and sampling of blood and BC fluid during operation. In a first stage, we characterized this simplified prototype by setting different functional parameters without the biological component. We observed an optimum exchange of fluids and metabolites. The Polyamix™ hollow fibers used allow adequate diffusive and convective mass transport. In order to evaluate the performance of these fibers against large size molecules we determined their permeability to plasma proteins. The experiments using FPLC showed that the pore size of the membranes used, with a cutoff value of 50 kDa, blocks the transfer of plasma proteins into the BC thus preventing damage of LMOs by the hypothetical patient's immune system proteins (antibodies, complement system).

After checking the system operation without any biological component, as a final step of the "*in vitro*" characterization of our BAL model, a validation step was performed, evaluating the performance of control and preserved LMOs in the MBR designed. The architecture chosen for the BAL we present here was not trivial; the BAL system in use in our laboratory, with isolated hepatocytes as biological component, was not suitable for LMOs which almost did not detoxify ammonia when applied to it (data not shown). As LMOs detoxification of ammonia on flat plates[11] was satisfactory, we decided to construct a "flat bottom" BAL to allow accommodating the tissue slices in a less crowded manner. In BAL devices designed to use LMOs, it is essential that the bioreactor architecture ensures a good viability of this biological component during the blood detoxification performance.

We observed that LDH releasing from LMOs cold preserved in Viaspan® was increasing with the perfusion time and this phenomenon was not observed for LMOs preserved in BG35 solution or controls. This fact can be attributed to a protective effect exerted by PEG 35000 kDa (key component of BG35 solution) on cell membranes[14,19,20].

Observation of ammonia depuration is an evidence of hepatic synthetic function and is an important feature to propose the device we present here for clinical application[21,22]. When this MBR was challenged with an ammonia overload it showed an effective detoxification of this detrimental metabolite, either when cold preserved or fresh LMOs were examined. LMOs cold preserved in both preservation solutions were able to detoxify a similar percentage of the initial dose as compared to the control group. Although LMOs cold preserved in ViaSpan® showed higher levels of LDH release after 120 min of reperfusion they were able to detoxify an ammonium overload as well as control and cold preserved in BG35 solution LMOs did. Our group had already shown that, immediately after 48 h of cold preservation, ATP levels were severely decreased but they were actively replenished during reperfusion[23,24]. This fact can explain the good ammonium detoxification performance observed and constitute an indication of LMOs conserved mitochondrial function after cold preservation. Histological evaluation of LMOs showed that although BG35 protect hepatic morphology better than ViaSpan® solution, both cold preservation solutions proved to be useful to preserve the biological component integrity in our flat-plate model of MBR.

To provide a clear idea of the amount of ammonia that

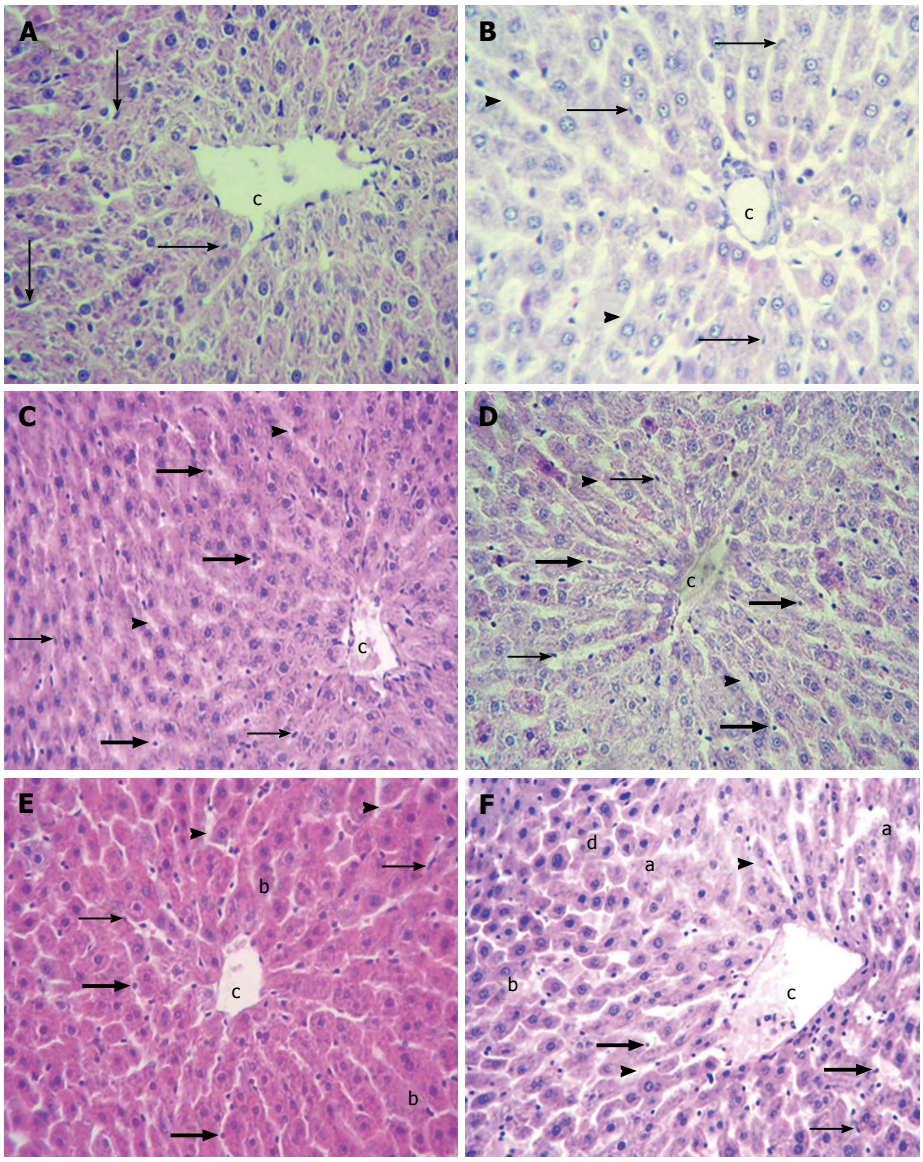

Figure 6 Liver microorgans histology. Hematoxylin-eosin. Samples were taken from control and preserved groups at the beginning (A, C and E) and at the end (t = 120 min) (B, D and F) of the experiment. Controls (A and B) showed normal hepatic parenchyma with fusiform endothelial cells (arrows) attached to perisinusoidal extracellular matrix and conserved hepatocyte cords. Sinusoids appeared dilated (bold arrow head) after 120 min. Liver microorgans (LMOs) preserved in BG35 (C and D) presented conserved hepatic architecture with fusiform (arrows) and rounded (bold arrows) endothelial cells. Sinusoids were dilated (bold arrow heads). LMOs preserved in ViaSpan® (E and F) had fusiform (arrows) and rounded (bold arrows) endothelial cells, dilated sinusoids (bold arrow head), and balonized hepatocytes (b) at the beginning of the experiment. F: After 120 min, blebs (a) and areas of hepatocyte trabecular disruption (d) were also found. Central Vein (CV). Magnification × 200. *n* = 6 LMOs independent preparations for each condition. c: Central vein.

LMOs were able to metabolize, we also determined the amount (μmol) of this compound detoxified per gram of wet tissue during reperfusion. Once again we found similar levels of ammonium detoxification between control LMOs and LMOs cold preserved in Viaspan® or BG35 solution. The ammonium concentration in blood of patients with acute liver failure (ALF) could be greater than 0.2 mmol/L and it should be considered that also there is a continuous infusion of this metabolite to blood flow. In our *in vitro* experiments we used a higher concentration (1 mmol/L) since we worked with a single initial dose of ammonium. In addition, Calligaris *et al*[25] showed that neither cell viability nor ammonium detoxification capacity of freshly isolated hepatocyte suspensions were affected by the concentration of the initial ammonium overload.

It is important to consider that in this work we tested two preservation solutions: ViaSpan® which is the gold standard in liver preservation[26,27] and BG35 that was design by our group specifically to suit cold preservation of LMOs, and the entire liver in the future. The use of BG35 solution for the cold storage of LMOs may facilitate liver research since one litter of ViaSpan® is about 3 time more expensive than the same volume of BG35[11,14].

The experimental MBR presented in this study relied on a simple design and was constructed using standard materials available in most laboratories. Due to these facts we foresee its employment as a useful tool to study the performance of LMOs submitted either

to preservation protocols or any other treatment or condition. Taking into account all the results previously shown, we have demonstrated that LMOs could be used as the biological component of the MBR designed, showing an adequate capacity to detoxify ammonia. We have also optimized the techniques to cold preserve this biocomponent to ensure its continuous availability, which is essential for any BAL to become a useful therapeutic tool for patients with ALF. As future prospects, these results encourage us to study other important liver functions, as transcription of albumin and clotting factors during reperfusion and to challenge it to treat acute liver failure of small animal models which will allow the measurement of bilirubin conjugation, blood clotting functions or intracranial pressure all important clinical prognostic predictors for ALF patients[28-30]. Also, to scale this MBR up and evaluate it in big animal models of ALF such as pigs.

ACKNOWLEDGMENTS

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COMMENTS

Background

Acute liver failure is a condition that sometimes is resolved spontaneously but in most cases requires liver transplantation. The regeneration capacity of the liver seems to be behind the cases were, after the first insult has disappeared, the organ recovers by itself. This has been shown to be a consequence of the amount of viable mass remaining in terms of tissue capacity to cope with the detoxification of harmful metabolites produced by the damage and to provide the needed quantities of essential liver produced molecules and factors. This is why many attempts have been pursued to help the patient´s liver to transit this acute failure and either recover or extend the time frame for a liver transplantation to be practical. In this sense, bioartificial liver (BAL) is thought as the better choice to accomplish this job but till now it is only performed by medical care teams that are able to obtain the biological component in the same unit making the practice limited to very few centers in the world.

Research frontiers

A choice for the optimal biological component for BAL devices, as looking forward to develop a tool ready to use worldwide, is not straightforward. Hepatic derived cell lines, whole animal livers (even "humanized" organs) and primary human or animal hepatocytes have been proposed and tested but none have proven to be easily translatable to health centers reality. The work presented here proposes the use of tissue slices [liver Microorgans (LMOs)] and their preservation for at least 48 h in a preservation solution designed by their group. The obtainment of this biological component presents much less technical difficulty than isolation of viable hepatocyes and it bares all the cellular types and a conserved micro-architecture compared to the liver itself. The authors also show the extension of the period of use of these LMOs from few hours to 2 d and they are certain that it could be increased more by tuning the composition of their BG35 solution further.

Innovations and breakthroughs

To date the reports found in the literature inform the use of isolated cells, either primary hepatocytes or continuous cell lines, or even whole pig livers and attempts have been made to cultivate the cellular component on artificial scaffolds mimicking extracellular matrices and micro-architecture. This biological components are used either fresh isolated or obtained directly by *in vitro* culturing. To the best of our knowledge, the authors are the only group using and combining tissue slices and cold preservation techniques to successfully apply these LMOs onto BAL devices. The authors are still working with the dimensions of a mini-prototype that should be scaled up to be used for human patients and this is the future challenge the authors have to undertake.

Applications

It follows that the application of their results would be the design of a BAL accessible on demand at low cost in health care centers for the treatment of patients, with either acute or chronic liver failure, for their recovery, or as a support until organ transplantation, and to ameliorate their quality of life in the process.

Peer-review

This is an *in vitro* study for demonstration of the bio-artificial liver with detoxification. An interesting study for research design and innovation of the device.

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ORIGINAL ARTICLE

Case Control Study

Pancreatic hyperechogenicity associated with hypoadiponectinemia and insulin resistance: A Japanese population study

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Abstract

AIM

To examine the relationship between pancreatic hyperechogenicity and risk factors for metabolic syndrome.

METHODS

A general population-based survey of lifestyle-related diseases was conducted from 2005 to 2006 in Japan. The study involved 551 participants older than 40 year of age. Data for 472 non-diabetic adults were included in the analysis. The measures included the demographic factors, blood parameters, results of a 75 g oral glucose tolerance test, and abdominal ultrasonography. The echogenicity of the pancreas and liver was compared, and then the subjects were separated into two groups: cases with pancreatic hyperechogenicity (n = 208) and cases without (controls, n = 264). The differences between both groups were compared using an unpaired t-test or Fisher's exact test. Multiple logistic regression analysis was used to determine the relationship between the pancreatic hyperechogenicity and clinical and bio-

chemical parameters.

RESULTS

Subjects with pancreatic hyperechogenicity had decreased serum adiponectin concentration compared to control subjects [8.9 (6.5, 12.8) *vs* 11.1 (7.8, 15.9), $P < 0.001$] and more frequently exhibited features of metabolic syndrome. Logistic regression analysis showed that the following variables were significantly and independently associated with pancreatic hyperechogenicity: Presence of hypoadiponectinemia, increased body mass index (BMI), higher homeostasis model assessment of insulin resistance (HOMA-IR) score, and presence of fatty liver. Similar associations were also observed in subjects with pancreatic hyperechogenicity without fatty liver. Multivariate association analysis of data from participants without fatty liver showed that hypoadiponectinemia was significantly associated with pancreatic hyperechogenicity (OR = 0.93, 95%CI: 0.90 - 0.97, $P < 0.001$). This association was independent of other confounding variables. Additionally, an increased BMI and higher HOMA-IR score were significantly associated with pancreatic hyperechogenicity.

CONCLUSION

Pancreatic hyperechogenicity is independently associated with increased BMI, insulin resistance, and hypoadiponectinemia in the general population.

Key words: Pancreatic hyperechogenicity; Metabolic syndrome; Obesity; Adiponectin; The Takahata study

Core tip: Pancreatic hyperechogenicity is related to aging. Several recent studies have reported that hepatic steatosis and increased body mass index (BMI) are predictors of a hyperechogenic pancreas. In the present study, fatty liver was also significantly associated with pancreatic hyperechogenicity. We performed additional analyses excluding participants with fatty liver in order to account for the effect of this condition. Our analyses showed that an increased BMI, higher homeostasis model assessment of insulin resistance score, and decreased adiponectin were also significantly associated with pancreatic hyperechogenicity.

Makino N, Shirahata N, Honda T, Ando Y, Matsuda A, Ikeda Y, Ito M, Nishise Y, Saito T, Ueno Y, Kawata S. Pancreatic hyperechogenicity associated with hypoadiponectinemia and insulin resistance: A Japanese population study. *World J Hepatol* 2016; 8(33): 1452-1458 Available from: URL: http://www.wjgnet.com/1948-5182/full/v8/i33/1452.htm DOI: http://dx.doi.org/10.4254/wjh.v8.i33.1452

INTRODUCTION

Pancreatic hyperechogenicity may be related to the aging process[1-3]. Previous studies have reported that increased echogenicity of the pancreas is associated with pancreatic lipomatosis[4,5]. Additionally, recent ultrasound studies have demonstrated pancreatic hyperechogenicity is correlated with obesity, hepatic steatosis[6,7], and insulin resistance[8]. To our knowledge, there are a limited number of reports examining the relationship between pancreatic hyperechogenicity and lifestyle-related risk factors.

Recent studies have demonstrated that adipose tissue not only stores fat but also functions as an endocrine organ by producing various adipocytokines such as adiponectin. Adiponectin is a peptide hormone that plays a key role in the development of insulin resistance associated with metabolic syndrome[9]. Adiponectin levels are correlated directly with insulin sensitivity and are decreased in obese individuals and patients with type 2 diabetes[10,11]. Recent studies have shown that there is an association between low concentrations of adiponectin and cancer development[12,13]. However, no report has examined the relationship between adiponectin concentration and pancreatic hyperechogenicity.

The aim of the present study was to examine the associations between pancreatic hyperechogenicity and risk factors for metabolic syndrome, including adiponectin concentration, in the general Japanese population.

MATERIALS AND METHODS

Study population

The study was part of an ongoing molecular epidemiological project utilizing the regional characteristics of the 21st Century Centers of Excellence program in Japan, which has been previously described[14]. The surveyed population was the entire population of adults aged over 40 year in the town of Takahata, Yamagata prefecture, located in northeastern Japan. There were 551 participants enrolled in the study between 2005 and 2006. All participants received a physical examination, blood tests, a 75-g oral glucose tolerance test (OGTT), and abdominal ultrasonography (US). We excluded 79 of the 551 participants for the following reasons: Poor US images of the pancreas (43 individuals); blood samples collected in a non-fasting state (27 individuals); or OGTT-diagnosed diabetes mellitus based on the American Diabetes Association criteria (9 individuals)[15]. Exclusion criteria included a history of diabetes or pancreatic disease. However, no participants met the exclusion criteria. The data collected from 472 subjects (201 males and 271 females) were included in the final analysis.

Measurements

We obtained information on current medication and lifestyle-related characteristics from all participants using a questionnaire. Trained study staff measured height, weight and systolic and diastolic blood pressure (BP) using standard methods. Body mass index (BMI) was calculated as weight (in kilograms) divided by height (in meters squared). Insulin resistance was evaluated using

the homeostasis model assessment of insulin resistance (HOMA-IR) method with the following equation: HOMA-IR = fasting insulin (μU/mL) × fasting glucose (mg/dL)/405. Venous blood was drawn the morning after an overnight fast. The serum and plasma were separated immediately and stored at -80 ℃ until analysis. The serum adiponectin concentrations were measured by an enzyme-linked immunosorbent assay as described previously[9]. The other biochemical blood parameters were determined by standard laboratory procedures in the General Laboratory of BML, Inc. (Saitama, Japan). All laboratory personnel were blinded to the status of the samples.

All participants underwent US and an OGTT during a single visit at the research center and within 2 mo of the physical examination and initial blood collection. Blood samples were obtained to determine plasma glucose in the basal period and 120 min after an oral glucose load in the morning following an overnight fast.

The participants fasted overnight for the US study, and the scans were performed by one of four experienced operators using either a Toshiba Nemio™ scanner or an Aloka SSD-3500 scanner with a 3.5-MHz convex transducer. The participants were scanned while lying supine. The images were recorded on a standard computer hard disk drive. The recorded images were analyzed and judged simultaneously by four experienced physicians blinded to the details of the subjects, as described in a previous report[5]. The echogenicity of the pancreatic body was compared with the liver. The subjects were separated into cases with pancreatic echogenicity higher than hepatic echogenicity (n = 208) and controls whose pancreatic echogenicity was equal to or lower than the liver (n = 264). The presence of fatty liver was defined as a US pattern consistent with evidence of increased ultrasonographic contrast between the hepatic and renal parenchyma, vessel blurring, and narrowing of the lumen of the hepatic veins. We used these criteria to divide the subjects into cases with (n = 206) or without (n = 266) fatty liver.

Statistical analysis

The distribution of the continuous variables was assessed for normality. If a normal distribution was evident, then the data were expressed as the means ± standard deviation. The data with a non-normal distribution were log-transformed for analysis and expressed as the median with 25^{th}/75^{th} percentiles. The case and control groups were compared using the unpaired t test or Fisher's exact test. The relationship between pancreatic hyperechogenicity and clinical and biochemical parameters was determined using multiple logistic regression analyses with the backward elimination method. The odds ratio (OR) and 95%CI were then calculated.

The parameters in the multiple logistic regression analysis were categorized using the following cutoff values: BMI (< 25 kg/m^2, ⩾ 25 kg/m^2), systolic BP (< 130 mmHg, ⩾ 130 mmHg), diastolic BP (< 85 mmHg, ⩾ 85 mmHg), fasting plasma glucose (< 110 mg/dL, ⩾ 110 mg/dL), HOMA-IR score (< 2.0, ⩾ 2.0), high-density lipoprotein (HDL) cholesterol (< 40 mg/dL, ⩾ 40 mg/dL), triglyceride (< 150 mg/dL, ⩾ 150 mg/dL), glutamic pyruvic transaminase (GPT) (< 35 IU/L, ⩾ 35 IU/L), pre-load plasma glucose in the OGTT (< 110 mg/dL, ⩾ 110 mg/dL) and post-load 2-h plasma glucose in the OGTT (< 140 mg/dL, ⩾ 140 mg/dL). The median values for serum insulin and pancreatic isoamylase were used as the cutoff points. Serum adiponectin was analyzed as a continuous variable. All data were analyzed using SPSS software (version 15.0, SPSS Inc., Chicago, IL, United States). All differences with P < 0.05 were considered statistically significant. The statistical methods of this study were reviewed by Yuko Nishise from the Faculty of Medicine, Yamagata University.

RESULTS

The baseline clinical and biochemical data for the cases and controls are shown in Table 1. The serum adiponectin levels were markedly lower in the cases than in the controls [8.9 (6.5-12.8) *vs* 11.1 (7.8-15.9), P < 0.001]. In addition, there were significant differences between the cases and controls for the following variables: Age, presence of fatty liver, weight, BMI, systolic BP, diastolic BP, serum insulin, fasting plasma glucose, HOMA-IR score, HDL cholesterol, total cholesterol, triglyceride, pancreatic isoamylase, GPT, preload plasma glucose in the OGTT, and 2-h plasma glucose.

Each parameter was dichotomized according to the cutoff points to further explore the relationship between pancreatic hyperechogenicity and other parameters. We then conducted multiple logistic regression analyses with the backward elimination method.

We first used an age-adjusted model to exclude the influence of aging. The results indicate that there was a significant negative association between decreased adiponectin levels and pancreatic hyperechogenicity (OR = 0.92, 95%CI: 0.88-0.95, P < 0.001). In addition, the presence of fatty liver, higher values of BMI, systolic BP, diastolic BP, serum insulin, HOMA-IR score, 2-h plasma glucose and lower pancreatic isoamylase levels were significantly associated with pancreatic hyperechogenicity (Table 2).

We next performed the analysis after adjustment for age, presence of fatty liver, BMI, systolic and diastolic BP, adiponectin, serum insulin, HOMA-IR, triglyceride, pancreatic isoamylase, GPT, and 2-h plasma glucose. The analysis showed that hypoadiponectinemia was significantly associated with pancreatic hyperechogenicity (OR = 0.9, 95%CI: 0.91-0.98, P = 0.004), independent of the other confounding variables. The presence of fatty liver, increased BMI, and higher HOMA-IR score were also significantly associated with pancreatic hyperechogenicity. In addition, decreased pancreatic isoamylase showed a weak relationship with pancreatic hyperechogenicity (Table 2).

We then performed further analyses to exclude the influence of fatty liver. The baseline clinical and

Table 1 Comparison of baseline characteristics between the pancreatic hyperechogenicity and control groups

Clinical parameters	Pancreatic hyperechogenicity (n = 208)	Controls (n = 264)	P value
Age (yr)	60.8 ± 9.4	56.9 ± 9.8	< 0.001
Fatty liver (fatty/non-fatty)	113/95	93/171	< 0.001
Sex (male/female)	86/122	115/149	0.640
Height (cm)	157.9 ± 8.4	159.3 ± 8.8	0.085
Weight (kg)	61.2 ± 9.4	56.2 ± 9.3	< 0.001
BMI (kg/m^2)	24.4 ± 2.6	22.1 ± 2.7	< 0.001
Systolic BP (mmHg)	135.9 ± 15.8	129.6 ± 15.7	< 0.001
Diastolic BP (mmHg)	83.1 ± 10.2	79.6 ± 10.2	< 0.001
Adiponectin (μg/mL)	8.9 (6.5-12.8)	11.1 (7.8-15.9)	< 0.001
Serum insulin (μU/mL)	4.7 (3.4-6.8)	3.6 (2.7-5.0)	< 0.001
Fasting plasma glucose (mg/dL)	94.5 ± 9.2	90.8 ± 9.7	< 0.001
HOMA-IR	1.1 (0.7-1.6)	0.8 (0.6-1.1)	< 0.001
High-density lipoprotein cholesterol (mg/dL)	58.4 ± 14.2	63.2 ± 15.4	0.001
Low-density lipoprotein cholesterol (mg/dL)	127.1 ± 36.1	123.8 ± 32.4	0.310
Total cholesterol (mg/dL)	205.9 ± 33.1	199.8 ± 34.4	0.049
Triglyceride (mg/dL)	96 (71-135)	81 (63-112)	< 0.001
Pancreatic isoamylase (U/L)	28 (23-34)	30 (25-37)	0.014
Glutamic oxaloacetic transaminase (IU/L)	23 (20-27)	22 (19-28)	0.706
Glutamic pyruvic transaminase (IU/L)	21 (17-28)	20 (15-26)	0.011
γ-glutamyl transpeptidase (IU/L)	24 (17-42)	22 (15-33)	0.070
Preload plasma glucose (OGTT) (mg/dL)	97.4 ± 9.9	93.1 ± 10.2	< 0.001
2-h plasma glucose (OGTT) (mg/dL)	114.6 ± 29.7	101.1 ± 26.7	< 0.001

Data are expressed as means ± SD, or median (25th; 75th percentiles). Unpaired t test or Fisher's exact test were used to compare the two groups. BMI: Body mass index; BP: Blood pressure; HOMA-IR: Homeostasis model assessment of insulin resistance; OGTT: Oral glucose tolerance test.

Table 2 Age-adjusted and multivariate odds ratios for pancreatic hyperechogenicity

Confounding factor	Pancreatic hyperechogenicity (n)	Controls (n)	Age-adjusted		Multivariate[3]	
			Odds ratio (95%CI)	P value	Odds ratio (95%CI)	P value
Non-fatty liver/fatty liver	95/113	171/93	2.6 (1.8-3.9)	< 0.001	1.77 (1.15-2.72)	0.009
BMI (kg/m^2), < 25/≥ 25	125/83	229/35	5.0 (3.1-8.0)	< 0.001	3.56 (2.17-5.83)	< 0.001
Systolic BP (mmHg), < 130/≥ 130	60/148	119/145	1.6 (1.1-2.5)	0.016		
Diastolic BP (mmHg), < 85/≥ 85	110/98	180/84	1.9 (1.3-2.7)	0.001		
Adiponectin (μg/mL)[1]			0.92 (0.88-0.95)	< 0.001	0.9 (0.91-0.98)	0.004
Serum insulin (μU/mL)[2], ≤ 4.0/> 4.0	83/125	153/111	2.2 (1.5-3.2)	< 0.001		
Fasting plasma glucose (mg/dL), < 110/≥ 110	198/10	251/13	0.8 (0.3-1.9)	0.593		
HOMA-IR, < 2.0/≥ 2.0	177/31	253/11	4.4 (2.1-9.1)	< 0.001	2.4 (1.1-5.1)	0.032
HDL cholesterol (mg/dL), ≥ 40/< 40	194/14	253/11	1.9 (0.8-4.5)	0.119		
Triglyceride (mg/dL), < 150/≥ 150	173/35	234/30	1.7 (1.0-2.9)	0.055		
Pancreatic isoamylase (U/L)[2], ≥ 30/< 30	89/119	143/121	1.7 (1.2-2.5)	0.004	2.08 (0.95-4.57)	0.068
GPT (IU/L), < 35/≥ 35	177/31	234/30	1.7 (1.0-2.9)	0.069		
Preload plasma glucose (OGTT) (mg/dL), < 110/≥ 110	189/19	247/17	1.1 (0.6-2.3)	0.710		
2-h plasma glucose (OGTT) (mg/dL), < 140/≥ 140	170/38	244/20	2.4 (1.3-4.3)	0.003		

[1]Serum adiponectin was analyzed as a continuous variable; [2]The median values for serum insulin and pancreatic isoamylase were used as the cutoff points; [3]Adjusted for the age, presence of fatty liver, BMI, systolic BP, diastolic BP, adiponectin, serum insulin, HOMA-IR, triglyceride, pancreatic isoamylase, GPT and 2-h plasma glucose. Odds ratios and 95%CI were estimated using the multiple logistic regression model with backward elimination. BMI: Body mass index; BP: Blood pressure; HOMA-IR: Homeostasis model assessment of insulin resistance; HDL: High-density lipoprotein; GPT: Glutamic pyruvic transaminase; OGTT: Oral glucose tolerance test.

biochemical data for cases and controls without fatty liver are shown in Table 3. The serum adiponectin levels were lower in cases than in controls [10.3 (7.6-14.6) *vs* 12.0 (8.6-17.0), P = 0.022]. Furthermore, there were significant differences between cases and controls for the following parameters: Age, weight, BMI, serum insulin, fasting plasma glucose, HOMA-IR score, HDL cholesterol, triglycerides, and preload plasma glucose in the OGTT.

We next performed multivariate association analyses of data from participants without fatty liver. The multivariate analysis showed that hypoadiponectinemia was significantly associated with pancreatic hyperechogenicity (OR = 0.93, 95%CI: 0.90-0.97, P < 0.001), independent of the other confounding variables. Additionally, an increased BMI and higher HOMA-IR score were also significantly associated with pancreatic hyperechogenicity (Table 4).

DISCUSSION

Studies examining digestive organ disease and altered

Table 3 Comparison of baseline characteristics between the pancreatic hyperechogenicity and control groups excluding participants with fatty liver

Clinical parameters	Pancreatic hyperechogenicity (n = 95)	Control (n = 171)	P value
Age (yr)	62 ± 9	58 ± 10	0.001
Sex (male/female)	44/51	76/95	0.769
Height (cm)	158.0 ± 8.0	158.1 ± 9.0	0.960
Weight (kg)	59.5 ± 9.4	53.9 ± 8.5	< 0.001
BMI (kg/m^2)	23.7 ± 2.6	21.5 ± 2.5	< 0.001
Systolic BP (mmHg)	133 ± 17	130 ± 16	0.135
Diastolic BP (mmHg)	82 ± 11	80 ± 10	0.180
Adiponectin (μg/mL)	10.3 (7.6-14.6)	12.0 (8.6-17.0)	0.022
Serum insulin (μU/mL)	4.0 (2.7-5.4)	3.3 (2.5-4.5)	0.004
Fasting plasma glucose (mg/dL)	93 ± 8	90 ± 10	0.026
HOMA-IR	0.9 (0.59-1.28)	0.71 (0.54-1.02)	0.002
High-density lipoprotein cholesterol (mg/dL)	60 ± 15	66 ± 15	0.001
Low-density lipoprotein cholesterol (mg/dL)	124 ± 35	122 ± 33	0.708
Total cholesterol (mg/dL)	202 ± 33	199 ± 36	0.464
Triglyceride (mg/dL)	87 (66-133)	76 (59-96)	0.005
Pancreatic isoamylase (U/L)	29 (24-35)	31 (25-37)	0.054
Glutamic oxaloacetic transaminase (IU/L)	22 (19-25)	23 (19-28)	0.189
Glutamic pyruvic transaminase (IU/L)	19 (16-23)	20 (15-25)	0.937
γ-glutamyl transpeptidase (IU/L)	21 (16-40)	21 (15-32)	0.266
Preload plasma glucose (OGTT) (mg/dL)	96 ± 9	92 ± 10	0.009
2-h plasma glucose (OGTT) (mg/dL)	107 ± 29	100 ± 28	0.063

Data are expressed as means ± SD, or median (25th; 75th percentiles). Unpaired t test or Fisher's exact test were used to compare the two groups. BMI: Body mass index; BP: Blood pressure; HOMA-IR: Homeostasis model assessment of insulin resistance; OGTT: Oral glucose tolerance test.

Table 4 Multivariate association analysis of clinical parameters for pancreatic hyperechogenicity excluding participants with fatty liver

	Multivariate[2]	
Confounding factor	Odds ratio (95%CI)	P value
BMI (kg/m^2)	3.89 (2.39-6.35)	< 0.001
Adiponectin (μg/mL)[1]	0.93 (0.90-0.97)	< 0.001
HOMA-IR	2.23 (1.02-4.89)	0.045

[1]Serum adiponectin was analyzed as a continuous variable; [2]Adjusted for age, BMI, diastolic BP, adiponectin, serum insulin, HOMA-IR, HDL cholesterol, triglyceride and pancreatic isoamylase. Odds ratios and 95%CI were estimated using the multiple logistic regression model with backward elimination. CI: Confidence interval; BMI: Body mass index; HOMA-IR: Homeostasis model assessment of insulin resistance.

secretion of adipocytokines caused by metabolic syndrome will improve our understanding of the mechanisms involved in pathophysiological conditions. It is possible that such investigations might lead to the development of preventive measures for diseases linked to metabolic syndrome.

Pancreatic hyperechogenicity is thought to be related to the aging process[1-3]. Our data showed that several features of metabolic syndrome, such as higher BMI, increased HOMA-IR score, and hypoadiponectinemia, were also independently associated with pancreatic hyperechogenicity.

This is the first study conducted in a general population that investigated the relationship between pancreatic hyperechogenicity and risk factors of metabolic syndrome, such as insulin resistance and serum adiponectin concentration. The main study findings were: (1) that serum adiponectin concentrations were markedly lower in subjects with pancreatic hyperechogenicity than in controls [8.9 (6.5-12.8) *vs* 11.1 (7.8-15.9), P < 0.001]; and (2) that decreased adiponectin levels were associated independently with pancreatic hyperechogenicity (OR = 0.9, 95%CI: 0.91-0.98, P = 0.004). Adiponectin is produced by adipose tissue, and a low adiponectin concentration is considered to be a key factor in the development of insulin resistance underlying metabolic syndrome[9-11].

Several studies have reported that increased echogenicity of the pancreas is related to lipomatosis of the pancreatic parenchyma[4,5]. Pancreatic lipomatosis is the most common histological change in the pancreas associated with age, obesity, and insulin resistance[6-8,16-19]. Recently, Raeder *et al*[5] evaluated the pancreatic fat content using US and magnetic resonance imaging and showed that pancreatic lipomatosis may reflect early events involved in the pathogenesis of diabetes and exocrine pancreatic dysfunction in non-diabetic children with mutations in carboxyl-ester lipase. In addition, Tushuizen and co-workers[20] measured the pancreatic fat content using proton magnetic resonance spectroscopy and found that pancreatic fat was inversely associated with β-cell function parameters in non-diabetic men. However, there was no association in their diabetic counterparts. The authors suggested that pancreatic fat content may contribute to β-cell dysfunction[20]. In our logistic regression analysis, we adjusted for age and pancreatic hyperechogenicity, which is a potential marker of lipomatosis. We found that these parameters were associated with higher serum insulin, HOMA-IR, and 2-h plasma glucose levels in the OGTT. There was

no association with fasting and preload plasma glucose concentrations in the OGTT (Table 2). It is possible that pancreatic hyperechogenicity with insulin resistance precedes the development of diabetes in the non-diabetic general population. Thus, further large-scale prospective studies are necessary to investigate whether pancreatic hyperechogenicity is an early pathological event in the diabetes disease process.

Several recent studies have reported that hepatic steatosis and increased BMI are predictors of a hyperechogenic pancreas[6,7]. In the present study, fatty liver was also significantly associated with pancreatic hyperechogenicity (OR = 1.77, 95%CI: 1.15-2.72, P = 0.009) (Table 2). Therefore, we performed additional analyses excluding participants with fatty liver in order to account for the effect of this condition in our results. The adjusted analysis showed that increased BMI, higher HOMA-IR score, and decreased adiponectin were also significantly associated with pancreatic hyperechogenicity (Table 4). This is the first study investigating the relationship between pancreatic hyperechogenicity and risk factors for metabolic syndrome by excluding the influence of fatty liver.

In this study, we used a simple and traditional method of assessing the severity of pancreatic hyperechogenicity, which was the comparison of echogenicity between the pancreatic body and the liver. However, this approach can potentially result in misdiagnosis of pancreatic hyperechogenicity if the extent of fatty liver is severe. We also performed additional analyses to exclude the influence of fatty liver. As shown in Tables 2 and 4, the results of our analyses that either included or excluded fatty liver, respectively, were similar and both showed increased BMI, higher HOMA-IR scores, and decreased adiponectin levels. However, our study had a limitation: No histological confirmation of pancreatic fat was possible.

There may be unknown factors that may cause the histological changes associated with obesity in addition to fat accumulation, fibrosis and functional changes in the exocrine pancreas. We recently demonstrated that intra-lobular fat accumulates in exocrine pancreatic tissue and that lipid droplets in acinar cells increase in Zucker diabetic fatty rats, which is an animal model of type 2 diabetes caused by the chronic intake of a high-fat diet. These conditions appear cause acinar cell injury and fibrosis[21]. Thus, additional clinical and experimental studies of the interrelationships between diabetes, metabolic syndrome and pancreatic injury should be conducted to clarify the pathogenesis of "non-alcoholic fatty pancreatic disease".

In conclusion, our study of a non-diabetic general population showed that pancreatic hyperechogenicity was independently associated with increased BMI, insulin resistance and hypoadiponectinemia.

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COMMENTS

Background

Pancreatic hyperechogenicity is thought to be related to the aging process. However, little is known about the association between pancreatic hyperechogenicity and other life-style related risk factors.

Research frontiers

Prior studies have reported that increased echogenicity of the pancreas is associated with pancreatic lipomatosis. Recent ultrasound studies have shown that pancreatic hyperechogenicity is correlated with obesity, hepatic steatosis, and insulin resistance.

Innovations and breakthroughs

This is the first study investigating the relationship between pancreatic hyperechogenicity and risk factors for metabolic syndrome by excluding the influence of fatty liver.

Applications

Pancreatic hyperechogenicity is independently associated with increased body mass index, insulin resistance, and hypoadiponectinemia in the general population. Pancreatic hyperechogenicity could be a useful marker of the metabolic syndrome.

Peer-review

Accept the manuscript for publication without significant corrections.

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ORIGINAL ARTICLE

Observational Study

Neglected features of lifestyle: Their relevance in non-alcoholic fatty liver disease

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Abstract

AIM

To investigated in non-alcoholic-fatty-liver-disease (NAFLD), with ultrasound (US)-detected fatty liver, and in a group of non-alcoholic and otherwise healthy subjects, relationship of neglected features of lifestyle with NAFLD and obesity.

METHODS

Five hundred and thirty-two NAFLD and 667 non-NAFLD healthy subjects, age 21-60 years were studied. Severity of liver steatosis was assessed by US bright liver score. The adherence to mediterranean diet score (AMDS) was assessed on the basis of a 1-wk recall computerized questionnaire which included a detailed physical activity reports (Baecke questionnaire). The western dietary profile score, as a simplified paradigm of unhealthy diet, a questionnaire quantifying sun exposure score and a sleep habits questionnaires provided a further comprehensive lifestyle assessment.

RESULTS

Body mass index (BMI), insulin resistance (HOMA), and triglycerides, poorer adherence to a mediterranean diet profile, sedentary habits, minor sun exposure and use of "western diet" foods are greater in NAFLD. Multiple

linear regression analysis, weighted by years of age, displays BMI, HOMA and AMDS as the most powerful independent predictors of fatty liver severity; however, also the physical activity score, the western diet habit and the sun exposure score are acting inside the model with significant independent effects.

CONCLUSION

Articulated clinical intervention, according to our results, are justified in NAFLD and can be pursued addressing by focused intervention nutritional profile, physical exercise mainly in open-air subsets for enhancing sun exposure and healthier sleep duration and rhythm.

Key words: Fatty liver; Ultrasound; Diet; Malnutrition; Sleep; Clinical risk management; Health psychology; Sun exposure; Obesity

Core tip: Non-alcoholic-fatty-liver-disease (NAFLD) is a multifactorial condition associated with malnutrition and, mainly, with obesity, sedentary life and insulin resistance; some neglected factor, such as sleep and sun exposure curtailment, along with D vitamin deficiency, are associated with NAFLD; articulated clinical intervention, according to our results, is justified in NAFLD and can be pursued addressing by focused intervention nutritional profile, open-air physical exercise for enhancing sun exposure and healthy behaviour targeted to improved sleep duration and rhythm.

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INTRODUCTION

Liver diseases, already in the past, were considered at least partly a consequence of unhealthy lifestyles and adverse environmental conditions, a concept that was very well addressed also by pathologists[1]. Lifestyle regards the use of the body functions related to physical exercise, exerted in work, love, leisure or sport, the quality and quantity of food, the sleep and rest rhythms, the exposure to hostile or unhealthy environmental factors, and other aspects, including fashion, clothing and non-sport leisure activity[2,3]. As in the past, the impact of the fashions and of beliefs based on alleged scientific statements and commercial information, namely publicity, is the key factor[4]. This framework, also by conditioning different lifestyles, reasonably affects the "establishment and maintenance of several diseases, including liver disease"[5]. In a very simplified manner today we tend to describe the lifestyles in medicine especially in terms of diet and physical inactivity or sedentary life, with a synergistic effect on body size - obesity - and on disease related with excessive food intake (atherosclerosis and liver disease)[6]. Marketing strategies focus much on some related aspects that have an influence on nutrition and physical activity, but also with trade repercussions, while neglecting and avoiding other modes of social behavior. Some of these factors, such as sleep duration[7,8], the sleeping patterns[9-12], including shift-work related effects[7], exposure to noise[13,14], the level of social alarm about events or situations[15], the possibility of urban mobility[16,17], may have determinant effects on nutritional profiles and exercise implementation. Communication and perception of risks, as traditionally recognized, are flanked by communication and induction of apparently neutral behavior that can behave as true risk factors for disease. The strong pressure towards practices aimed at optimizing physical fitness and dietary methods aimed at healthy foods often involves forms of orthorexia[18]; such strategies are widely used to gain and maintain niches of food and fitness markets. All this would be irrelevant, except that, as in the case of prevention of obesity and fatty liver, and probably also in the field of atherosclerotic, neurodegenerative and cancer diseases, dietary caloric intake and a sedentary lifestyle are not the only factors exerting independent synergistic effects[6]. In fact, even the dietary profiles[19], methods of exercise implementation[20,21], and other related factors, such as sleep deprivation[4], D vitamin deficiency and exposure to sunlight[22], environmental noise[16], and reasonably also others, seem to be part of an interrelated group of neglected risk factors, which only now are beginning to be studied more methodically.

Aim of our research is to investigate if some of the above mentioned neglected behavioural factors, concurrently with nutritional and physical exercise profile, may be associated or contribute independently as factors related to fatty liver in a group of non-alcoholic and otherwise healthy subjects with ultrasound (US)-detected fatty liver.

MATERIALS AND METHODS

Patients

Five hundred thirty-two non-alcoholic-fatty-liver-disease (NAFLD) and 667 non-NAFLD subjects (women 749, men 450, total 1199), age 21-60 years, without relevant acute or chronic disease, as below detailed in the exclusion criteria, were studied. These patients were consecutively referred to the same out-patients public medical unit (day-hospital) for lifestyle-nutritional prescription addressed to the management of minor digestive disease (mainly gastro-esophageal reflux syndrome or irritable bowel syndrome), overweight or obesity. The subjects were enrolled throughout January 2008-December 2015, were all patients first-time visitors, had not had previous referral or intervention in our unit, and were studied by a comprehensive US assessment (liver-abdomen, heart, thyroid and lung),

according to our current practice[3]. Exclusion criteria: (1) all patients with signs of moderate-severe congestive heart failure, previous myocardial infarction, idiopatic myocardiopathy, pericarditis, malignancies; (2) severe chronic liver disease, apart from the lone finding of bright liver; abnormal aminotransferase levels at the beginning of this study, defined as alanine transaminase (ALT) > 30 IU/L in men and ALT > 19 IU/L in women; acute or chronic virus hepatitis, which were excluded by concurrent laboratory assays, as below detailed; (3) any history of diabetes mellitus (fasting glucose ⩾ 126 mg/dL or HbA1c ⩾ 6.5%) or drug intake of anti-diabetic drugs, particularly metformin; (4) extreme obesity [body mass index (BMI) ⩾ 40] and underweight bad-nourished profile (BMI < 18.5 or serum albumin < 3 g/dL); (5) acute and/or chronic infectious, rheumatic or autoimmune disease; and (6) alcohol abuse (exceeding 20 g/d on a weekly base); renal insufficiency, *i.e.,* glomerular filtration rate < 90 mL/min per 1.73 m^2 and/or proteinuria > 0.10 g/d. According to these exclusion criteria 1508 further subjects, potentially but only partially eligible, are excluded by this study.

Laboratory/imaging methods

The severity of liver steatosis was assessed by US bright liver score (BLS), graded 1-3: grade 0 was the absence of bright liver, *i.e.,* a normal pattern[23], BLS was and previously validated by US-guided fine needle aspirate biopsy by 20 Gauge Menghini's needles[3]; GE echo color Doppler machines (GE Logiq 5/Vivid7 Expert US manufactured by GE Medical Systems, Milwaukee, WI, United States), high resolution, equipped with real-time convex, phased array and linear scan transducers, were used throughout this study.

Routine laboratory tests included virus hepatitis (hepatitis A, B and C virus, *i.e.*, HAV, HBV and HCV) and cancer biomarkers (Alpha-fetoprotein, CEA, Ca125, Ca 19-9, Ca15-3), thyroid hormones FT3 FT4, thyroid-stimulating hormone, aspartate aminotransferase, ALT, γ-glutamyl transpeptidase, ferritin, total protein, and albumin. Mediterranean diet adherence profile was assessed by the adherence to mediterranean diet score (AMDS) on the basis of a 1-wk recall computerized questionnaire[3,5] using a pictogram-based method of visualizing dietary intake, descriptive also of the size of the single portion; pictograms includes also items for the quantification of physical activity, which is otherwise quantified by detailed physical activity reports (Baecke questionnaire)[5]. The Western Dietary Profile score, as a simplified paradigm of unhealthy diet, was assessed submitting a specific questionnaire, which is reported in Appendix; also the Baecke's physical activity questionnaire is briefly described in appendix, and subsequently the total score was studied by statistical analysis. The questionnaires submitted for quantifying sun exposure score, used mainly as an index of the open air activity and sleep habits questionnaires are routinely included within the context of a comprehensive lifestyle assessment, and detailed in appendix. The study and the manuscript were approved by the institutional review board of the project office. No conflict of interest is to be declared for this invited manuscript. Written informed consent was obtained from each patient prior to the clinical data recording and before the US procedure, allowing the use of information for teaching and clinical research. Detail that might disclose the identity of the subjects under study is carefully omitted in any part of the study.

Statistical analysis

Comparison of data between the two groups of patients, NAFLD *vs* controls, is reported and differences assessed by Student's *t* test. Subsequently: (1) receiver operating characteristic (ROC) curve analysis of data of controls *vs* NAFLD subjects is used for defining the optimal cut-offs which may distinct the two group. The performance of each measure in prediction of NAFLD was evaluated by ROC curve. The area under the ROC curve and the 95%CI were used as indexes of accuracy. The optimal cut-off value was determined with maximum sum of sensitivity and specificity. For the purpose of identifying such thresholds, the measures used were BMI, HOMA, AMDS, western diet score (WDS), Physical activity Baecke's total score, sun exposure score, and sleep daily hours, calculated on a weekly base; (2) contingency tables and odds ratio of NAFLS *vs* non-NAFLD were calculated, according to each defined cut-off; and (3) MLR analysis, weighted by age, using BMI, HOMA, AMDS, WDS, physical activity baecke's total score, Sun exposure score, sleep hours *vs* BLS score of fatty liver was at last performed.

RESULTS

The two groups of patients were comparable for age (Table 1), while other measures, such as BMI, HOMA and Triglycerides are greater in NAFLD. Comparison of data between the two groups of patients, NAFLD *vs* controls, is reported in detail (Table 1): A poorer adherence to a mediterranean diet profile, greater sedentary habits and greater use of "western diet" foods are the main differences. Moreover, liver size and, obviously, detection of fatty liver are the main US feature distinctive of the two groups. The ROC curve analysis graph of the data of controls *vs* NAFLD subjects for BMI, HOMA, HDL Cholesterol is displayed in Figure 1.

The most suitable thresholds distinctive of NAFLD *vs* controls are, in our population: BMI ⩾ 26.40, HOMA ⩾ 1.87, HDL < 54.50, TGL ⩾ 94, AMDS < 34, WDS ⩾ 15.5, physical activity Baecke's total score < 41.5, Sun exposure score SES < 34.5, and sleep daily hours, calculated on a weekly base sleep hours < 8.0. Contingency tables and Odds ratio were calculated for NAFLD *vs* controls, according to above defined thresholds. Greater prevalence of overweight-obesity, insulin resistance, increased triglycerides and low HDL cholesterol, poor adherence

Table 1 Differences between non-alcoholic-fatty-liver-disease and control patients

	NAFLD (*n* = 532)	Controls (*n* = 667)	*P* vaule
Age, yr	48.11 ± 9.00	48.60 ± 8.70	0.343
Systolic blood pressure (mmHg)	124.53 ± 9.71	121.21 ± 10.80	< 0.0001
Diastolic blood pressure (mmHg)	78.84 ± 6.72	76.50 ± 6.73	< 0.0001
BMI, kg/m^2	30.49 ± 5.55	24.44 ± 3.72	< 0.0001
HOMA	2.89 ± 1.76	1.80 ± 1.28	< 0.0001
eGFR	82.49 ± 14.15	82.15 ± 17.44	0.714
Total cholesterol, mg/dL	205.17 ± 37.16	207.09 ± 38.82	0.387
HDL cholesterol, mg/dL	51.67 ± 15.85	61.45 ± 16.41	< 0.0001
Triglycerides, mg/dL	109.08 ± 42.41	95.23 ± 58.59	< 0.0001
LDL cholesterol, mg/dL	131.98 ± 33.45	126.59 ± 37.29	0.009
γ-GT (U/L)	33.24 ± 29.40	26.03 ± 21.95	< 0.0001
AST (U/L)	20.77 ± 5.91	21.01 ± 7.10	0.530
ALT (U/L)	15.65 ± 4.60	10.40 ± 4.88	< 0.0001
Alkaline phosphatase (U/L)	68.37 ± 18.49	72.75 ± 43.42	0.030
Serum albumin (g/dL)	4.62 ± 0.39	4.53 ± 0.40	< 0.0001
Lifestyle items			
AMDS	32.21 ± 0.91	34.91 ± 0.61	< 0.0001
Baecke - physical activity total score	39.82 ± 3.60	41.43 ± 3.32	< 0.0001
Western diet score	22.84 ± 7.87	12.73 ± 2.48	< 0.0001
Sun exposure score	31.43 ± 3.89	35.73 ± 5.25	< 0.0001
Sleep hours	7.86 ± 1.31	7.90 ± 1.23	0.552

BMI: Body mass index; HOMA-IR: Homoeostasis model insulin resistance; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; γ-GT: γ-glutamyl transpeptidase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; AMDS: Adherence mediterranean diet score; NAFLD: Non-alcoholic-fatty-liver-disease; eGFR: Estimated glomerular filtration rate.

to mediterranean diet profile, greater use of Western diet food, greater sedentary life habits and minor sun exposure, open air time were observed (Table 2).

Multiple Linear regression analysis (Table 3), weighted by years of age for avoiding age as a potential confounding factor, using the same items as predictors of the severity of fatty liver, assessed by US as BLS, confirms the significance of the chosen model, displaying BMI, HOMA and AMDS as the most powerful predictors of fatty liver severity; also the physical activity score, the western diet habit and the sun exposure score are still inside the model, with significant independent effects. The number of sleep hours does not show any significant linear effect in the model. Nonetheless, in a separate analysis, sleep hours display a U shaped behaviour, showing a greater relationship with more severe fatty liver at the two extremes of the curve: Few and many hours of sleep are both associated with more severe fatty liver.

DISCUSSION

Currently, overweight and obesity are the most established associated factors of NAFLD, and are considered, even with some limitation, actual risk factors and putative, indirect causative factors[2,3]. Nonetheless, other and quite neglected factors were and are studied: Most of

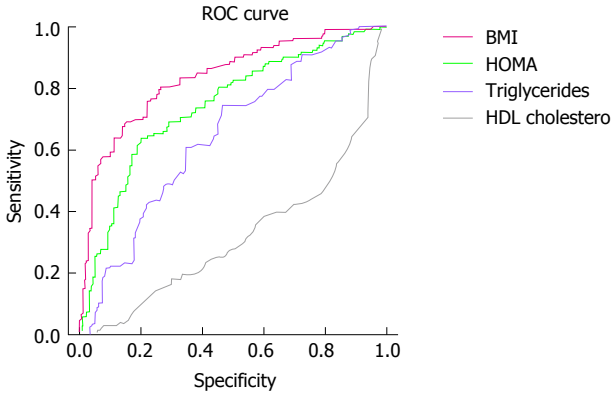

Figure 1 Receiver operating characteristic curves of body mass index, homoeostasis model insulin resistance, Triglycerides and high-density lipoprotein - cholesterol. The performance of each measure in the prediction of NAFLD is evaluated by the receiver operating characteristic (ROC) curve. The area under the ROC curve (AUROC) and the 95%CI are used as indexes of accuracy. The optimal cutoff value is determined as the maximum sum of sensitivity and specificity. Accordingly, BMI displays the greater accuracy for predicting NAFLD in comparison of HOMA, Triglycerides and HDL-Cholesterol. The cutoffs are used as thresholds for the calculation of odds of NAFLD, as reported in Table 2. BMI: Body mass index; HOMA: Homoeostasis; HDL: High-density lipoprotein; NAFLD: Non-alcoholic-fatty-liver-disease.

them are related to behaviour, such as physical activity[5], sleep habits[4] and Sun exposure, this last with a likely effects on vitamin D status[22]. Nutrition has a qualitative profile, and not only a quantitative one, *i.e.,* not only caloric intake, so that the association of unhealthy dietary habits, apart the abuse of alcohol, is associated with unhealthy liver and, notably, NAFLD. This is confirmed in our study in which we observe that, apart the greater BMI, also a poorer adherence to mediterranean diet profile[5], widely and since several years used as a proxy of healthy diet, strongly predicts the occurrence of NAFLD, independently from overweight. Also the almost reciprocal western diet profile displays an unfavourable relationship for the occurrence of NAFLD. This is confirmed in our study by the significant difference of averages, with a greater WDS in NAFLD (Table 1), by the greater odds of NAFLD associated with greater BMI and western diet habits, and with lower adherence to mediterranean diet (Table 2). Moreover, by a model of multivariate analysis (Table 3) the effects of BMI, mediterranean diet and western diet are independently operating, addressing clearly to the opposite effects of mediterranean diet (favourable) and of western diet and overweight (detrimental). Concurrently with nutritional profiles and BMI, sedentary life, assessed quantitatively as physical activity score, displays the same effects: A better physical exercise profile is associated with a lower prevalence (Table 2) and severity of bright liver score (Table 3), as assessed in NAFLD by liver US. Physical activity score is overall poorer in NAFLD *vs* controls (Table 1). The same association is observed for the sun exposure score, which is greater in controls (Table 1) and which may indicate, apart a greater open air life, also a better D vitamin status, important because vitamin D deficiency is associated with NAFLD[22]. Differently from

Table 2 Pearson's χ^2 and odds ratio

	NAFLD	Controls	χ^2	*P* value	OR	95%CI
BMI ⩾ 26.40	408	167	316.385[1]	< 0.0001	9.851	7.546-12.861
BMI < 26.40	124	500				
HOMA ⩾ 1.87	368	211	167.011[1]	< 0.0001	4.849	3.792-6.202
HOMA < 1.87	164	456				
HDL ⩾ 54.50	204	400	55.358[1]	< 0.0001	0.415	0.329-0.524
HDL < 54.50	328	267				
TGL ⩾ 94	324	240	73.775[1]	< 0.0001	2.771	2.191-3.506
TGL < 94	208	427				
AMDS ⩾ 34	32	650	1008.831[1]	< 0.0001	0.002	0.001-0.003
AMDS < 34	500	17				
BAECKE ⩾ 41.5	181	354	43.468[1]	< 0.0001	0.456	0.360-0.577
BAECKE < 41.5	351	313				
WDS ⩾ 15.5	399	97	445.981[1]	< 0.0001	17.629	13.174-23.590
WDS < 15.5	133	570				
SES ⩾ 34.5	111	348	122.788[1]	< 0.0001	0.242	0.187-0.313
SES < 34.5	421	319				
Sleep hours ⩾ 8	319	370	2.592[1]	0.107	1.210	0.959-1.527
Sleep hours < 8	208	292				

[1]Indicates the thresholds calculated by ROC analysis used as cut-offs for comparison between groups with lower measures (BMI, HOMA, AMDS, WDS, SES, BAECKE) *vs* groups with greater measures. BMI: Body mass index; HOMA-IR: Homoeostasis model insulin resistance; HDL: High-density lipoprotein; TGL: Triglycerides; AMDS: Adherence to mediterranean diet score; WDS: Western diet score; SES: Sun Exposure Score; BAECKE: Baecke's physical activity questionnaire total score.

Table 3 Multiple linear regression of variables

Predictors	*R*	R^2	*F*	Sig.	β	*P* value
	0.965	0.932	2309.1	< 0.0001		
BMI, kg/m^2					-0.448	< 0.0001
HOMA					-0.393	< 0.0001
AMDS					-1.398	< 0.0001
Baecke					-0.074	< 0.0001
WDS					0.069	< 0.0001
Sun exposure score					-0.044	< 0.0001
Sleep hours					-0.008	0.296

Weighted Least Squares Regression - Weighted by Age. Baecke's physical activity questionnaire total score and sleep hours *vs* the severity of NAFLD (included in this analysis as a categorical variable with all 3 severity grades), assessed by ultrasound as bright liver score. BMI: Body mass index; HOMA-IR: Homoeostasis model insulin resistance; AMDS: Adherence to mediterranean diet score; WDS: Western diet score; NAFLD: Non-alcoholic-fatty-liver-disease.

the observation reported in youngsters[4], sleep hours do not show any significant relationship with NAFLD.

We must acknowledge several limitations of our study. First, the overall, comparison between NAFLD patients and controls (Table 1) does not display extreme differences, even if they are statistically significant, when considering sleep hours, sun exposure, AMDS and physical activity. There are very different features considering the greater score of Western Diet profile pattern in NAFLD. These even small differences between NAFLD and controls become more relevant within the model that takes into account all the co-variates, so that we must still consider them as relatively important features regarding NAFLD, even envisaging a size effect in the group studied.

Second limitation is that our eligibility criteria were rather strict, resulting in a population without significant co-morbidities, since all patients with diabetes and/or even minimally elevated ALT levels were excluded. It is possible that the analyzed lifestyle measures might work differently in a more comprehensive NAFLD cohort that includes other associated diseases. Scope of the study was to investigate NAFLD as an almost-isolated disease, and even with these restrictions association of recognized and neglected aspects of lifestyle are seemingly operating.

Modification over the time of healthier nutritional and behavioural profiles is a very articulated topic of investigation, which includes also the need of assessing the process of erosion of traditionally cohesive family and community relationships[24] with effects on health and mortality. Such studies have a counterpart in the current societal efforts aimed at the preservation of traditional habits, and even clinical conditions, such as high hemoglobin levels[25] which often are credited as healthier. Many animal models have been studied in which dietary variations produce liver injury, and by extrapolation, malnutrition, particularly deficiencies of protein and vitamins has long been considered an important factor in human cirrhosis when no evidence existed for another aetiology; by contrast, weight reduction through low-calorie diets or starvation reduces the steatosis resulting from obesity[1]. Malnutrition was in the last century, and now again, the key of many disease and, notably of liver disease, with its paradigm of fatty liver evolving toward fibrosis. Apart the pioneering studies on lifestyle changes[26] we are still on the starting blocks because each aspect of lifestyle is studied, and thereafter assessed and managed as

an individual factor. Despite the great attention which is devoted in Europe to healthier environment and to urban mobility, using the paradigm of smart city, few or no research are at the moment published and available, even if elsewhere there is already a move in this sense also by comprehensive approach focused to clinical risk assessment and management[2]. The important most recent reviews appropriately address benefits of healthy diet and exercise on NAFLD[27] both in adults[28] and in children[29], even if other factors, genetic[30], behavioural and environmental should not be neglected[31,32]. The opportunity for the medicine are relevant since articulated clinical intervention, which, according to our results, are justified, can be pursued with a focus on nutritional profile, physical exercise mainly open-air for enhancing sun exposure and improving sleep duration and rhythm[33], cultural and traditional medicine issues and, comprehensively, the quality of life[34-39]. The pre-requisite is that both medical doctor and patient should not be mucking around in search of the magic bullet, and instead try to take seriously and with a strategy the road of lasting lifestyle change. Individual, professional and societal benefits are the outcomes that can be reached[2].

COMMENTS

Background

In a very simplified manner today the authors tend to describe the lifestyles in medicine especially in terms of diet and physical inactivity or sedentary life, with a synergistic effect on body size - obesity - and on disease related with excessive food intake (atherosclerosis and liver disease).

Research frontiers

Many animal models have been studied in which dietary variations produce liver injury, and by extrapolation, malnutrition; particularly deficiencies of protein and vitamins has long been considered an important factor in human cirrhosis when no evidence existed for another aetiology; by contrast, weight reduction through low-calorie diets or starvation reduces the steatosis resulting from obesity.

Innovations and breakthroughs

This is confirmed in their study in which they observe that, apart the greater BMI, also a poorer adherence to mediterranean diet profile, widely and since several years used as a proxy of healthy diet, strongly predicts the occurrence of non-alcoholic-fatty-liver-disease (NAFLD), independently from overweight. Also the almost reciprocal western diet profile displays an unfavourable relationship for the occurrence of NAFLD. This is confirmed in our study by the significant difference of averages, with a greater western diet score in NAFLD, by the greater odds of NAFLD associated with greater body mass index and western diet habits, and with lower adherence to mediterranean diet.

Applications

The opportunity for the medicine is relevant since articulated clinical intervention, which, according to their results, are justified, can be pursued with a focus on nutritional profile, physical exercise mainly open-air for enhancing sun exposure and improving sleep duration and rhythm, cultural and traditional medicine issues and, comprehensively, the quality of life. The pre-requisite is that both medical doctor and patient should not be mucking around in search of the magic bullet, and instead try to take seriously and with a strategy the road of lasting lifestyle change. Individual, professional and societal benefits are the outcomes that can be reached.

Peer-review

The manuscript of "Neglected features of lifestyle: Their relevance in non-alcoholic fatty liver disease" is very interesting.

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ORIGINAL ARTICLE

Prospective Study

Can platelet count/spleen diameter ratio be used for cirrhotic children to predict esophageal varices?

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Author contributions: Sezer OB and Özçay F designed the manuscript; Sezer OB, Çelik D and Özçay F collected the data; Sezer OB and Özçay F substantially contributed to the conception or design; Tutar N performed all of the ultrasonographic examinations; Sezer OB wrote the manuscript; Sezer OB and Özçay F gave final approval and agreed to be accountable for all aspects of the work, ensuring that questions relating to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Institutional review board statement: This study was reviewed and approved by the Ethics Committee of Baskent University.

Clinical trial registration statement: Study registration information number of our study is KA11/11252.

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

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Abstract

AIM

To determine the laboratory and radiologic parameters, including the platelet count (PC)-to-spleen diameter (SD) ratio as a non-invasive marker that may predict the presence of esophageal varices (EV) in children with cirrhosis.

METHODS

Eighty-nine patients with cirrhosis, but without a history of variceal bleeding were prospectively included. The children were grouped into 6-12 and 12-18 years of age groups. These groups were also divided into 2 subgroups (presence and absence of EV). All of the patients underwent a complete biochemical and radiologic evaluation. The PC (n/mm^3)-to-SD (mm) ratio was calculated for each patient.

RESULTS

Sixty-nine of 98 (70.4%) patients had EV. The presence of ascites in all age groups was significantly associated

with the presence of EV. There were no differences in serum albumin levels, PC, SD and the PC-to-SD ratio between the presence and absence of EV groups in both age groups ($P > 0.05$).

CONCLUSION

Laboratory and radiologic parameters, including the PC-to-SD ratio as a non-invasive marker (except for the presence of ascites), was inappropriate for detecting EV in children with cirrhosis.

Key words: Esophageal varices; Variceal bleeding; Platelet count-to-spleen diameter ratio; Children

Core tip: Laboratory and radiologic parameters, including the platelet count (PC)-to-spleen diameter (SD) ratio were investigated in children with cirrhosis as a non-invasive marker that may predict the presence of esophageal varices (EV). This study is the first study to assess the PC-to-SD ratio in children with cirrhosis for detecting EV according to age groups This study demonstrated that the parameters, other than the presence of ascites, were inappropriate for detecting EV in children with cirrhosis.

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INTRODUCTION

Esophageal variceal bleeding is among the most serious consequences of chronic liver disease[1]. Approximately two-thirds of children with cirrhosis have esophageal varices (EV), and the mortality associated with a variceal bleeding episode is 20%-35%[1-4]. Prevention of bleeding from a ruptured EV has become one of the main goals in the follow-up of these patients. Although a consensus has been reached for adults, there is no formal recommendation for endoscopic screening in children with cirrhosis[5].

Esophagogastroduodenoscopy (EGD) is the present reference standard diagnostic test for EV. Nevertheless, only 50%-70% of cirrhotic patients have varices on the first EGD and < 30% have large varices and/or the red wale sign (high-risk EV for bleeding) in adults and children[6-8]. Because of the relatively low prevalence of varices that require primary prophylaxis, the cost, inconvenience, and morbidity associated with endoscopic surveillance may not be justified for all patients with cirrhosis. To reduce the increasing burden on endoscopy units and prevent unnecessary harm to patients, researchers have attempted to identify parameters for non-invasive prediction of EV[9]. Several reports have identified non-invasive variables that may predict the presence of EV in childhood and have shown predictive factors for bleeding risk, such as hypoalbuminemia, the Child-Pugh score, an increased spleen diameter (SD), a low platelet count (PC), the PC-to-SD ratio, the clinical prediction rule, and the aspartate aminotransferase-to-platelet ratio index[6,7,10]. For this purpose, the PC-to-SD ratio was investigated to predict the presence of EV in adult patients with cirrhosis[11-14]. Chawla *et al*[15] concluded that the PC-to-SD ratio is elegant, simple and inexpensive, and it may become a helpful tool to limit the number of endoscopies for primary prophylaxis in adult patients with portal hypertension. Therefore, we conducted this study to investigate laboratory and radiologic parameters, including the PC-to-SD ratio, as predictors of EV in children with cirrhosis.

MATERIALS AND METHODS

All children (6-18 years of age) who had been diagnosed with cirrhosis in the outpatient clinics of the Paediatric Gastroenterology Hepatology and Nutrition at Baskent University, Ankara, Turkey, were included in this prospective study. The diagnosis of cirrhosis was made based on laboratory, radiologic, and physical examination findings or by liver histology in the absence of clear clinical signs of liver cirrhosis. Demographic characteristics (age, gender and underlying disease), blood chemistry evaluations, international normalized ratio, and Child-Pugh scores were recorded for each patient.

Patients with a clinical history of upper digestive hemorrhage, band ligation, sclerosing therapy, transjugular intrahepatic portosystemic stent shunt, surgery for portal hypertension, hepatic encephalopathy, and use of beta-blockers or other vasoactive drugs were excluded from the study.

The children were further grouped into 6-12 and 12-18 year age groups. These groups were divided into two sub-groups (EV-present and -absent) based on the EGD. The EGD was performed by the same paediatric endoscopists in our endoscopy unit using a video endoscope (Olympus GIF-XP 240; Tokyo, Japan or Fujinon EG 590W videoendoscopy; Tokyo, Japan). EV were classified according to the Baveno IV criteria[16,17] and American Association for the Study of Liver Diseases practice guidelines[18] as no, small, and large varices. EV were also classified according to the bleeding risk as high risk and non-high risk using varices diameters and red sign parameters.

The spleen bipolar diameter and presence of ascites were evaluated by ultrasonography (Siemens Sonoline Antares 4.1 MHz or 9.4 MHz probe; Siemens Medical Solutions United States, Inc., Issaquah, WA, United States) by the same radiologist.

The study design was approved by the Ethics Committee of our hospital (Study No. KA11/11252). Before enrollment, written informed consent was obtained from the primary caretaker of each patient.

We used SPSS software (version 16.0; SPSS, Inc.,

Table 1 Laboratory and ultrasonographic data in the age group of 6-12 years

	Patient with varices (n = 29)	Control (n = 13)	P value
Mean age (yr)	9.7 ± 2.0	10.0 ± 1.9	0.595
Gender (% female)	45	38.5	0.384
INR	1.5 ± 0.5	1.4 ± 0.5	0.860
ALT (IU/L)	57.3 ± 50.8	44.3 ± 29.3	0.210
AST (IU/L)	79.1 ± 71.5	53.1 ± 32.9	0.241
Total bilirubin (mg/dL)	3.5 ± 6.0	2.5 ± 4.7	0.618
Albumin (mg/dL)	3.9 ± 0.6	4.2 ± 0.6	0.231
Ultrasonographic ascites (%)	27.5%	0%	0.037
Spleen diameter (mm)	167.3 ± 39.1	151.3 ± 32.4	0.206
Platelet count (thousand/mm^3)	129000 ± 53519	153000 ± 97798	0.312
Platelet count/spleen diameter	976.6 ± 793.5	1062.4 ± 718.0	0.741
Child-Pugh score	6.3 ± 1.5	5.7 ± 1.4	0.193

INR: International normalized ratio; ALT: Alanine transaminase; AST: Aspartate transaminase.

Table 2 Laboratory and ultrasonographic data in the age group of 12-18 years

	Patient with varices (n = 40)	Control (n = 16)	P value
Mean age (yr)	14.2 ± 1.7	13.5 ± 1.2	0.161
Gender (% female)	48	56	0.591
INR	1.3 ± 0.3	1.4 ± 0.6	0.347
ALT (IU/L)	86.5 ± 76.1	60.5 ± 67.2	0.250
AST (IU/L)	115.2 ± 124.2	105.5 ± 231.8	0.842
Total bilirubin (mg/dL)	5.0 ± 10.4	5.3 ± 12.5	0.931
Albumin (mg/dL)	3.8 ± 0.7	3.9 ± 0.7	0.757
Ultrasonographic ascites (%)	35%	6%	0.028
Spleen diameter (mm)	181.4 ± 37.2	150.3 ± 34.2	0.389
Platelet count (thousand/mm^3)	103000 ± 55867	115000 ± 65472	0.499
Platelet count/spleen diameter	733.9 ± 737.4	830.78 ± 553.5	0.637
Child-Pugh score	6.9 ± 1.9	6.2 ± 1.8	0.214

INR: International normalized ratio; ALT: Alanine transaminase; AST: Aspartate transaminase.

Chicago, IL, United States) for statistical analysis. Data are expressed as the mean and standard deviation and proportions. For comparison of categorical variables, Fisher's exact test or a χ^2 test was used. Differences between numeric variables were tested with a Mann-Whitney U-test. Values of $P < 0.05$ were considered to indicate statistically significant differences.

RESULTS

Ninety-eight children with cirrhosis were included in this study. The ages of the children ranged from 6-18 years (median age, 12.16 ± 2.70 years). Forty-six children were females (46.9%) and 52 were males (53.1%).

The etiology of cirrhosis was cryptogenic cirrhosis (n = 40), Wilson's disease (n = 35), progressive familial intrahepatic cholestasis (2), sclerosing cholangitis (n = 4), Budd-Chiari syndrome (n = 4), tyrosinemia (n = 3), glycogen storage disease (n = 3), autoimmune hepatitis (n = 2), hepatitis B infection (n = 2), Allagille syndrome (n = 2), and alfa1-antitrypsin deficiency (n = 1). Sixty-one patients were Child-Pugh class A, 29 were class B, and 8 were class C.

In this study, 69 children (70.4%) were shown to have EV based on the first EGD and 29 children (29.6%) were shown not to have EV.

Fifty-five of the 69 patients had small EV (79.7%) and 14 patients (20.3%) had large EV. There were 11 children (15.9%) with red wale signs (seven children had large EV and four children had small EV). Therefore, 18 of the 69 patients with EV (26.1%) had high-risk EV for bleeding according to the presence of large varices and/or red sign (six patients in the 6-12 year age group, and 12 patients in the 12-18 year age group).

There were no differences in age and gender between the EV-present and -absent sub groups in both age groups ($P > 0.05$). In the two age groups, a higher percentage of ascites was observed among the EV-present group than the EV-absent group (Tables 1 and 2). We did not find a statistically significant difference in the PC-to-SD ratio between patients with large and small varices (636.9 ± 256.5 and 894.1 ± 844.4, respectively; $P = 0.89$).

We did not find a significant difference for serum albumin, PC, SD and the PC-to-SD ratio between the EV-present and -absent varices sub-groups in both age groups ($P > 0.05$; Tables 1 and 2).

DISCUSSION

Despite advances in diagnosis and treatment, bleeding from EV is one of the major causes of morbidity and mortality among patients with cirrhosis. Hence, preventing the first episode of variceal bleeding may reduce mortality and morbidity.

In this prospective study involving children 6-18 years of age with cirrhosis, we found that only the presence of ascites is associated with the presence of EV. There have been several studies identifying non-invasive variables that may predict the presence of EV in children[6,7,10]. The first study, in which the predictive risk factors were evaluated by Fagundes *et al*[6] in a pediatric group [median age at the time of first EGD was 6 years (age range, 0.7-17.6 years)], showed that children with cirrhosis and splenomegaly were nearly 15-fold more likely to have EV compared with children with cirrhosis but without splenomegaly. Fagundes *et al*[6] concluded that hypoalbuminemia, splenomegaly, and a PC < 130000/mm^3 were predictors for the presence of EV, spleen size was not measured by ultrasonography. The second study, conducted by Gana *et al*[7], derived a non-invasive clinical prediction rule capable of identifying children with EV. In this study, 17 of 51 children (< 18 years of age) with liver disease or portal vein thromboses were shown

to have EV, and hypoalbuminemia was shown to be an independent variable for the presence of EV. In the same study[7], a higher percentage of ascites, increased spleen length, and lower PC (cut-off value = 115000/mm^3) were reported among children with EV. Further, the PC-to-spleen length-for age Z score ratio was significantly lower among the EV-present group[7].

Fagundes *et al*[6] and Gana *et al*[7] reported lower albumin levels among children with EV. Our results were not in agreement with the findings of these two studies. A possible explanation may be the difference in etiologic factors in our patients.

A recent study involving 103 patients with a diagnosis of chronic liver disease or extrahepatic portal vein obstruction (mean age, 8.9 ± 4.7 years) showed a significantly higher spleen length and lower PC (cut-off value = 115000/mm^3) among children with EV than children without EV[10]. In the same study, it was reported that a PC-to-spleen size (cm) ratio < 1.0 discriminated between patients with and without EV, despite a lack of statistical significance based on logistic regression. The authors suggested that the lack of statistical significance was explained by the age and gender differences in spleen size.

Based on the findings of these three studies[6,7,10], low PC and increased spleen length are logical parameters by which to determine EV in children with cirrhosis. In addition, Gana *et al*[7] and Adami *et al*[10] reported that PC (cut-off value = 115000/mm^3) was the best predictor of EV.

In the current study, we did not find a significant difference for PC, SD and the PC-to-SD ratio between the EV-present and -absent sub-groups in both age groups. A possible explanation is the heterogeneity of patients studied. Another explanation is the lack of children with portal vein thromboses in the current study. The three studies investigating risk factors for EV included children with cirrhosis and portal vein tromboses[6,7,10]. It is well-known that portal vein thrombosis is a risk factor for splenomegaly and thrombocytopenia. The PC loses discriminatory power because of multi-causality (such as autoimmune events, myelotoxic effects of viruses, or reduced synthesis of thrombopoietin) as a consequence of progressive liver dysfunction; however, in children with portal vein thromboses, thrombocytopenia is directly related to portal hypertension, as well as the development of varices[19].

One of the most important limitations of our study was the small number of patients; however, this study was the first study to assess the PC-to-SD ratio in children in two age groups with cirrhosis as a means to detect EV. We consider the PC, SD and PC-to-SD ratio to lack suitability as non-invasive markers for detecting EV in children with cirrhosis. Further studies on this subject with larger sample sizes are required to assess the importance of the PC, SD and PC-to-SD ratio in cirrhotic children with or without portal vein thrombosis.

COMMENTS

Background

Esophageal variceal (EV) bleeding is among the most serious consequences of chronic liver disease. Approximately two-thirds of children with cirrhosis have EV and the mortality associated with a variceal bleeding episode is 20%-35%. Identification of children with cirrhosis who are at high risk for EV using a non-invasive test is important to reduce the need for endoscopy. The authors' goal was to investigate laboratory and radiologic parameters, including the platelet count (PC)-to-spleen diameter (SD) ratio to predict the presence of EV in children with cirrhosis.

Research frontiers

To reduce the increasing burden on endoscopy units and prevent unnecessary harm to patients with cirrhosis, researchers have attempted to identify parameters for the non-invasive prediction of EV.

Innovations and breakthroughs

A few studies have shown that a low PC and PC-to-SD ratio may predict the presence of EV in patients with cirrhosis. In their study, the authors did not find a significant difference in the PC, SD and PC-to-SD ratio between the EV-present and -absent sub-groups in both age groups of children.

Applications

The PC-to-SD ratio is not an appropriate index with which to predict EV in children with cirrhosis. This may indicate that endoscopy remains the ideal choice for detecting EV in children with cirrhosis.

Terminology

Esophageal varices are abnormal, enlarged veins which generally occur in patients with serious liver diseases. The vessels can leak blood, or even rupture, thus causing life-threatening bleeding.

Peer-review

It is helpful for clinical doctors to perform endoscopic examination promptly.

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ORIGINAL ARTICLE

Prospective Study

Elevation of serum urokinase plasminogen activator receptor and liver stiffness in postoperative biliary atresia

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Author contributions: Honsawek S designed the study; Udomsinprasert W, Honsawek S and Jirathanathornnukul N performed the research; Udomsinprasert W, Honsawek S, Jirathanathornnukul N, Chongsrisawat V and Poovorawan Y analyzed the data; Chongsrisawat V and Poovorawan Y examined all the patients and collected the clinical data; Udomsinprasert W and Honsawek S wrote the paper; Honsawek S revised the manuscript for final submission.

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Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

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Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at sittisak.h@chula.ac.th. Participants gave informed consent for data sharing.

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Abstract

AIM

To investigate serum urokinase-type plasminogen activator receptor (uPAR) and liver stiffness in biliary atresia (BA) and examine the correlation of circulating uPAR, liver stiffness, and clinical outcomes in postoperative BA children.

METHODS

Eighty-five postKasai BA children and 24 control subjects were registered. Circulating uPAR was measured using enzyme-linked immunosorbent essay. Liver stiffness was analyzed using transient elastography.

RESULTS

BA children had significantly greater circulating uPAR and

liver stiffness scores than control subjects ($P < 0.001$). Circulating uPAR and liver stiffness were substantially higher in jaundiced BA children than non-jaundiced BA children ($P < 0.001$). In addition, circulating uPAR was positively associated with serum aspartate aminotransferase ($r = 0.507$, $P < 0.001$), alanine aminotransferase ($r = 0.364$, $P < 0.001$), total bilirubin ($r = 0.559$, $P < 0.001$), alkaline phosphatase ($r = 0.325$, $P < 0.001$), and liver stiffness scores ($r = 0.508$, $P < 0.001$).

CONCLUSION

Circulating uPAR and liver stiffness values were greater in BA children than healthy controls. The increased circulating uPAR was associated with liver dysfunction in BA. As a consequence, serum uPAR and liver stiffness may be used as noninvasive biomarkers indicating the progression of liver fibrosis in postKasai BA.

Key words: Biliary atresia; Jaundice; Liver stiffness; Severity; Urokinase plasminogen activator receptor

Core tip: Urokinase plasminogen activator receptor (uPAR) is known to be a substantial factor in the etiopathogenesis of hepatic inflammation and liver fibrogenesis. This study is the first to show that circulating uPAR is more elevated in biliary atresia (BA) children than in control subjects, and that circulating uPAR is correlated with the degree of jaundice and liver fibrosis in biliary atresia. Elevated serum uPAR is positively correlated with the severity of liver stiffness in postKasai BA children. Hence, serum uPAR could be used as a biological parameter indicating the progression and prognosis of liver fibrosis in BA children.

Udomsinprasert W, Honsawek S, Jirathanathornnukul N, Chongsrisawat V, Poovorawan Y. Elevation of serum urokinase plasminogen activator receptor and liver stiffness in postoperative biliary atresia. *World J Hepatol* 2016; 8(33): 1471-1477 Available from: URL: http://www.wjgnet.com/1948-5182/full/v8/i33/1471.htm DOI: http://dx.doi.org/10.4254/wjh.v8.i33.1471

INTRODUCTION

Biliary atresia (BA) is a severe chronic cholestatic liver disease of unknown etiology in young infants. The estimated incidence of BA varies from 1 in 8000 to 1 in 20000 live births, with a high frequency in Asians[1]. Affected newborns exhibit evidence of biliary obstruction within the first few months of life. BA is manifested by impaired liver function and fibroinflammatory obliterative cholangiopathy of both intrahepatic and extrahepatic bile ducts[2,3]. Extrahepatic BA is the most common form of ductal cholestasis. BA patients initially develop neonatal jaundice due to hepatic cholestasis and progress to hepatic fibrosis, which result in biliary cirrhosis[1-3]. Even though no medical therapies exist, sequential treatment strategy involving surgical Kasai portoenterostomy and liver transplantation is the only option for the most affected children. Nonetheless the precise pathogenesis of BA has yet to be determined, a number of theories regarding the etiology of BA include toxin exposure, virus-mediated inflammation, abnormal inflammatory response, defective morphogenesis, genetic mutation, and immunological dysregulation[4].

Urokinase-type plasminogen activator receptor (uPAR, CD87) is a cellular membrane receptor that attachs to urokinase-type plasminogen activator (uPA) with high affinity, through promoting the pericellular activation of plasminogen[5]. The involvement of uPA, its receptor (uPAR), and plasminogen activator inhibitor-1 (PAI-1) in regulation of cell adhesion, migration, proliferation, differentiation, and cell survival has recently demonstrated[6]. uPAR is expressed by a wide range of immune cells and endothelial cells, which contribute to the etiopathogenesis of hepatic inflammation and liver fibrogenesis[7,8]. Once inflammation is activated, uPAR is released from the cell membrane by proteolytic enzymes to produce soluble uPAR[9]. In recent years, previous studies have investigated that elevated circulating uPAR levels have been observed in acute liver failure, chronic liver diseases, and nonalcoholic fatty liver diseases[10-12].

It has been previously shown that certain cytokines and growth factors play possible parts in the etiopathology of biliary atresia[13-16]. The measurements on circulating uPAR and liver stiffness of BA have never been documented. We hypothesized that circulating uPAR and liver stiffness could be more elevated in BA patients than in control subjects and circulating uPAR would be associated with the disease severity and clinical outcomes in postKasia biliary atresia. Hence, the purpose of the current research is to determine circulating uPAR and liver stiffness measurements and to investigate the plausible correlation of circulating uPAR, liver stiffness, and clinical outcomes in postoperative biliary atresia children.

MATERIALS AND METHODS

The present study was approved by the Institutional Review Board on Human Research of the Faculty of Medicine, Chulalongkorn University, and was conducted in compliance with the ethical guidelines of the Declaration of Helsinki. All parents of children were informed of the study's purpose and of any interventions involved in the current study. Written informed consent was derived from the parents prior to the subjects entering the study.

Study population

Eighty-five BA children (39 girls and 46 boys with mean age of 9.0 ± 0.6 years) and 24 normal control subjects (11 girls and 13 boys with mean age of 8.5 ± 0.5 years) were enrolled in the study. None of them had undergone

liver transplantation. Healthy controls attending the Well Baby Clinic at our institution for vaccination had normal physical findings and no underlying disease. BA children were classified into two groups according to their serum total bilirubin (TB): Non-jaundiced BA children (TB < 2 mg/dL, n = 46) and persistent jaundiced BA children (TB ⩾ 2 mg/dL, n = 39).

Laboratory methods

Samples of peripheral venous blood were collected from every participant, and were kept at -80 ℃ for subsequent measurement. The quantitative assessment of serum uPAR was performed by using commercially available enzyme-linked immunosorbent essay (Quantikine, R and D Systems, Minneapolis, MN, United States). According to the manufacturer's protocol, recombinant human uPAR standards and serum samples were added into each well, which has been pre-coated with specific antibody to uPAR. After incubating for 2 h at room temperature, every well was washed thoroughly with wash buffer. Then, uPAR conjugate was pipetted into each well and incubated for 2 h at room temperature. After 4 washes, substrate solution was added into the wells and the microplate was incubated for 30 min at room temperature with protection from light. Lastly, the reaction was stopped by the stop solution and the optical density was determined using an automated microplate reader at 450 nm. A standard optical density-concentration curve was drawn for the determination of uPAR concentration. The liver function tests including serum albumin,TB, direct bilirubin, aspatate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) were measured using a Hitachi 912 (Roche Diagnostics, Basel, Switzerland) automated machine at the central laboratory of our hospital.

Liver stiffness measurement

Transient elastography (Fibroscan, Echosens, Paris, France) measured the liver stiffness between 25 to 65 mm from the skin surface, which is approximately equivalent to the volume of a cylinder of 1 cm diameter and 4 cm length. The measurements were performed by placing a transducer probe of Fibroscan on the intercostal space at the area of the right lobe of the liver with patients lying in a dorsal decubitus position with maximum abduction of the right arm. The target location for measurement was a liver portion that was at least 6 cm thick, and devoid of major vascular structures. The measurements were performed until 10 validated results had been obtained with a success rate of at least 80%. The median value of 10 validated scores was considered the elastic modulus of the liver, and it was expressed in kilopascals (kPa).

Statistical analysis

Statistical analysis was executed by using the SPSS version 22.0 statistical software package (SPSS Inc., Chicago, IL, United States). Comparisons of demographic and clinical outcomes between groups were performed using χ^2 and Student's unpaired t-test when appropriate. Correlation between numerical data was obtained using Pearson's correlation coefficient (r). Data were presented as mean ± SEM of the mean. A two-tailed P-value of less than 0.05 was taken to indicate statistical significance.

RESULTS

Comparison between BA children and control subjects

Eighty-five postoperative biliary atresia children and 24 ethnically matched unaffected volunteers were prospectively recruited in the current work. The baseline features of BA children and control subjects are presented in Table 1. There was no significant difference of age and gender between case and control groups. However, circulating uPAR values were substantially greater in BA children than in control subjects (6085.9 ± 400.7 pg/mL *vs* 4754.5 ± 294.9 pg/mL, P = 0.01) (Figure 1). Moreover, BA group had notably greater liver stiffness values than control group (28.7 ± 2.7 kPa *vs* 4.1 ± 0.2 kPa, P < 0.001).

Differences between jaundiced group and non-jaundiced group of BA children

BA children were subdivided into jaundiced group (n = 39) and non-jaundiced group (n = 46). The clinical characteristics and biochemical features of patients according to jaundice status are illustrated in Table 2. Jaudiced BA children exhibited remarkably greater serum uPAR levels than non-jaundiced BA children (7373.5 ± 684.6 pg/mL *vs* 4994.2 ± 400.9 pg/mL, P = 0.003) (Figure 2). Furthermore, mean liver stiffness measurement of jaundiced BA group was greatly increased compared with that of non-jaundiced BA group (46.2 ± 3.7 kPa *vs* 13.9 ± 2.0 kPa, P < 0.001).

Subsequent investigation revealed that circulating uPAR was directly associated with serum AST (r = 0.507, P < 0.001), ALT (r = 0.364, P < 0.001), TB (r = 0.559, P < 0.001), ALP (r = 0.325, P < 0.001), and liver stiffness values (r = 0.508, P < 0.001) in BA children (Figure 3). However, circulating uPAR concentration was negatively associated with serum albumin level (r = -0.666, P < 0.001) (Figure 3).

DISCUSSION

Biliary atresia is a chronic progressive fibroinflammatory liver disorder with mysterious etiology. The etiopathology of BA currently remains elusive and it seems that multiple factors may contribute to the development of BA. Yet today, Kasai operation has been proved as the most effective option of surgical treatment. Without surgery, children with biliary atresia will finally die due to biliary cirrhosis and liver failure[1]. Recently, circulating uPAR levels have been shown to be involved in chronic liver disorders, including chronic hepatitis B and C, liver cirrhosis, and hepatocellular carcinoma[17-20]. Based on

Table 1 Demographic data, biochemical characteristics, and liver stiffness scores of biliary atresia patients and healthy controls

Variables	BA (n = 85)	Controls (n = 24)	P value
Age (yr)	9.0 ± 0.6	8.5 ± 0.5	0.2
Gender (female:male)	39:46	11:13	0.4
Albumin (g/dL)	4.2 ± 0.1	-	NA
Total bilirubin (mg/dL)	2.7 ± 0.4	-	NA
Direct bilirubin (mg/dL)	2.3 ± 0.4	-	NA
AST (IU/L)	143.7 ± 11.9	-	NA
ALT (IU/L)	137.1 ± 12.5	-	NA
ALP (IU/L)	449.2 ± 34.0	-	NA
Liver stiffness (kPa)	28.7 ± 2.7	4.1 ± 0.2	< 0.001
uPAR (pg/mL)	6085.9 ± 400.7	4754.5 ± 294.9	0.01

The data was expressed as mean ± SEM. BA: Biliary atresia; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; uPAR: Urokinase-type plasminogen activator receptor; NA: Not applicable.

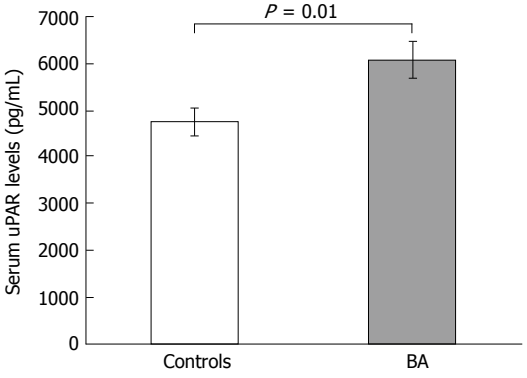

Figure 1 Comparison of serum urokinase-type plasminogen activator receptor levels in biliary atresia patients and healthy controls. uPAR: Urokinase-type plasminogen activator receptor; BA: Biliary atresia.

Table 2 Comparison of biliary atresia patients without and with jaundice

Variables	BA patients with jaundice (n = 39)	BA patients without jaundice (n = 46)	P-value
Age (yr)	9.5 ± 0.9	8.6 ± 0.9	0.4
Gender (female:male)	18:21	21:25	0.5
Albumin (g/dL)	3.8 ± 0.1	4.5 ± 0.1	< 0.001
Total bilirubin (mg/dL)	5.1 ± 0.7	0.5 ± 0.1	< 0.001
Direct bilirubin (mg/dL)	4.5 ± 0.6	0.2 ± 0.1	< 0.001
AST (IU/L)	210.4 ± 17.2	84.7 ± 10.2	< 0.001
ALT (IU/L)	195.9 ± 19.9	85.1 ± 10.7	< 0.001
ALP (IU/L)	599.7 ± 52.8	313.0 ± 32.0	< 0.001
Liver stiffness (kPa)	46.2 ± 3.7	13.9 ± 2.0	< 0.001
uPAR (pg/mL)	7373.5 ± 684.6	4994.2 ± 400.9	0.003

The data are expressed as mean ± SEM. BA: Biliary atresia; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; uPAR: Urokinase-type plasminogen activator receptor; NA: Not applicable.

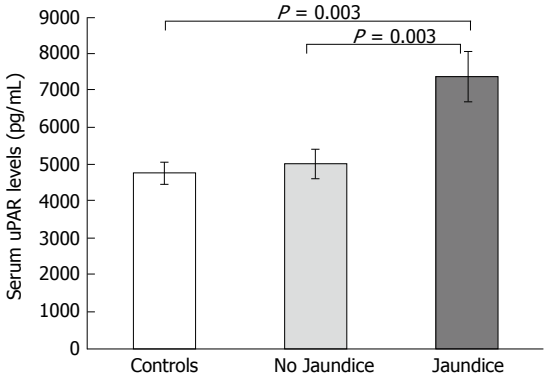

Figure 2 Comparison of serum urokinase-type plasminogen activator receptor levels in biliary atresia patients without jaundice and with jaundice. uPAR: Urokinase-type plasminogen activator receptor.

our experience, there is no report about circulating uPAR and hepatic fibrosis in various degrees of postoperative biliary atresia.

The present study is the first to show that circulating uPAR and liver fibrosis values were significantly higher in children suffering from BA than in control subjects. Additionally, circulating uPAR in jaundiced BA children was markedly increased with respect to that in non-jaundiced BA children. Elevated circulating uPAR levels were directly associated with total bilirubin, AST, ALT, ALP in post Kasai BA children, suggesting that circulating uPAR is related to degree of jaundice BA children. Furthermore, the degree of jaundice is possibly linked to the severity of intrahepatic biliary obliteration. Both AST and ALT are extensively used as biochemical parameters of hepatic abnormality indicating liver cell injury. Hence, the findings imply that uPAR could have a plausible role in the mechanism of liver cell injury in postoperative biliary atresia, and it would be associated with the severity of bile duct obliteration.

The present investigation demonstrated that circulating uPAR was more pronounced in biliary atresia children than control subjects. In accordance with this observation, Sjöwall *et al*[10] reported that circulating uPAR was increased in subjects with non-alcoholic fatty liver disease and associated with the severity of fibrosis. Moreover, uPAR expressions in liver tissue samples have been documented in subjects with hepatocellular carcinoma as shown by Morita *et al*[21]. In addition, Zimmermann *et al*[12] reported that circulating uPAR was substantially elevated in subjects with chronic liver diseases compared with controls and were closely correlated with liver function and fibrosis.

In light of our findings, certain hypotheses could explain high circulating uPAR in jaundiced biliary atresia children. Firstly, the release of uPAR in the injured liver could be accountable for the increased circulating uPAR. Secondly, the elevation of circulating uPAR may be ascribed to the unbalance between uPAR synthesis and uPAR clearance. The reduction of uPAR destruction in BA children with liver fibrosis may lead to the elevated circulating uPAR. Decreased pre-systemic hepatic metabolism might explain the increased uPAR levels in serum BA children with hepatic dysfunction. Besides, other tissues outside the liver could synthesize and

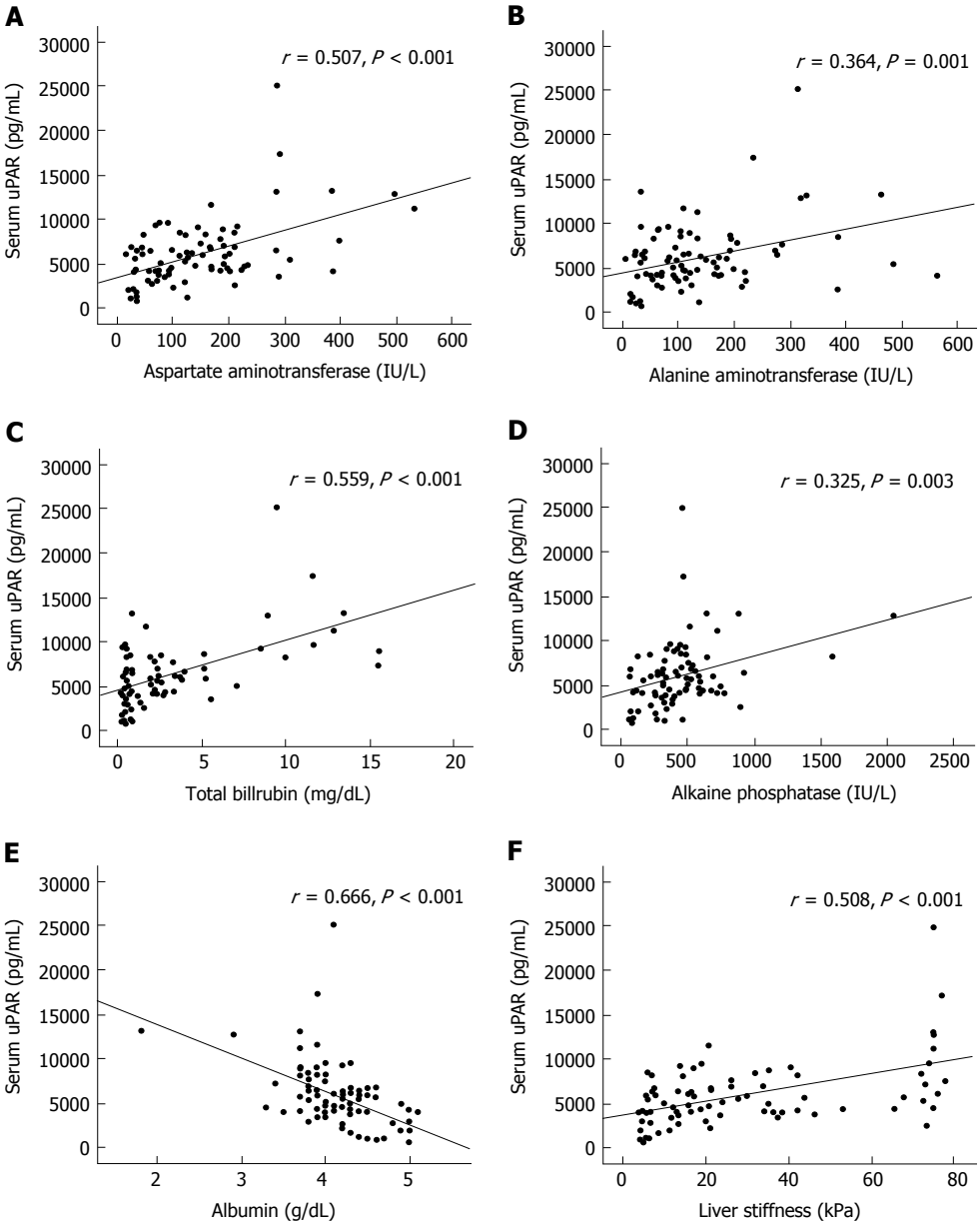

Figure 3 Scatter diagram and regression analysis in biliary atresia patients. uPAR levels are correlated with (A) serum aspartate aminotransferase (B) serum alanine aminotransferase (C) serum total bilirubin (D) serum alkaline phosphatase (E) serum albumin and (F) liver stiffness. uPAR: Urokinase-type plasminogen activator receptor.

release uPAR into the blood. The rising serum level of uPAR is likely attributable to the results of hepatocellular injury and further liver fibrosis. Whether increment of serum uPAR in BA children indicates low destruction, high production, or both remain obscure. Additional research will be needed to clarify the molecular basis leading to increased circulating uPAR.

Several caveats need to be acknowledged in this study. First, relatively small sample size of enrolled subjects limits the statistical power of our findings. Second, the cross-sectional study precludes definite information regarding causal relationships. In addition, inadequate assessment of various confounders such as comorbidity must be considered. To address these challenges, future studies should collect prospective measurements of these data to preclude bias and reverse causation. Moreover, the present investigation was restricted to the subjects under follow-up at our institution. Accordingly, our results may not be generalized across different populations. Finally, hepatic expression of uPAR has not been investigated. Further studies on immunohistochemistry of uPAR from liver tissues might provide better knowledge on molecular mechanisms of uPAR in biliary atresia.

To sum up, our study illustrated that circulating uPAR and liver stiffness measurement were markedly higher in biliary atresia children than in control subjects. Circulating uPAR was more elevated in jaundiced BA children compared to non-jaundiced BA children. Furthermore, elevated serum uPAR was correlated with hepatic dysfunction and outcome parameters. Circulating uPAR

and liver stiffness values might be used as noninvasive biological markers indicating the progression and prognosis of hepatic fibrosis in postoperative biliary atresia children. Although underlying mechanisms of the cause and effect relationships remain elusive, there is abundant room for the definite role of uPAR in the etiopathogenesis of hepatic fibrosis in BA.

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COMMENTS

Background

Biliary atresia (BA) is a severe chronic cholestatic liver disease of unknown etiology in young infants. The exact pathogenesis of BA remains a matter of debate. Circulating urokinase plasminogen activator receptor (uPAR) has arisen as a promising biochemical marker of certain disorders, such as liver injury and fibrosis. Although recent reports suggest a potential applicability for the measurement of circulating uPAR in liver fibrosis, the assessments on circulating uPAR and liver stiffness of BA have never been documented.

Research frontiers

Recent evidences demonstrate the significance of urokinase plasminogen activator receptor in hepatitis, liver fibrosis, and liver failure. The current study shows that circulating uPAR levels are more elevated in BA children than in control subjects. Moreover, uPAR level is correlated with liver stiffness, and clinical outcomes in postoperative BA.

Innovations and breakthroughs

BA children exhibited significantly higher circulating uPAR and liver stiffness values than control subjects. Circulating uPAR and liver stiffness values were more pronounced in jaundiced BA children than in non-jaundiced BA children. Additionally, elevated circulating uPAR levels were associated with hepatic dysfunction and clinical outcomes.

Applications

Increased circulating uPAR and liver stiffness values were was associated with hepatocellular dysfunction in postKasai children affected with BA. As a consequence, circulating uPAR and liver stiffness measurements could be used as noninvasive biological markers indicating the progression and prognosis of liver fibrogenesis in BA children.

Terminology

uPAR also known as CD87, is a multidomain membrane protein that has a role in the regulation of cell migration, proliferation, and survival and is expressed by diverse immune cells and endothelial cells, which contribute to the etiopathogenesis of hepatic inflammation and liver fibrogenesis.

Peer-review

Great paper that needs to be published. uPAR is known to be a substantial factor in the etiopathogenesis of hepatic inflammation and liver fibrogenesis.

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SYSTEMATIC REVIEWS

Bibliometric analysis of top 100 cited articles in nonalcoholic fatty liver disease research

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Author contributions: Zhang TS, Qin HL, Wang T, Li HT, Li H and Xiang XH prepared the manuscript; Xia SH and Xiang XH contributed to the conception of this work, revised and approved the manuscript.

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Abstract

AIM

To identify and assess the research situation of top 100 cited articles in nonalcoholic fatty liver disease (NAFLD).

METHODS

The global scientific research articles in the Science Citation Index-Expanded relevant to NAFLD were retrieved and listed according to their citation times from the most to the least. The 100 most frequently cited original articles were selected to systematically evaluate their bibliometric parameters including times cited, publication year, journals, subject categories, and the highly related concepts of NAFLD, which reflected the history and current situation, publication distribution of leading countries and institutes as well as the research hotspots of NAFLD.

RESULTS

Top 100 cited articles in NAFLD were published from 1965 to 2015 with a citation ranging of 227 to 2151 times since publication, in which the United States was the most predominant country and Mayo Clin was the most productive institution. The majority of the top 100 cited articles were concentrated in SCI subject category of Gastroenterology and Hepatology. Hepatology and Gastroenterology is the top journal that published over half 100 top-cited articles. The significant peak of top cited articles present in the first half of the 2000s while the highest mean number of citation presents in first half of the 1980s. In addition, concepts related to pathology characteristics, epidemiology and medicalization, metabolic syndrome and its combination of symptoms including insulin resistance, biomarkers

of lipid metabolism and obesity are listed as the highly related concepts.

CONCLUSION

The 100 top-cited articles marked with the leading countries, institutions, journals, hotspots and development trend in NAFLD field that could provide the foundation for further investigations.

Key words: Bibliometrics; Top-cited articles; Metabolic syndrome; Prevalence; Medicalization; Nonalcoholic fatty liver disease

Core tip: Bibliometrics was used to quantitatively analyze top 100 cited articles from the database of the Science Citation Index Expanded to reveal the global publication trends about nonalcoholic fatty liver disease (NAFLD). This study is the first global look at the history and current situation of NAFLD research to assess the performances of leading countries/territories and institutes and research hotspots of this disease. The performances and research hotspots are related to the potential pathogenesis of NAFLD. Incidence and prevalence as well as treatment progress for NAFLD were systematically reviewed, and their relationships with global performances results were also discussed.

Zhang TS, Qin HL, Wang T, Li HT, Li H, Xia SH, Xiang XH. Bibliometric analysis of top 100 cited articles in nonalcoholic fatty liver disease research. *World J Hepatol* 2016; 8(33): 1478-1488 Available from: URL: http://www.wjgnet.com/1948-5182/full/v8/i33/1478.htm DOI: http://dx.doi.org/10.4254/wjh.v8.i33.1478

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is defined by liver fat deposition with a concentration of hepatic triglycerides exceeding 5% of liver weight in the absence of excessive alcohol intake. NAFLD is an umbrella term used to describe a histological spectrum ranging from simple steatosis to nonalcoholic steatohepatitis (NASH). NASH is virtually indistinguishable histologically from alcoholic steatohepatitis, which is designated the disease with inflammation and liver cell injury in some NAFLD patients[1]. It was thought that hepatic fatty change was a kind of benign lesions previously. However, the recent research showed that about 10%-30% of NAFLD could evolve into NASH, accompanying by fibrosis, cirrhosis, liver failure and even hepatocellular carcinoma[2]. NAFLD patients are more likely to be accompanied with obesity, diabetes, cardiovascular and cerebrovascular diseases to increase death and disability rate. Owing to the high morbidity rate of obesity and metabolic syndrome worldwide, NAFLD has become the leading cause of chronic liver disease[1]. It is time to identify and evaluate the high citation articles to get insight into history and current situation of NAFLD research.

Citation rank list has been often used in medicine to characterize works with the remarkable intellectual influence[3]. Many highly cited articles have stimulated further standard-breaking investigations and discussions[4]. However, the bibliometric analysis of the most influential articles in NAFLD field remains unexploited. As the most frequently used source database for a broad review of scientific value in a specific research field, Science Citation Index-Expanded (SCI-Expanded) from Thomson Reuters is a highly effective research tool for evaluating scientific performance and tracking evolution trends. In this study, bibliometric method was applied to analyze the citation times, publication year, countries and institutes, journals, subspecialty, and key words of the 100 most cited articles in NAFLD field in SCI-Expanded from 1965 to 2015.

MATERIALS AND METHODS

The data were obtained from the SCI-Expanded from the Institute for Scientific Information, which indexed 8618 major journals with citation references across 176 categories in science edition in 2015. The keywords for bibliography retrieval in database consisted of "nonalcoholic steatohepatitis", "nonalcoholic fatty liver disease", and their heteromorphic form and abbreviation limited in liver or hepatology fields. Papers were listed according to their citation times from the most to the least. Only the top 100 original articles from the most citation list were included for further analysis. The retrieve process of the top 100 citied articles was shown in Figure 1. In detail, the retrieved data for statistical process were imported to Excel 2010. According to JCR in 2014 (available in June 2015), the reported impact factor (IF) of each journal was referred. The 100 top cited articles were assessed by decreasing orders of articles and citation. Bibliometric parameters including publication productions of countries and institutes with five indexes including total, independent, collaborative, first author, and corresponding author articles; distribution of journals and subspecialties; top 10 of most cited articles were assessed.

Furthermore, the most frequent key words and concepts were also discussed. Part of concepts such as "NAFLD" and "NASH" were abandoned since they completely overlap with the study content. Highly related concepts including all concepts from the Gene Ontology (GO) and the Medical Subject Headings (MeSH) were categorized by semantic search technology using GoPubMed® search engine (http://www.gopubmed.org/web/gopubmed/).

RESULTS

Publication year

After screening, 8828 meaningful articles related to NAFLD were retrieved in the period of 1965 to 2015. It

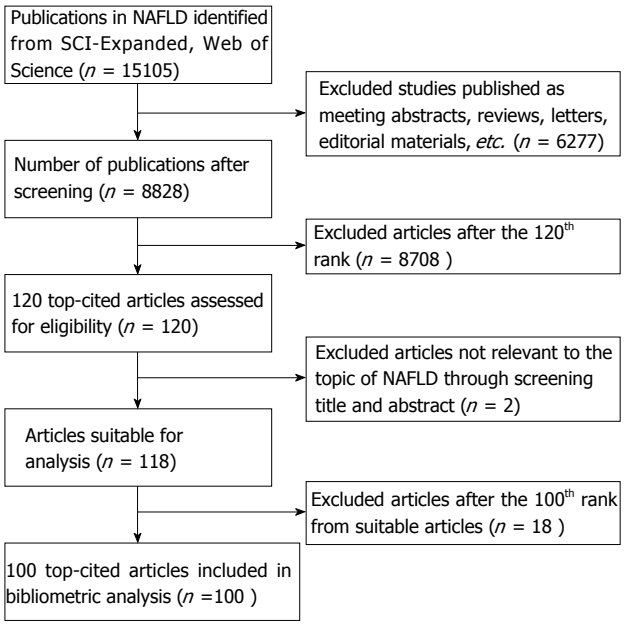

Figure 1 Flow chart of the selection process for the top 100 cited in nonalcoholic fatty liver disease. NAFLD: Nonalcoholic fatty liver disease.

can be seen that the number of total articles increased at an exponential rate, which entered an exponential growth phase since 2004 (Figure 2). A power exponential function can describe the growth curve: $Y = 1 \times 10^{-233}e^{0.2701x}$, $R^2 = 0.9668$.

The publication years of the top 100 cited articles in NAFLD field spanned from 1980 to 2012 with a citation ranging from 227 to 2151 times since publication. The majority of top 100 cited articles (74%) were concentrated in the 2000s (Figure 3). The most cited article published by Kleiner DE (National Cancer Institute, United States) in 2005 was cited 2151 times according to the SCI-Expanded database (Table 1).

Publication distribution of countries and institutes

The top 100 cited articles were originated from 19 countries. The most productive country was the United States (55), followed sequentially by Italy (20), Australia (14), France (9), United Kingdom (7). The rest of the countries had less than four publications (Table 2). The numbers in the brackets refer to the publication number (similarly hereinafter).

Twelve institutions published more than 4 top cited articles. Mayo Clin (12) ranked the first place in NAFLD research, followed by University of Bologna (9), University of Turin (9), The University of Sydney (7) and University of California, San Diego (6). And the rest of the Institutes such as University of Texas, Saint Louis University and Virginia Commonwealth University contributed five each to the top 100 cited articles (Table 3).

Subspecialties and journals

According to the JCR in 2014, the top 100 articles of NAFLD were scattered in 13 SCI subject categories (Table 4). These main subspecialties were Gastroenterology and Hepatology (71), Endocrinology and Metabolism (7), General and Internal Medicine (6), Research and Experimental Medicine (4) and Science and Technology (4).

The top 100 articles were distributed in 25 journals including professional journals and other disciplines journals. Eleven (44%) journals published 2 or more articles (Table 5), among which the most productive journal was *Hepatology* (42), followed by *Gastroenterology* (16), *Am J Gastroenterol* (5), *J Hepatol* (5), *J Clin Invest* (4), *Proc Natl Acad Sci USA* (3) and *J Clin Endocrinol Metab* (3).

The most frequently cited articles

As elaboration of all the top 100 cited articles is difficult, the top 10 citation articles were further discussed instead. United States (7), Italy (2) and Australia (1) respectively published the top 10 most frequently cited articles (Table 1). Three in ten focused on epidemiological subjects to investigate the regional and ethnic differences and explore the genetic mechanism implied in NAFLD, which were published respectively in the year of 1990 (864 citations), 2004 (1320 citations) and 2005 (974 citations) (Table 1). Other three articles discussed the pathogenic role of metabolic syndrome where insulin resistance and obesity were repeatedly mentioned. The rest of articles analyzed NAFLD from the clinical and histological aspect, among which two were about the histological grading and staging of NAFLD.

Highly related concepts

Highly related concepts of the top 100 cited papers from GO and MeSH with frequency more than 10 times were listed in Table 6. The analysis indicated that multisystem metabolic syndrome and its related key words (obesity, insulin resistance, *etc.*) occupied a majority of proportion. Some key words discussed histological and pathology characteristics of NAFLD including hepatic steatosis, fibrosis, biopsies, *etc*. Noteworthy, the topic of epidemiology covering prevalence, male/men, female/women, middle aged and adolescent was also involved in frequent concepts (Table 7).

DISCUSSION

This paper used bibliometrics method to evaluate top 100 cited articles to reveal the global publication performance of NAFLD. The high citation articles can reflect the development evolution direction and scientific level in the NADLD research field to a certain extent.

Publication trends and distribution of NAFLD-related literature

In recent five decades, exponential increase of published articles reflects the globally development trend of NAFLD. In line with the increased prevalence of obesity, diabetes, and hyperlipemia, NAFLD has been increasing worldwide over recent half century[5]. As a result of modern

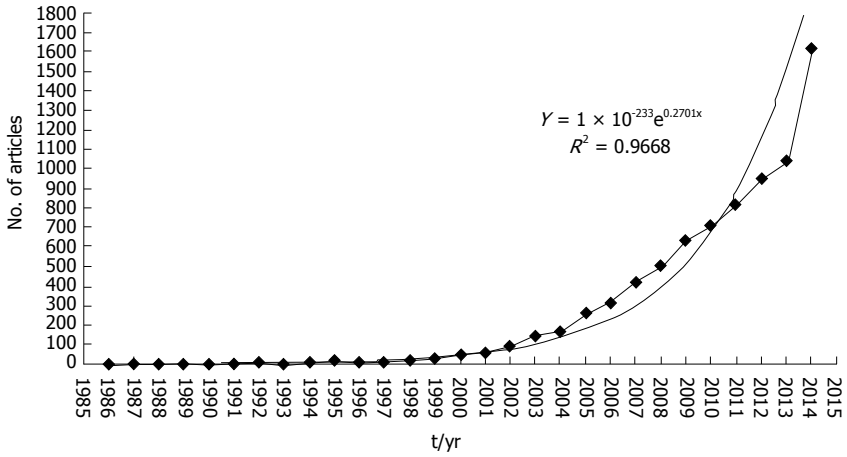

Figure 2 Number of global SCI Journal articles varies with time. Remarks: Fitting equation during 1985-2015 is: $Y = 1 \times 10^{-233}e^{0.2701x}$, $R^2 = 0.9668$. In the equation, Y is the number of accumulation articles and X is the sequence number of year. It indicated that research on NAFLD entered an exponential growth phase since 2004. NAFLD: Nonalcoholic fatty liver disease.

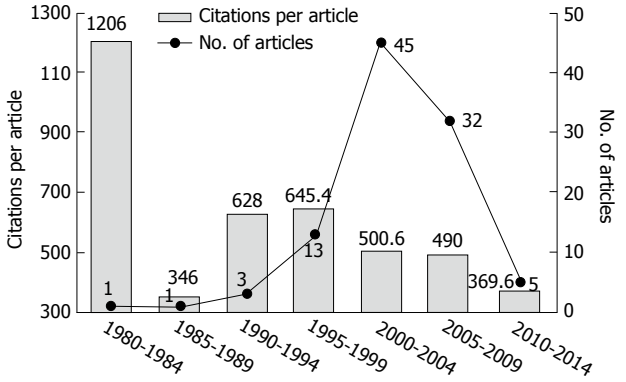

Figure 3 Number of the top 100 cited papers in nonalcoholic fatty liver disease per five year and the mean of the citation of the top cited paper with five years bin.

sedentary and over-nutrition lifestyle which makes a very large population fall risk of NAFLD, research on NAFLD would develop more rapidly in the near future[6].

East Asian countries/territories such as Japan, China (mainland), South Korea and Taiwan occupied an important place in NAFLD research and their importance tended to be more and more obvious. This might owe to the rising prevalence of NAFLD in Asia recently as well as the growth of economic power and the advance of scientific research which prompted these countries/territories to invest more in research to prevent and control NAFLD[6]. A global scientific review covered total articles relevant to NAFLD from 1986 to 2013 were performed to analyze distribution of publication number and found that Japan, China (mainland) and South Korea ranked second, fourth and ninth respectively among the most productive country/territories[7]. However, only six of top 100 cited papers originate these countries/territories. It shows that the quality and influence of research in NAFLD need to improve for East Asian countries.

It was found that most of the 100 most cited papers were published in 2000s (74 articles), while the most of high citation times per articles distributed in 1990s. These distributions suggested that the older paper had the more citation times[8]. The opinions in 1990s and 2000s were neither too old to be outdated nor too nearly to be cited. Actually, academic community has recognized that the real importance and influence of a work often can't be precisely assessed for at least 2 decades after it is published[9].

The research hotspots of NAFLD

Highly related concepts and top keywords could partly reflect the profile of hotspots in NAFLD research. GoPubMed® search engine connect text (abstracts from the MEDLINE database) to background knowledge in the form of semantic networks of concept categories, which is done by meaning and not by keywords only. These results are approximately consistent with our contemporaneous bibliometric analysis in high frequency keywords that covered total articles relevant to NAFLD[7].

Potential pathogenesis: According to highly related concepts list, a cluster of pathogenesis related keywords occupied a majority of high frequency words mentioned by NAFLD researches. The research hotspots extracted using bibliometrics analysis informs the underlying pathogenesis of NAFLD. The results indicated that multisystem metabolic syndrome and its combination of symptoms including insulin resistance, obesity as well as oxidative stress and dyslipoproteinemia played a vital role in the pathogenesis of NAFLD. In fact, although pathogenesis of NAFLD remains elusive, the severity of NAFLD seems to increase in parallel with the features of metabolic syndrome[10-12]. NAFLD/NASH is increasingly regarded as a hepatic manifestation of metabolic syndrome. However, considering that not all patients with NAFLD/NASH suffer from one of these conditions[1], still uncertain pathogenesis of NAFLD might hinder the people and needs to be explored[13].

Table 1 The information of top 100 cited articles in nonalcoholic fatty liver disease

Rank	Title of article	Journal	First author/institute	Year	Times cited
1	Design and validation of a histological scoring system for nonalcoholic fatty liver disease	*Hepatology*	Kleiner DE/NCI, United States	2005	2151
2	Nonalcoholic steatohepatitis: A proposal for grading and staging the histological lesions	*Am J Gastroenterol*	Brunt EM/Saint Louis University, United States	1999	1609
3	Nonalcoholic fatty liver disease: A spectrum of clinical and pathological severity	*Gastroenterology*	Matteoni CA/Cleveland Clin Fdn, United States	1999	1506
4	Prevalence of hepatic steatosis in an urban population in the United States: Impact of ethnicity	*Hepatology*	Browning JD/Univ Texas, United States	2004	1320
5	Non-alcoholic steatohepatitis - Mayo-Clinic experiences with A hitherto unnamed disease	*Mayo Clin Proc*	Ludwig J/Mayo Clin, United States	1980	1206
6	Nonalcoholic fatty liver, steatohepatitis, and the metabolic syndrome	*Hepatology*	Marchesini G/Università di Bologna, Bologna, Italy	2003	1134
7	Nonalcoholic fatty liver disease - a feature of the metabolic syndrome	*Diabetes*	Marchesini G/Univ Bologna, Italy	2001	1072
8	The natural history of nonalcoholic fatty liver disease: A population-based cohort study	*Gastroenterology*	Adams LA/Mayo Clin, United States	2005	974
9	Nonalcoholic steatohepatitis: Association of insulin resistance and mitochondrial abnormalities	*Gastroenterology*	Sanyal AJ/Virginia Commonwealth Univ, United States	2001	935
10	The natural-history of nonalcoholic steatohepatitis - a follow-up-study of 42 patients for up to 21 yr	*Hepatology*	Powell EE/University of Queensland, Australia	1990	864
11	Independent predictors of liver fibrosis in patients with nonalcoholic steatohepatitis	*Hepatology*	Angulo P/Mayo Clin, United States	1999	802
12	Sources of fatty acids stored in liver and secreted *via* lipoproteins in patients with nonalcoholic fatty liver disease	*J Clin Invest*	Donnelly KL/Univ Minnesota, United States	2005	801
13	Nonalcoholic steatohepatitis - an expanded clinical entity	*Gastroenterology*	Bacon BR/St. Louis UNIV, United States	1994	756
14	Association of nonalcoholic fatty liver disease with insulin resistance	*Am J Med*	Marchesini G/Univ Bologna, United States	1999	736
15	Long-term follow-up of patients with NAFLD and elevated liver enzymes	*Hepatology*	Ekstedt M/Linkoping Univ Hosp, Sweden	2006	719
16	Expanding the natural history from cryptogenic cirrhosis to of nonalcoholic steatohepatitis: Hepatocellular carcinoma	*Gastroenterology*	Bugianesi E/Univ Turin, Italy	2002	712
17	The utility of radiological imaging in nonalcoholic fatty liver disease	*Gastroenterology*	Saadeh S/Inova Fairfax Hosp, United States	2002	708
18	The fat-derived hormone adiponectin alleviates alcoholic and nonalcoholic fatty liver diseases in mice	*J Clin Invest*	Xu AM/Univ Auckland, China	2003	696
19	Nonalcoholic fatty liver disease: Predictors of nonalcoholic steatohepatitis and liver fibrosis in the severely obese	*Gastroenterology*	Dixon JB/Monash Univ, Australia	2001	666
20	A placebo-controlled trial of pioglitazone in subjects with nonalcoholic steatohepatitis	*N Engl J Med*	Belfort R/Univ Texas, Italy	2006	662
21	Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease	*Nature Genet*	Romeo S/Univ Texas, United States	2008	614
22	NASH and insulin resistance: Insulin hypersecretion and specific association with the insulin resistance syndrome	*Hepatology*	Chitturi S/Univ Sydney, Australia	2002	610
23	Sampling variability of liver biopsy in nonalcoholic fatty liver disease	*Gastroenterology*	Ratziu V/Grp Hosp Pitie Salpetriere, France	2005	572
24	Fat accumulation in the liver is associated with defects in insulin suppression of glucose production and serum free fatty acids independent of obesity in normal men	*J Clin Endocrinol Metab*	Seppala-Lindroos A/Univ Helsinki, Finland	2002	563
25	Beyond insulin resistance in NASH: TNF-alpha or adiponectin?	*Hepatology*	Hui JM/Westmead Hosp, Australia	2004	552
26	Magnetic resonance spectroscopy to measure hepatic triglyceride content: Prevalence of hepatic steatosis in the general population	*Am J Physiol -Endocrinol Metab*	Szczepaniak, LS/Univ Texas, United States	2005	551
27	Pioglitazone, Vitamin E or Placebo for Nonalcoholic Steatohepatitis	*N Engl J Med*	Sanyal AJ/Virginia Commonwealth Univ, United States	2010	550
28	The natural history of nonalcoholic fatty liver: A follow-up study	*Hepatology*	Teli MR/Univ Newcastle, United Kingdom	1995	544
29	Mechanism of hepatic insulin resistance in non-alcoholic fatty liver disease	*J. Biol. Chem.*	Samuel VT/Yale Univ, Australia	2004	537
30	Obesity increases sensitivity to endotoxin liver injury: Implications for the pathogenesis of steatohepatitis	*Proc Natl Acad Sci USA*	Yang SQ/Johns Hopkins Univ, United States	1997	504
31	Prevalence of fatty liver in children and adolescents	*Pediatrics*	Schwimmer JB/Univ Calif San Diego, United States	2006	454
32	Diabetes increases the risk of chronic liver disease and hepatocellular carcinoma	*Gastroenterology*	El-Serag HB/Houston Dept Vet Affairs Med Ctr, United States	2004	452

33	Hepatocyte apoptosis and Fas expression are prominent features of human nonalcoholic steatohepatitis	*Gastroenterology*	Feldstein AE/Mayo Clin, United States	2003	451
34	Prevalence of and risk factors for nonalcoholic fatty liver disease: The Dionysos Nutrition and Liver Study	*Hepatology*	Bedogni G/Fondo Studio Malattie Fegato ONLUS, Italy	2005	449
35	CYP2E1 and CYP4A as microsomal catalysts of lipid peroxides in murine nonalcoholic steatohepatitis	*J Clin Invest*	Leclercq IA/Univ Sydney, United States	2000	435
36	Probiotics and antibodies to TNF inhibit inflammatory activity and improve nonalcoholic fatty liver disease	*Hepatology*	Li ZP/Johns Hopkins Univ, United States	2003	433
37	Increased hepatic iron concentration in nonalcoholic steatohepatitis is associated with increased fibrosis	*Gastroenterology*	George DK/Royal Brisbane Hosp, Australia	1998	431
38	Clinical and histologic spectrum of nonalcoholic fatty liver disease associated with normal ALT values	*Hepatology*	Mofrad P/Virginia Commonwealth Univ, U United States	2003	427
39	The NAFLD fibrosis score: A noninvasive system that identifies liver fibrosis in patients with NAFLD	*Hepatology*	Angulo P/Mayo Clin, United Kingdom	2007	425
40	A pilot study of ploglitazone treatment for nonalcoholic steatohepatitis	*Hepatology*	Promrat K/NIDDK, United States	2004	410
41	Improved nonalcoholic steatohepatitis after 48 wk of treatment with the PPAR-gamma ligand rosiglitazone	*Hepatology*	Neuschwander-Tetri BA/St. Louis Univ, United States	2003	406
42	Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity	*Nature*	Henao-Mejia J/Yale Univ, United States	2012	399
43	Liver pathology and the metabolic syndrome X in severe obesity	*J Clin Endocrinol Metab*	Marceau P/SUNY Hlth Sci Ctr, Canada	1999	389
44	The metabolic syndrome as a predictor of nonalcoholic fatty liver disease	*Ann Intern Med*	Hamaguchi M/Asahi Univ, Japan	2005	387
45	Metformin in non-alcoholic steatohepatitis	*Lancet*	Marchesini G/Univ Bologna, Italy	2001	376
46	Nonalcoholic steatohepatitis, insulin resistance, and metabolic syndrome: Further evidence for an etiologic association	*Hepatology*	Pagano G/Univ Turin, Italy	2002	373
47	Metabolic profiling reveals a contribution of gut microbiota to fatty liver phenotype in insulin-resistant mice	*Proc Natl Acad Sci USA*	Dumas ME/Univ London Imperial Coll Sci Technol & Med, United Kingdom	2006	361
48	Hepatic cytochrome p450 2E1 is increased in patients with nonalcoholic steatohepatitis	*Hepatology*	Weltman MD/Westmead Hosp, Sweden	1998	355
49	The histological course of nonalcoholic fatty liver disease: A longitudinal study of 103 patients with sequential liver biopsies	*J Hepatol*	Adams LA/Mayo Clin, United States	2005	349
50	Nonalcoholic steatohepatitis - A study of 49 patients	*Hum Pathol*	Lee RG/Oregon Health Sciences University, United States	1989	346
51	Prevalence of Nonalcoholic Fatty Liver Disease and Nonalcoholic Steatohepatitis Among a Largely Middle-Aged Population Utilizing Ultrasound and Liver Biopsy: A Prospective Study	*Gastroenterology*	Williams CD/Brooke Army Med Ctr, United States	2011	343
52	Free fatty acids promote hepatic lipotoxicity by stimulating TNF-alpha expression *via* a lysosomal pathway	*Hepatology*	Feldstein AE/Mayo Clin, United States	2004	336
53	*In vivo* assessment of liver cell apoptosis as a novel biomarker of disease severity in nonalcoholic fatty liver disease	*Hepatology*	Wieckowska A/Cleveland Clin Fdn, United States	2006	330
54	Therapeutic effects of restricted diet and exercise in obese patients with fatty liver	*J Hepatol*	Ueno T/Kurume University School of Medicine, Japan	1997	329
55	Gene expression of tumor necrosis factor alpha and TNF-receptors, p55 and p75, in nonalcoholic steatohepatitis patients	*Hepatology*	Crespo J/Hosp Univ Marques Valdecilla, Spain	2001	327
56	Inhibiting triglyceride synthesis improves hepatic steatosis but exacerbates liver damage and fibrosis in obese mice with nonalcoholic steatohepatitis	*Hepatology*	Yamaguchi K/Duke Univ, United States	2007	324
57	Nonalcoholic fatty liver disease: Improvement in liver histological analysis with weight loss	*Hepatology*	Dixon JB/Monash Univ, Australia	2004	324
58	The role of small intestinal bacterial overgrowth, intestinal permeability, endotoxaemia, and tumour necrosis factor alpha in the pathogenesis of non-alcoholic steatohepatitis	*Gut*	Wigg AJ/Queen Elizabeth Hosp, Australia	2001	324
59	Intrahepatic fat, not visceral fat, is linked with metabolic complications of obesity	*Proc Natl Acad Sci USA*	Fabbrini E/Washington Univ, Greece	2009	323
60	Ursodeoxycholic acid or clofibrate in the treatment of non-alcohol-induced steatohepatitis: A pilot study	*Hepatology*	Laurin J/Mayo Clin, United States	1996	317
61	Vitamin E treatment of nonalcoholic steatohepatitis in children: A pilot study	*J Pediatr*	Lavine JE/Univ Calif San Diego, United States	2000	312
62	A randomized controlled trial of metformin *vs* vitamin E or prescriptive diet in nonalcoholic fatty liver disease	*Am J Gastroenterol*	Bugianesi E/Univ Bologna, Italy	2005	309
63	Ursodeoxycholic acid for treatment of nonalcoholic steatohepatitis: Results of a randomized trial	*Hepatology*	Lindor KD/Mayo Clin, Canada	2004	305
64	Deletion of NEMO/IKK gamma in liver parenchymal cells causes steatohepatitis and hepatocellular carcinoma	*Cancer Cell*	Luedde T/Univ Cologne, Belgium	2007	285
65	NAFLD may be a common underlying liver disease in patients with hepatocellular carcinoma in the United States	*Hepatology*	Marrero JA/Univ Michigan, United States	2002	283

66	Vitamin E and vitamin C treatment improves fibrosis in patients with nonalcoholic steatohepatitis	*Am J Gastroenterol*	Harrison SA/Univ Texas, United States	2003	281
67	High glucose and hyperinsulinemia stimulate connective tissue growth factor expression: A potential mechanism involved in progression to fibrosis in nonalcoholic steatohepatitis	*Hepatology*	Paradis V/Hop Bicetre, France	2001	281
68	Prevalence of obesity and diabetes in patients with cryptogenic cirrhosis: A case-control study	*Hepatology*	Poonawala A/Johns Hopkins Univ, United States	2000	281
69	Insulin resistance-associated hepatic iron overload	*Gastroenterology*	Mendler MH/Hop Pontchaillou, France	1999	281
70	Free fatty acids induce JNK-dependent hepatocyte lipoapoptosis	*J Biol Chem*	Malhi H/Mayo Clin, United States	2006	280
71	Dietary habits and their relations to insulin resistance and postprandial lipemia in nonalcoholic steatohepatitis	*Hepatology*	Musso G/Univ Turin, Italy	2003	279
72	Cytokines and NASH: A pilot study of the effects of lifestyle modification and vitamin E	*Hepatology*	Kugelmas M/Univ Louisville, United States	2003	275
73	Nonalcoholic fatty liver disease and risk of future cardiovascular events among type 2 diabetic patients	*Diabetes*	Targher G/Osped Sacro Cuore don G Calabria, Italy	2005	271
74	A lipidomic analysis of nonalcoholic fatty liver disease	*Hepatology*	Puri P/Virginia Commonwealth Univ, United States	2007	269
75	The Incidence and Risk Factors of Hepatocellular Carcinoma in Patients with Nonalcoholic Steatohepatitis	*Hepatology*	Ascha MS/Cleveland Clin, United States	2010	268
76	Prevalence of nonalcoholic fatty liver disease and its association with cardiovascular disease among type 2 diabetic patients	*Diabetes Care*	Targher G/Osped Sacro Cuore don Calabria, United Kingdom	2007	268
77	Burden of liver disease in the United States: Summary of a workshop	*Hepatology*	Kim WR/Mayo Clin, United States	2002	266
78	Plasma Endotoxin Concentrations In Patients With Alcoholic And Nonalcoholic Liver-Disease - Reevaluation With An Improved Chromogenic Assay	*J Hepatol*	Fukui H/ROBERT BOSCH KRANKENHAUS, Germany	1991	264
79	Histopathology of pediatric nonalcoholic fatty liver disease	*Hepatology*	Schwinnner JB/Univ Calif San Diego, USA	2005	262
80	A position statement on NAFLD/NASH based on the EASL 2009 special conference	*J Hepatol*	Ratziu V/Azienda USL Modena, Italy	2010	259
81	Increased intestinal permeability in obese mice: New evidence in the pathogenesis of nonalcoholic steatohepatitis	*Am J Physiol-Gastroint Liver Physiol*	Brun P/Univ Padua, Italy	2007	258
82	Endothelial dysfunction and cardiovascular risk profile in nonalcoholic fatty liver disease	*Hepatology*	Villanova N/Alma Mater Studiorum Univ Bologna, Italy	2005	258
83	Defective hepatic mitochondrial respiratory chain in patients with nonalcoholic steatohepatitis	*Hepatology*	Perez-Carreras M/Hosp Univ 12 Octubre, Spain	2003	254
84	Survival, liver failure, and hepatocellular carcinoma in obesity-related cryptogenic cirrhosis	*Hepatology*	Ratziu V/Hop La Pitie Salpetriere, France	2002	254
85	A pilot study of a thiazolidinedione, troglitazone, in nonalcoholic steatohepatitis	*Am J Gastroenterol*	Caldwell SH/Univ Virginia, United States	2001	247
86	Hepatocyte-specific Pten deficiency results in steatohepatitis and hepatocellular carcinomas	*J Clin Invest*	Horie Y/Akita Univ, Japan	2004	240
87	Randomized Controlled Trial Testing the Effects of Weight Loss on Nonalcoholic Steatohepatitis	*Hepatology*	Promrat K/Brown Univ, United States	2010	239
88	Insulin resistance in chronic hepatitis C: Association with genotypes 1 and 4, serum HCV RNA level, and liver fibrosis	*Gastroenterology*	Moucari R/Hop Beaujon, France	2008	239
89	Betaine, a promising new agent for patients with nonalcoholic steatohepatitis: Results of a pilot study	*Am J Gastroenterol*	Abdelmalek MF/Mayo Clin, United States	2001	239
90	Steatosis in chronic hepatitis C: Relative contributions of obesity, diabetes mellitus, and alcohol	*Hepatology*	Monto A/Univ Calif San Francisco, United States	2002	237
91	Therapeutic efficacy of an angiotensin Ⅱ receptor antagonist in patients with nonalcoholic steatohepatitis	*Hepatology*	Yokohama S/Dokkyo Univ, Japan	2004	235
92	Hepatic-Effects Of Dietary Weight-Loss In Morbidly Obese Subjects	*J Hepatol*	Andersen T/Univ Copenhagen, Denmark	1991	236
93	Rosiglitazone for nonalcoholic steatohepatitis: One-year results of the randomized placebo-controlled fatty liver improvement with rosiglitazone therapy trial	*Gastroenterology*	Ratziu V/Univ Paris, France	2008	234
94	Diagnosis of Fibrosis and Cirrhosis Using Liver Stiffness Measurement in Nonalcoholic Fatty Liver Disease	*Hepatology*	Wong VWS/Hop Haut Leveque, China	2010	232
95	Increased hepatocyte CYP2E1 expression in a rat nutritional model of hepatic steatosis with inflammation	*Gastroenterology*	Weltman MD/Univ Sydney, Australia	1996	230
96	Effect of steatohepatitis associated with irinotecan or oxaliplatin pretreatment on resectability of hepatic colorectal metastases	*J Am Coll Surg*	Fernandez FG/Washington Univ, United States	2005	229
97	Adiponectin and its receptors in non-alcoholic steatohepatitis	*Gut*	Kaser S/Univ Innsbruck Hosp, Spain	2005	229
98	Long-term outcomes of cirrhosis in nonalcoholic steatohepatitis compared with hepatitis C	*Hepatology*	Hui JM/Univ Sydney, Australia	2003	229

99	Noninvasive markers of fibrosis in nonalcoholic fatty liver disease: Validating the European liver fibrosis panel and exploring simple markers	*Hepatology*	Guha IN/Guha, United Kingdom	2008	228
100	Plasma adiponectin in nonalcoholic fatty liver is related to hepatic insulin resistance and hepatic fat content, not to liver disease severity	*J Clin Endocrinol Metab*	Bugianesi E/Univ Turin, Italy	2005	227

NAFLD: Nonalcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis; TNF: Tumor necrosis factor; ALT: Alanine aminotransferase; PPAR: Peroxisome proliferator activated receptor; HCV: Hepatitis C virus.

Table 2 Countries of origin of the top 100 articles in nonalcoholic fatty liver disease

Rank	Nation	TP	FP	SP	CP	RP	TC
1	United States	55	48	45	10	49	26975
2	Italy	20	13	11	9	15	5567
3	Australia	14	10	8	6	9	4767
4	France	9	6	6	3	7	1861
5	United Kingdom	7	5	2	5	2	1826
6	Japan	4	4	4	0	4	1191
7	Spain	3	3	2	1	2	810
8	Sweden	2	2	1	1	1	1074
9	China	2	2	0	2	0	928
10	Canada	2	2	0	2	0	694
11	Germany	2	1	1	1	1	264
12	Finland	1	1	1	0	1	563
13	Greece	1	1	0	1	0	323
14	Belgium	1	1	0	1	0	285
15	Denmark	1	1	1	0	1	236
16	New Zealand	1	0	0	1	1	0
17	Austria	1	0	0	1	1	0
18	South Africa	1	0	0	1	0	0

TP: The number of total 100 top-cited articles; FP: The number of first author articles; SP: The number of single-country articles; CP: The number of internationally collaborative articles; RP: The number of corresponding author articles in total 100 top-cited articles; TC: Total citation of first author articles; Rank: According to the order of TP firstly and TC secondly. As for New Zealand Austria and South Africa, the country with more citation of corresponding author articles took precedence.

Table 3 Top productive institutions list with top 100 cited articles in nonalcoholic fatty liver disease

Rank	Institution	TP	FP	SP	CP	RP	TC
1	Mayo Clinic	12	12	8	4	11	5950
2	University of Bologna	9	5	1	8	5	3627
2	University of Turin	9	4	1	8	4	1591
4	The University of Sydney	7	4	1	6	2	1504
5	University of California, San Diego	6	3	0	6	3	1028
6	University of Texas	5	5	1	4	4	3428
7	Saint Louis University	5	3	3	2	3	2771
8	Virginia Commonwealth University	5	4	2	3	2	2181
9	Westmead Hospital	4	2	0	4	3	907
10	Washington University	4	2	0	4	1	552
11	University of Paris	4	1	0	4	1	234
12	University of California, San Francisco	4	1	1	3	0	237
13	National Cancer Institute	4	1	0	4	1	2151
14	MetroHealth Medical Center	4	0	0	4	0	0

TP: The number of total 100 top-cited articles; TP: The number of total 100 top-cited articles; FP: The number of first author articles; SP: The number of single-country articles; CP: The number of internationally collaborative articles; RP: The number of corresponding author articles in total 100 top-cited articles; TC: Total citation of first author articles; Rank: According to the order of TP firstly and TC secondly. As for National Cancer Institute and Metrohlth Med Ctr, the institute with more corresponding author articles took precedence.

Epidemic studies: Concepts related to epidemiology such as humans, male/men, female/women, middle aged and adolescent make up another high frequency concepts cluster, which might be closely involved in the accelerating incidence of this disease. The morbidity rate of NAFLD has doubled during last 20 years, whereas the morbidity rate of other chronic liver diseases has remained stable or even decreased. Epidemic investigations of NAFLD primarily focus on human genetic and metabolic studies[14]. Several epidemiological investigations such as case series, familial and twin studies have widely revealed the function of heritability[15]. Noteworthy, in comparison to high-risk population of NAFLD clustering around middle-aged and elderly adults before, younger age trend has gradually shown especially in Asian countries during the last two decades. Following the epidemics of childhood obesity, NAFLD as the most common form of chronic liver disease in adolescents has become a reality[16].

Medicalization progress: Medicalization is also a high frequency concepts cluster. Lack of uniformed diagnosis regulation and no established therapy remains a hindrance to be broken through in this field. NASH is characterized by hepatocellular damage, lobular necroinflammation and fibrogenesis. The early diagnosis of advanced fibrosis in NAFLD is therefore crucial[17,18]. The liver biopsy remains the most reliable diagnostic method to appropriately evaluate the severity of liver fibrosis. Facing to limitations of this invasive technique in current use, a number of experimental biomarkers have been developed in order to predict the degree of liver fibrosis[19]. Moreover, as a promising method for evaluation of patients with NAFLD, nuclear medicine through liver scintigraphy has recently been proposed[20].

Preventing existing comorbidities such as metabolic disorders, cardiovascular or cerebrovascular events are the primary target for NAFLD treatment, while the secondary goal of NAFLD therapy is reversal of hepatic steatosis[21-23]. Lifestyle modification such as weight loss and balanced diet remains the main way of management in NAFLD/NASH. In addition, the benefit of nutritional supplementation on disease progression has attracted growing interest[24]. Most recent data has evidenced the effects of nutrients and dietary bioactive compounds intake (*i.e.*, long-chain PUFA, Vitamin E,

Table 4 Most frequent subspecialties with the top 100 cited articles in nonalcoholic fatty liver disease

Rank	Subject categories	No. of articles	Total citation
1	Gastroenterology and Hepatology	71	33290
2	Endocrinology and Metabolism	7	3341
3	General and Internal Medicine	6	3917
4	Research and Experimental Medicine	4	2172
5	Science and Technology	4	1587
6	Biochemistry and Molecular Biology	2	817
7	Physiology	2	809
8	Pediatrics	2	766
9	Genetics and Heredity	1	614
10	Pathology	1	346
11	Cell Biology	1	285
12	Oncology	1	285
13	Surgery	1	229

Remarks: In the situation of equal numbers of articles, the subspecialties with more total citation took precedence.

Table 5 Journal distribution of top 100 cited articles in nonalcoholic fatty liver disease

Rank	Journal	No. of articles	Total citation	Impact factor (2014)
1	*Hepatology*	42	18867	11.055
2	*Gastroenterology*	16	9490	16.716
3	*Am J Gastroenterol*	5	2685	10.755
3	*J Hepatol*	5	1437	11.336
5	*J Clin Invest*	4	2172	13.215
6	*Proc Natl Acad Sci USA*	3	1188	9.674
7	*J Clin Endocrinol Metab*	3	1179	3.457
8	*Diabetes*	2	1343	8.095
9	*New Engl J Med*	2	1212	55.873
10	*J Biol Chem*	2	817	4.573
11	*Gut*	2	553	14.66

Remarks: In the situation of equal numbers of articles, the journals with more total citation took precedence.

Vitamin D, minerals and polyphenols) on the modulation of molecular mechanisms leading to fat accumulation, oxidative stress, inflammation and liver fibrosis in NAFLD patients[25]. In the field of pharmaceutical therapies, a wide range of drugs have been applied in clinical trials, including antioxidants, lipid lowering agents, and rennin-angiotensin system blockers[26-28]. Up to the present, lifestyle modification is the main clinical recommendation as an initial step. Although promising results have shown that long-term insulin sensitizers such as metformin, rosiglitazone, and thiazolidinediones are effective in NAFLD therapy, there are no approved drugs[29-31].

In conclusion, it is important to acknowledge the top 100 cited articles because they marked with the leading countries, institutions, journals, hotspots, past and current trends in NAFLD field that could provide the foundation for further investigations. Highly related concepts of the top 100 cited papers in NAFLD suggest that pathogenesis mainly related to metabolic syndrome, epidemiology, and medicalization including diagnosis and treatment are attracting ever-growing attention.

Table 6 High frequency key words in the top 100 cited articles in nonalcoholic fatty liver disease (frequency > 2)

Rank	Key word	Frequency
1	Hepatic steatosis	4
1	Obesity	4
3	Fibrosis	3
4	Metabolic syndrome	2
4	Insulin resistance	2
4	Biopsies	2
4	Intestinal bacteria	2
4	Endotoxin	2

Table 7 Highly related concepts of the top 100 articles in nonalcoholic fatty liver disease categorized by GoPubMed® search engine

Rank	Highly related concepts	Frequency	Rank	Highly related concepts	Frequency
1	Fatty liver	97	24	Wounds and injuries	15
2	Male	91	25	Aspartate Aminotransferases	14
3	Humans	89	26	Mice	14
4	Female	84	27	Carcinoma, Hepatocellular	13
5	Middle aged	72	28	Tumor necrosis factor-alpha	12
6	Patients	71	29	Multivariate analysis	12
7	Fibrosis	59	30	Prospective studies	12
8	Biopsy	45	31	Follow-up studies	12
9	Liver	45	32	Hepatitis C	11
10	Obesity	42	33	cell killing	11
11	Aged	36	34	cytolysis	11
12	Insulin	35	35	Medicalization	11
13	Serum	32	36	Metabolic syndrome X	10
14	Body mass index	31	37	Fatty acids, nonesterified	10
15	Syndrome	25	38	Aspartic acid	10
16	Risk Factors	24	39	Hypoglycemic agents	10
17	Alanine transaminase	23	40	Homeostasis	10
18	Alanine transaminase activity	19	41	Severity of illness index	10
19	Pathogenesis	19	42	Men	10
20	Prevalence	18	43	Personal autonomy	10
21	Hepatocytes	17	44	Women	10
22	Alanine	16	45	Adolescent	10
23	Triglycerides	15			

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COMMENTS

Background

Due to the increasing prevalence of obesity and metabolic syndrome worldwide,

nonalcoholic fatty liver disease (NAFLD) becomes the leading cause of chronic liver disease. The rapid growth of NAFLD research recently drives top cited articles in the field to be identified and bibliometric analysis to assess the history and current situation, publication distribution of leading countries and institutes as well as the research hotspots of NAFLD.

Research frontiers

A systematic review in 2015 covered total articles relevant to NAFLD from Science Citation Index-Expanded (SCI-Expanded) showed article amount has appeared to geometric growth in recent decades. However, bibliometric result from total articles is not sufficient to indicate the evolution and direction in NAFLD research. The citation times by other authors has been used as a measurable comparison to evaluate the academic impact of an article in its subject field. To date, there have no top cited articles analysis were carried out in NAFLD field.

Innovations and breakthroughs

This paper summarized the current findings from the analysis of the top 100 cited articles in NAFLD field. It is the first global look at the history and current situation of NAFLD research to assess the performances of leading countries/territories and institutes and research hotspots of this disease. In terms of the number of published 100 top-cited articles in NAFLD, United States was the most predominant country and Mayo Clin was the most productive institution. Highly related concepts of the top 100 cited papers in NAFLD suggest that pathogenesis (mainly related to metabolic syndrome), epidemiology, and medicalization (including diagnosis and treatment) are attracting ever-growing attention.

Applications

Top 100 cited articles marked with the leading countries, institutions, journals, hotspots, past and current trends in NAFLD field that could provide the foundation for further investigations. Medical bibliometric analysis on top 100 cited articles is expected to provide a reference for the researchers to get involved in NAFLD area.

Terminology

The articles involved in bibliometric analysis were collected based on online version of SCI-Expanded from Thomson Reuters. Keywords for bibliography retrieval in database consisted of "nonalcoholic steatohepatitis" and "nonalcoholic fatty liver disease".

Peer-review

This study retrieved the top 100 cited articles in the field of NAFLD and determined the country of origin, peak of highly-cited articles and international collaborations. The present study is very interesting on a high prevalent chronic liver disease.

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