World Journal of

Gastroenterology

World Journal of Gastroenterology

A peer-reviewed, online, open-access journal of gastroenterology and hepatology

Editorial Board

2014-2017

Hitoshi Maruyama, *Chiba*
Atsushi Masamune, *Sendai*
Hiroyuki Matsubayashi, *Suntogun*
Akihisa Matsuda, *Inzai*
Hirofumi Matsui, *Tsukuba*
Akira Matsumori, *Kyoto*
Yoichi Matsuo, *Nagoya*
Y Matsuzaki, *Ami*
Toshihiro Mitaka, *Sapporo*
Kouichi Miura, *Akita*
Shinichi Miyagawa, *Matumoto*
Eiji Miyoshi, *Suita*
Toru Mizuguchi, *Sapporo*
Nobumasa Mizuno, *Nagoya*
Zenichi Morise, *Nagoya*
Tomohiko Moriyama, *Fukuoka*
Kunihiko Murase, *Tusima*
Michihiro Mutoh, *Tsukiji*
Akihito Nagahara, *Tokyo*
Hikaru Nagahara, *Tokyo*
Hidenari Nagai, *Tokyo*
Koichi Nagata, *Shimotsuke-shi*
Masaki Nagaya, *Kawasaki*
Hisato Nakajima, *Nishi-Shinbashi*
Toshifusa Nakajima, *Tokyo*
Hiroshi Nakano, *Kawasaki*
Hiroshi Nakase, *Kyoto*
Toshiyuki Nakayama, *Nagasaki*
Takahiro Nakazawa, *Nagoya*
Shoji Natsugoe, *Kagoshima City*
Tsutomu Nishida, *Suita*
Shuji Nomoto, *Naogya*
Sachiyo Nomura, *Tokyo*
Takeshi Ogura, *Takatsukishi*
Nobuhiro Ohkohchi, *Tsukuba*
Toshifumi Ohkusa, *Kashiwa*
Hirohide Ohnishi, *Akita*
Teruo Okano, *Tokyo*
Satoshi Osawa, *Hamamatsu*
Motoyuki Otsuka, *Tokyo*
Michitaka Ozaki, *Sapporo*
Satoru Saito, *Yokohama*
Naoaki Sakata, *Sendai*
Ken Sato, *Maebashi*
Toshiro Sato, *Tokyo*
Tomoyuki Shibata, *Toyoake*
Tomohiko Shimatani, *Kure*
Yukihiro Shimizu, *Nanto*
Tadashi Shimoyama, *Hirosaki*
Masayuki Sho, *Nara*
Ikuo Shoji, *Kobe*
Atsushi Sofuni, *Tokyo*
Takeshi Suda, *Niigata*
M Sugimoto, *Hamamatsu*
Ken Sugimoto, *Hamamatsu*
Haruhiko Sugimura, *Hamamatsu*
Shoichiro Sumi, *Kyoto*
Hidekazu Suzuki, *Tokyo*
Masahiro Tajika, *Nagoya*
Hitoshi Takagi, *Takasaki*
Toru Takahashi, *Niigata*
Yoshihisa Takahashi, *Tokyo*
Shinsuke Takeno, *Fukuoka*
Akihiro Tamori, *Osaka*
Kyosuke Tanaka, *Tsu*
Shinji Tanaka, *Hiroshima*

Atsushi Tanaka, *Tokyo*
Yasuhito Tanaka, *Nagoya*
Shinji Tanaka, *Tokyo*
Minoru Tomizawa, *Yotsukaido City*
Kyoko Tsukiyama-Kohara, *Kagoshima*
Takuya Watanabe, *Niigata*
Kazuhiro Watanabe, *Sendai*
Satoshi Yamagiwa, *Niigata*
Takayuki Yamamoto, *Yokkaichi*
Hiroshi Yamamoto, *Otsu*
Kosho Yamanouchi, *Nagasaki*
Ichiro Yasuda, *Gifu*
Yutaka Yata, *Maebashi-city*
Shin-ichi Yokota, *Sapporo*
Norimasa Yoshida, *Kyoto*
Hiroshi Yoshida, *Tama-City*
Hitoshi Yoshiji, *Kashihara*
Kazuhiko Yoshimatsu, *Tokyo*
Kentaro Yoshioka, *Toyoake*
Nobuhiro Zaima, *Nara*

Jordan
Khaled Ali Jadallah, *Irbid*

Kuwait
Islam Khan, *Kuwait*

Lebanon
Bassam N Abboud, *Beirut*
Kassem A Barada, *Beirut*
Marwan Ghosn, *Beirut*
Iyad A Issa, *Beirut*
Fadi H Mourad, *Beirut*
AIa Sharara, *Beirut*
Rita Slim, *Beirut*

Lithuania
Antanas Mickevicius, *Kaunas*

Malaysia
Huck Joo Tan, *Petaling Jaya*

Mexico
Richard A Awad, *Mexico City*
Carlos R Camara-Lemarroy, *Monterrey*
Norberto C Chavez-Tapia, *Mexico City*
Wolfgang Gaertner, *Mexico City*
Diego Garcia-Compean, *Monterrey*
Arturo Panduro, *Guadalajara*
OT Teramoto-Matsubara, *Mexico City*
Felix Tellez-Avila, *Mexico City*
Omar Vergara-Fernandez, *Mexico City*
Saúl Villa-Trevino, *Cuidad de México*

Morocco
Samir Ahboucha, *Khouribga*

Netherlands
Robert J de Knegt, *Rotterdam*
Tom Johannes Gerardus Gevers, *Nijmegen*
Menno Hoekstra, *Leiden*
BW Marcel Spanier, *Arnhem*
Karel van Erpecum, *Utrecht*

New Zealand
Leo K Cheng, *Auckland*
Andrew Stewart Day, *Christchurch*
Jonathan Barnes Koea, *Auckland*
Max Petrov, *Auckland*

Nigeria
Olufunmilayo Adenike Lesi, *Lagos*
Jesse Abiodun Otegbayo, *Ibadan*
Stella Ifeanyi Smith, *Lagos*

Norway
Trond Berg, *Oslo*
Trond Arnulf Buanes, *Krokkleiva*
Thomas de Lange, *Rud*
Magdy El-Salhy, *Stord*
Rasmus Goll, *Tromso*
Dag Arne Lihaug Hoff, *Aalesund*

Pakistan
Zaigham Abbas, *Karachi*
Usman A Ashfaq, *Faisalabad*
Muhammad Adnan Bawany, *Hyderabad*
Muhammad Idrees, *Lahore*
Saeed Sadiq Hamid, *Karachi*
Yasir Waheed, *Islamabad*

Poland
Thomas Brzozowski, *Cracow*
Magdalena Chmiela, *Lodz*
Krzysztof Jonderko, *Sosnowiec*
Anna Kasicka-Jonderko, *Sosnowiec*
Michal Kukla, *Katowice*
Tomasz Hubert Mach, *Krakow*
Agata Mulak, *Wroclaw*
Danuta Owczarek, *Kraków*
Piotr Socha, *Warsaw*
Piotr Stalke, *Gdansk*
Julian Teodor Swierczynski, *Gdansk*
Anna M Zawilak-Pawlik, *Wroclaw*

Portugal
Marie Isabelle Cremers, *Setubal*
Ceu Figueiredo, *Porto*
Ana Isabel Lopes, *LIsbon*
M Paula Macedo, *Lisboa*
Ricardo Marcos, *Porto*
Rui T Marinho, *Lisboa*
Guida Portela-Gomes, *Estoril*

Switzerland
Gieri Cathomas, *Liestal*
Jean Louis Frossard, *Geneve*
Christian Toso, *Geneva*
Stephan Robert Vavricka, *Zurich*
Dominique Velin, *Lausanne*

Thailand
Thawatchai Akaraviputh, *Bangkok*
P Yoysungnoen Chintana, *Pathumthani*
Veerapol Kukongviriyapan, *Muang*
Vijittra Leardkamolkarn, *Bangkok*
Varut Lohsiriwat, *Bangkok*
Somchai Pinlaor, *Khaon Kaen*
D Wattanasirichaigoon, *Bangkok*

Trinidad and Tobago
B Shivananda Nayak, *Mount Hope*

Tunisia
Ibtissem Ghedira, *Sousse*
Lilia Zouiten-Mekki, *Tunis*

Turkey
Inci Alican, *Istanbul*
Mustafa Altindis, *Sakarya*
Mutay Aslan, *Antalya*
Oktar Asoglu, *Istanbul*
Yasemin Hatice Balaban, *Istanbul*
Metin Basaranoglu, *Ankara*
Yusuf Bayraktar, *Ankara*
Süleyman Bayram, *Adiyaman*
Ahmet Bilici, *Istanbul*
Ahmet Sedat Boyacioglu, *Ankara*
Züleyha Akkan Cetinkaya, *Kocaeli*
Cavit Col, *Bolu*
Yasar Colak, *Istanbul*
Cagatay Erden Daphan, *Kirikkale*
Mehmet Demir, *Hatay*
Ahmet Merih Dobrucali, *Istanbul*
Gülsüm Ozlem Elpek, *Antalya*
Ayse Basak Engin, *Ankara*
Eren Ersoy, *Ankara*
Osman Ersoy, *Ankara*
Yusuf Ziya Erzin, *Istanbul*
Mukaddes Esrefoglu, *Istanbul*
Levent Filik, *Ankara*
Ozgur Harmanci, *Ankara*
Koray Hekimoglu, *Ankara*
Abdurrahman Kadayifci, *Gaziantep*
Cem Kalayci, *Istanbul*
Selin Kapan, *Istanbul*
Huseyin Kayadibi, *Adana*
Sabahattin Kaymakoglu, *Istanbul*
Metin Kement, *Istanbul*
Mevlut Kurt, *Bolu*
Resat Ozaras, *Istanbul*
Elvan Ozbek, *Adapazari*

Cengiz Ozcan, *Mersin*
Hasan Ozen, *Ankara*
Halil Ozguc, *Bursa*
Mehmet Ozturk, *Izmir*
Orhan V Ozkan, *Sakarya*
Semra Paydas, *Adana*
Ozlem Durmaz Suoglu, *Istanbul*
Ilker Tasci, *Ankara*
Müge Tecder-ünal, *Ankara*
Mesut Tez, *Ankara*
Serdar Topaloglu, *Trabzon*
Murat Toruner, *Ankara*
Gokhan Tumgor, *Adana*
Oguz Uskudar, *Adana*
Mehmet Yalniz, *Elazig*
Mehmet Yaman, *Elazig*
Veli Yazisiz, *Antalya*
Yusuf Yilmaz, *Istanbul*
Ozlem Yilmaz, *Izmir*
Oya Yucel, *Istanbul*
Ilhami Yuksel, *Ankara*

United Kingdom
Nadeem Ahmad Afzal, *Southampton*
Navneet K Ahluwalia, *Stockport*
Yeng S Ang, *Lancashire*
Ramesh P Arasaradnam, *Coventry*
Ian Leonard Phillip Beales, *Norwich*
John Beynon, *Swansea*
Barbara Braden, *Oxford*
Simon Bramhall, *Birmingham*
Geoffrey Burnstock, *London*
Ian Chau, *Sutton*
Thean Soon Chew, *London*
Helen G Coleman, *Belfast*
Anil Dhawan, *London*
Sunil Dolwani, *Cardiff*
Piers Gatenby, *London*
Anil T George, *London*
Pasquale Giordano, *London*
Paul Henderson, *Edinburgh*
Georgina Louise Hold, *Aberdeen*
Stefan Hubscher, *Birmingham*
Robin D Hughes, *London*
Nusrat Husain, *Manchester*
Matt W Johnson, *Luton*
Konrad Koss, *Macclesfield*
Anastasios Koulaouzidis, *Edinburgh*
Simon Lal, *Salford*
John S Leeds, *Aberdeen*
JK K Limdi, *Manchester*
Hongxiang Liu, *Cambridge*
Michael Joseph McGarvey, *London*
Michael Anthony Mendall, *London*
Alexander H Mirnezami, *Southampton*
J Bernadette Moore, *Guildford*
Claudio Nicoletti, *Norwich*
Savvas Papagrigoriadis, *London*
Sylvia LF Pender, *Southampton*
David Mark Pritchard, *Liverpool*
James A Ross, *Edinburgh*
Kamran Rostami, *Worcester*
Xiong Z Ruan, *London*
Frank I Tovey, *London*
Dhiraj Tripathi, *Birmingham*

Vamsi R Velchuru, *Great Yarmouth*
Nicholas T Ventham, *Edinburgh*
Diego Vergani, *London*
Jack Westwood Winter, *Glasgow*
Terence Wong, *London*
Ling Yang, *Oxford*

United States
Daniel E Abbott, *Cincinnati*
Ghassan K Abou-Alfa, *New York*
Julian Abrams, *New York*
David William Adelson, *Los Angeles*
Jonathan Steven Alexander, *Shreveport*
Tauseef Ali, *Oklahoma City*
Mohamed R Ali, *Sacramento*
Rajagopal N Aravalli, *Minneapolis*
Hassan Ashktorab, *Washington*
Shashi Bala, *Worcester*
Charles F Barish, *Raleigh*
P Patrick Basu, *New York*
Robert L Bell, *Berkeley Heights*
David Bentrem, *Chicago*
Henry J Binder, *New Haven*
Joshua Bleier, *Philadelphia*
Wojciech Blonski, *Johnson City*
Kenneth Boorom, *Corvallis*
Brian Boulay, *Chicago*
Carla W Brady, *Durham*
Kyle E Brown, *Iowa City*
Adeel A Butt, *Pittsburgh*
Weibiao Cao, *Providence*
Andrea Castillo, *Cheney*
Fernando J Castro, *Weston*
Adam S Cheifetz, *Boston*
Xiaoxin Luke Chen, *Durham*
Ramsey Cheung, *Palo Alto*
Parimal Chowdhury, *Little Rock*
Edward John Ciaccio, *New York*
Dahn L Clemens, *Omaha*
Yingzi Cong, *Galveston*
Laura Iris Cosen-Binker, *Boston*
Joseph John Cullen, *Iowa*
Mark J Czaja, *Bronx*
Mariana D Dabeva, *Bronx*
Christopher James Damman, *Seattle*
Isabelle G De Plaen, *Chicago*
Punita Dhawan, *Nashville*
Hui Dong, *La Jolla*
Wael El-Rifai, *Nashville*
Sukru H Emre, *New Haven*
Paul Feuerstadt, *Hamden*
Josef E Fischer, *Boston*
Laurie N Fishman, *Boston*
Joseph Che Forbi, *Atlanta*
Temitope Foster, *Atlanta*
Amy E Foxx-Orenstein, *Scottsdale*
Daniel E Freedberg, *New York*
Shai Friedland, *Palo Alto*
Virgilio George, *Indianapolis*
Ajay Goel, *Dallas*
Oliver Grundmann, *Gainesville*
Stefano Guandalini, *Chicago*
Chakshu Gupta, *St. Joseph*
Grigoriy E Gurvits, *New York*

World Journal of *Gastroenterology*

Contents

Contents

Contents

ABOUT COVER

Editorial board member of *World Journal of Gastroenterology*, Saúl Villa-Trevino, MD, PhD, Emeritus Professor, Senior Scientist, Departamento de Biología Celular, Centro de Investigación y de Estudios Avanzados del IPN (Cinvestav), Cuidad de México 07360, Mexico

AIMS AND SCOPE

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a peer-reviewed open access journal. *WJG* was established on October 1, 1995. It is published weekly on the 7th, 14th, 21st, and 28th each month. The *WJG* Editorial Board consists of 1375 experts in gastroenterology and hepatology from 68 countries.

The primary task of *WJG* is to rapidly publish high-quality original articles, reviews, and commentaries in the fields of gastroenterology, hepatology, gastrointestinal endoscopy, gastrointestinal surgery, hepatobiliary surgery, gastrointestinal oncology, gastrointestinal radiation oncology, gastrointestinal imaging, gastrointestinal interventional therapy, gastrointestinal infectious diseases, gastrointestinal pharmacology, gastrointestinal pathophysiology, gastrointestinal pathology, evidence-based medicine in gastroenterology, pancreatology, gastrointestinal laboratory medicine, gastrointestinal molecular biology, gastrointestinal immunology, gastrointestinal microbiology, gastrointestinal genetics, gastrointestinal translational medicine, gastrointestinal diagnostics, and gastrointestinal therapeutics. *WJG* is dedicated to become an influential and prestigious journal in gastroenterology and hepatology, to promote the development of above disciplines, and to improve the diagnostic and therapeutic skill and expertise of clinicians.

INDEXING/ABSTRACTING

World Journal of Gastroenterology (*WJG*) is now indexed in Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, Digital Object Identifier, and Directory of Open Access Journals. The 2015 edition of Journal Citation Reports® released by Thomson Reuters (ISI) cites the 2015 impact factor for *WJG* as 2.787 (5-year impact factor: 2.848), ranking *WJG* as 38 among 78 journals in gastroenterology and hepatology (quartile in category Q2).

FLYLEAF

EDITORS FOR THIS ISSUE

EDITORIAL

Long term follow-up and outcome of liver transplantation from hepatitis B surface antigen positive donors

Roberto Ballarin, Alessandro Cucchetti, Francesco Paolo Russo, Paolo Magistri, Matteo Cescon, Umberto Cillo, Patrizia Burra, Antonio Daniele Pinna, Fabrizio Di Benedetto

Roberto Ballarin, Paolo Magistri, Fabrizio Di Benedetto, Hepatopancreatobiliary Surgery and Liver Transplant Unit, University Hospital "Policlinico", University of Modena and Reggio Emilia, 41124 Modena, Italy

Alessandro Cucchetti, Matteo Cescon, Antonio Daniele Pinna, Department of Medical and Surgical Sciences, DIMEC, S. Orsola-Malpighi Hospital, Alma Mater Studiorum, University of Bologna, 40138 Bologna, Italy

Francesco Paolo Russo, Umberto Cillo, Patrizia Burra, Gastroenterology/Multivisceral Transplant Unit, Department of Surgery, Oncology and Gastroenterology, Padua University Hospital, 35128 Padua, Italy

Paolo Magistri, Department of Medical and Surgical Sciences and Translational Medicine, Sapienza, University of Rome, 00185 Rome, Italy

Author contributions: Ballarin R designed the research; Ballarin R, Cucchetti A, Russo FP and Burra P performed the research; Ballarin R, Cucchetti A, Russo FP, Burra P and Magistri P analyzed the data; Ballarin R and Magistri P wrote the paper; Cillo U, Pinna AD and Di Benedetto F critically revised the manuscript; all the authors contributed to this manuscript.

Conflict-of-interest statement: None of the authors has conflict of interest related to this publication.

Correspondence to: Roberto Ballarin, MD, PhD, Hepato-pancreatobiliary Surgery and Liver Transplant Unit, University Hospital "Policlinico", University of Modena and Reggio Emilia, Via del Pozzo 71, 41124 Modena, Italy. ballarinroberto@hotmail.com
Telephone: +39-59-4224740

Abstract

Liver transplant for hepatitis B virus (HBV) currently yields excellent outcomes: it allows to rescue patients with an HBV-related advanced liver disease, resulting in a demographical modification of the waiting list for liver transplant. In an age of patient-tailored treatments, in liver transplantation as well the aim is to offer the best suitable graft to the patient who can benefit from it, also expanding the criteria for organ acceptance and allocation. With the intent of developing strategies to increase the donor pool, we set-up a multicenter study involving 3 Liver Transplant Centers in Italy: patients undergoing liver transplantation between March 03, 2004, and May 21, 2010, were retrospectively evaluated. 1408 patients underwent liver transplantation during the study period, 28 (2%) received the graft from hepatitis B surface antigen positive (HBsAg)-positive deceased donors. The average follow-up after liver transplantation was 63.7 mo [range: 0.1-119.4; SD ± 35.8]. None Primary non-function, re-liver transplantation, early or late hepatic artery thrombosis occurred. The 1-, 3- and 5-year graft and patient survival resulted of 85.7%, 82.1%, 78.4%. Our results suggest that the use of HBsAg-positive donors liver grafts is feasible, since HBV can be controlled without affecting graft stability. However, the selection of grafts and the postoperative antiviral therapy should be managed appropriately.

Core tip: With the intent of developing strategies to increase the donor pool, we set-up a multicenter study involving 3 Liver Transplant Centers in Italy between March 2004 and May 2010. 1408 patients underwent liver transplantation during the study period, and 28 received the graft from hepatitis B surface antigen positive (HBsAg)-positive deceased donors. None primary non-function, re-liver transplantation, early or late hepatic artery thrombosis occurred. Our results show that transplantation of grafts from deceased HBsAg positive donors is feasible and this represents a way to expand the donor pool, especially in the high-endemic areas where a large proportion of patients are highly viremic and HBeAg positive.

INTRODUCTION

Epidemiology of hepatitis B and hepatocellular carcinoma

Hepatitis B virus (HBV) prevalence is different from a geographical region to another (Figure 1): currently, in Northern Europe, United States, Canada and Australia it ranges from 0.1% to 2%, while in central and Eastern Europe, as well as in Mid East, India, Central and Southern America, it is between 3% and 7%. Finally, the highest incidence, ranging from 10% to 20%, is registered in Africa and Easter Countries.

Notably, the incidence of hepatocellular carcinoma (HCC) in the same regions mirrors the prevalence of HBV. In Europe, Japan and North America HBV is responsible for 10%-15% of HCC cases, while conversely, in Asia and Africa, HBV is associated to 70% of cases. According to several studies, the relative risk of developing a tumor is close to 100-fold in HBV carriers *vs* non-carriers[1].

Liver transplantation for HBV

Liver transplant for HBV currently yields excellent outcomes, but in 1983, before the introduction of HBV immune globulin (HBIg) and antiviral therapy, a United States National Institute of Health consensus conference recommended against transplant for HBV because of the poor outcomes from severe recurrent liver disease. The first studies showed HBIg and HBIg plus lamivudine to improve graft and patient survival[2]. Subsequently, successful suppression of HBV DNA before transplant by Adefovir resulted in improved pre- and posttransplant survival[3]. More recently, the use of the more potent antiviral agent, entecavir, entirely prevented post-transplant recurrence, even in some patients with prior lamivudine resistance[4]. Whereas the original protocols utilized a lifetime administration of HBIg to maintain a blood titer high enough to prevent reinfection, and this was supplemented with lamivudine and now more potent antiviral agents, newer protocols have reduced the time of administration of the HBIg to 1 year with continued antiviral administration indefinitely after, or even use Entecavir or Tenofovir as a single agent to achieve an undetectable pretransplant viral load and maintain this indefinitely afterward[3].

Liver transplant for hepatitis B virus (HBV) currently yields excellent outcomes: it allows to rescue patients with an HBV-related advanced liver disease, resulting in a demographical modification of the waiting list for liver transplant. In a review of the Scientific Registry of Transplant Recipients (SRTR) database of registrants to the liver transplant list in the United States from 1985 to 2006, the overall number of registrants for HBV began declining after 1998 when oral antiviral therapy was first introduced[5]. Of the main indications for transplant owing to HBV (advanced liver disease, acute liver failure, and HCC), only HCC was increasing in number; registrants for advanced liver disease was declining most rapidly. This trend should continue; the data suggest that those with an early response to antiviral treatment with Tenofovir for acute severe reactivation of HBV have improved non-transplant survival (57% *vs* 13% for placebo-treated patients)[6].

However, antiviral therapy did not influence survival for those with acute liver failure owing to *de novo* HBV infection in a North American cohort of patients with acute liver failure[7]. It will likely be at least another decade until the incidence of HCC owing to HBV-induced liver disease begins to significantly decline, and this in part will be owing to treatment of HBV (as well as immunization of populations that began in the early 1990s). Eventually the choice of treatment to prevent HBV reinfection must take into account treatment efficacy, patient adherence, and cost.

Extended criteria for organ acceptance

The unmatched demand and supply rate between organs for transplantation is well known. As a matter of fact, we observed during the last decade a similar annual rate of donors in Europe and United States, while an increase of the "demand" for liver transplantation has been reported, in terms of new patients added in the waiting lists, longer mean waiting time and drop-out rate. Moreover, the lack of organs led to the exclusion from the waiting list of many
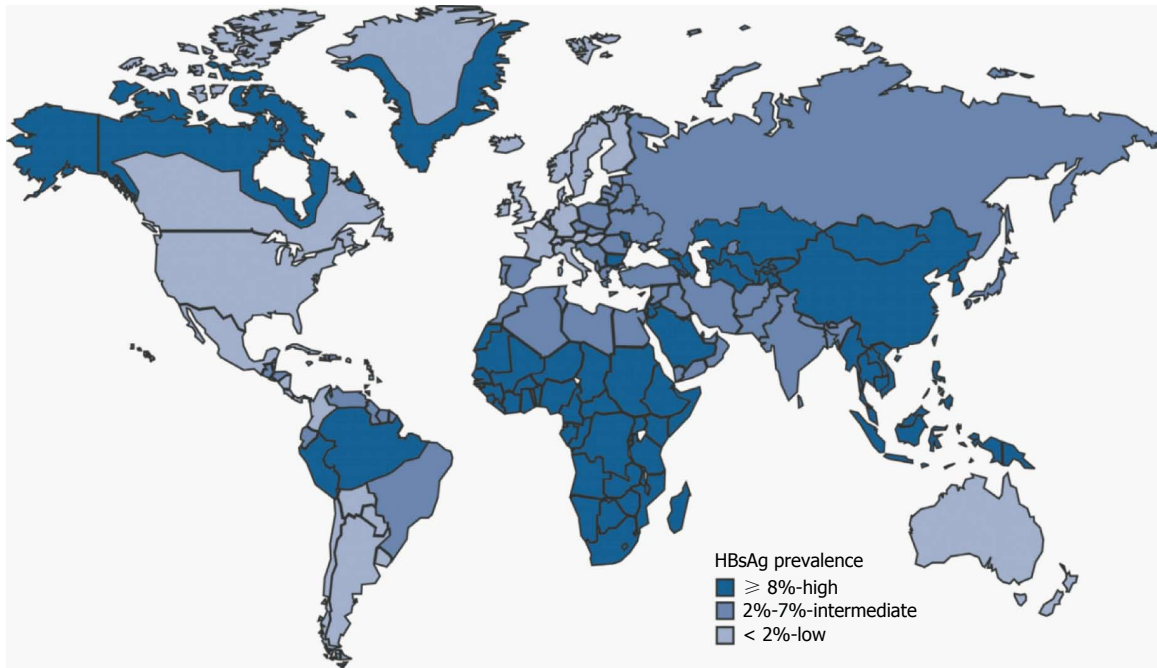
Figure 1 Geographic distribution of chronic hepatitis B virus infection-worldwide (2005). For multiple countries, estimates of prevalence of hepatitis B surface antigen (HBsAg), a marker of chronic hepatitis B virus infection, are based on limited data and may not reflect current prevalence in countries that have implemented childhood hepatitis B vaccination; prevalence may vary within countries. Source: Centers for Disease Control and Prevention (http://www.cdc.gov).

patients who can benefit from a transplant[8,9].

In an age of patient-tailored treatments, in liver transplantation as well the aim is to offer the best suitable graft to the patient who can benefit from it. In Europe and in the United States is estimated that almost 10% to 30% of patients listed for liver transplant dies before organ availability[8]. In the United States status I patients *i.e.,* patients entering in the waiting list at the highest medical urgency, reported a 12 folds increased risk of death while on the list compared with those entering at the two lowest categories of urgency[10]. Data from Scandinavia between 1990 and 2001 show that the mortality rate among patients waiting for liver transplant was 16%, while 27% of patients listed for a highly urgent liver transplantation failed to get the graft[11].

For many patients with a severe clinical status needing urgent transplant, the so-called marginal organ donor can provide a chance of cure. Patients that never obtained a transplant due to their clinical characteristics may as well benefit from a marginal donor, overcoming the problem of organ shortage.

The terms extended donor or expanded donor (ECD) mean changes in donor acceptability criteria, which not justifies the negative connotations of these terms. Although criteria to select organs for donation were revised and modified over years, this evolution did not affect neither patients' nor organs' survival. Characteristics of donor and recipient, together with allocation scheme, organ procurement and transplant procedure define the "ideal organ". Moreover, marginal donors can allow to obtain comparable survival rates when an appropriate allocation is ruled out.

Criteria and terms for certified suitability of organ donors: Assumptions and operational strategies in Italy
In 2001 a national committee of experts nominated by the Italian National Transplant Centre (Centro Nazionale Trapianti-CNT) released a document for all personnel involved in the evaluation process of potential organ donor. The Commettee was made up of infectious disease experts, immunologists, clinical experts, surgeons, coordinators, anatomopathologists, medical examiners and oncologists. During the preparation phase, which lasted one year, the text underwent a series of changes and supplements, resulting in a final version shared with the scientific community and approved by the Italian National Transplant Centre as technical annex (guidelines) to the Ministry Decree of August 2, 2002[12].

These Guidelines focus on two main aspects: (1) The definition of acceptable/unacceptable risks for donor suitability or single organ utilization; and (2) the establishment of practical steps for the risk evaluation process.

The first aim was to identify the different risk levels and as a result five risk levels have been defined: (1) unacceptable risk; (2) increased but acceptable risk; (3) calculated risk; (4) not assessable risk; and (5) standard risk.

Unacceptable risk: The donor classified under this category should be excluded from donation and no organ can be used for transplantation. For example, HIV1 or 2 positive donors fall into this category, as well as HBsAg and HDV contemporaneous seropositivity. Neoplastic diseases represents an unacceptable risk

with the following exceptions: carcinoma *in situ*, basal cell carcinoma, cutaneous squamous cell carcinoma without metastases, carcinoma *in situ* of the cervix, carcinoma *in situ* of vocal cords, urothelial papillary carcinoma (T0 according to the TNM classification). Eventually, systemic infections caused by agents for which treatments are not feasible and documented prior disease must also be considered as exclusion criteria.

Increased but acceptable risk: This category includes organs that can be used in case of urgency or particular clinical conditions of recipients. In these cases, even when the evaluation process shows the presence of pathogens or transmissible disease, organ utilization is allowed in the light of a risk benefit assessment. Patients struck by fulminant hepatitis, or retransplants for liver primary non function, or patients who underwent hepatectomy for trauma with complete organ function loss are included in this category.

Calculated risk: Includes all cases where the presence of a specific pathogen or a serological status of the donor (HBsAg$^+$, or anti-HCV$^+$ or HBcAb$^+$) is compatible with transplantation recipients with the same disease or serological status, independently from recipient's health conditions.

Not assessable risk: Includes cases for which the evaluation process does not allow an appropriate risk assessment for transmittable diseases for lack of one or more assessment elements (*e.g.*, failure to collect an accurate medical history for lack of relatives, unavailability of microbiology data despite a well-grounded suspicion of infectious pathology).

Standard risk: Includes cases for which the evaluation process did not identify any risk factor for transmittable disease. It is the most frequent condition in the assessment of donors and grafts.

The national guidelines also identify some special conditions that concern two main aspects, namely neoplastic and infectious risks.

About infections, special attention should be paid to the following cases: donor with HCV infection; donor with HBV infection (HBsAg positivity); donors with anticore IgG antibodies against B virus (HBcAb). In such cases the guidelines impose the adoption of the following procedures.

HBsAg positive donor: If a donor turns out to be HBsAg positive, transplantation is allowed in a HBsAg positive recipient, after informed consent, provided that the following conditions are met: (1) the donor has a negative HDV antigen, negative IgM anti HDV antibodies, negative IgG anti HDV antibodies or with a titre < 1:100 or below the significant level according to the assay used; the absence of IgM anti HDV does not exclude delta virus chronic infection; (2) the liver recipient is not co-infected by delta virus; and (3) the patient follow-up can be monitored on the basis of a common national protocol established by the National Transplant Centre and to record data on a National Registry.

HBsAg negative donor: If the recipient is HBsAg negative, he has no anti-HBV antibodies or has a protective anti-HBsAg titre ($\geqslant$ 10 mUI/mL), transplantation can be performed, after informed consent, when the following conditions are met: (1) the donor has a negative HDV antigen, negative IgM anti HDV antibodies, negative IgG anti HDV antibodies or with a titre < 1:100 or below the significant level according to the used assay; and (2) the patient follow-up can be monitored on the basis of a common national protocol established by the National Transplant Centre and to record data on a National Registry.

As a supplement to these measures, the Italian National Transplant Centre has deemed as proper to support further transplant network health workers, through adhoc developed information tools and an expert task force (second opinion) for evaluation of doubtful cases.

Study design
With the intent of developing strategies to increase the donor pool, we set-up a multicenter study involving 3 Liver Transplant Centers in Italy: the Universities of Modena, Bologna and Padova. The study was approved by the institutional review boards at each center. Patients undergoing liver transplantation between March 2004, and May 2010, were retrospectively evaluated. Among 1408 patients who underwent liver transplanation during the study period, 28 (2%) received the graft from HBsAg-positive deceased donors. All subjects were informed of the possible risks, consented to enter the study and signed a written form. For each HBsAg case we collected general clinical features and data regarding the transplantation, including MELD score and ischemia time. Then we retrospectively analyzed post-operative data, namely immunosuppressive therapy, histological evidence of HBV recurrence and antiviral therapy, and episodes of acute rejection.

The Italian regulations issued by the CNT allow HBsAg positive HDV negative recipients, HBcAb positive HDV negative patients, and HBV negative subjects with severe end-stage liver disease and a low life expectancy, to receive grafts from HBsAg positive HDV negative donors. Liver biopsy during organ procurement drives the evaluation on graft status, together with the serovirological complete assessment of HBV and HCV status, including HBV DNA. Moreover, Ishak score $\leqslant$ 1 and low inflammation, together HDV negative test in both donor and recipient, are required. HBV viral load, liver function test and age are not considered as exclusion criteria.

We performed liver biopsies routinely pre- and

postperfusion in all cases. All the centers performed a liver biopsy protocol at months 6 and 12. However, all centers performed liver biopsies whenever biochemical or clinical signs of liver dysfunction became evident.

There was agreement on the definition of HBV recurrence as the contemporary presence of serum HBV-DNA and graft histology with evidence of lymphocytic infiltrates suggestive of recurrent HBV infection. An experienced pathologist is required for this evaluation, in order to avoid confusion with acute cellular rejection signs, like absence of endothelitis and cholangitis. Ishak score and the Knodell modified HAI were used to stage the disease, giving to each biopsy a HAI inflammatory grade (scale of 0-18), a fibrosis stage (scale of 0-6), and a total score combining the previous 2. Steatosis score was recorded as none (0%), mild (1%-30%), moderate (31%-60%), or severe (61%-100%), according to the degree of steatosis noted in the biopsy.

We performed a standard antiviral prophylaxis in all patients, independently from serovirological profile.

All HBsAg-positive recipients were on antiviral treatment with nucleos(t)ide analogues before liver transplantation and continued the same antiviral therapy with the addition of HBV-specific immunoglobulins (HBIg) after liver transplantation. The HBsAg-negative recipients began a similar combined treatment after LT, with lamivudine (LMV) and HBIg.

HBIg administration consisted of 10000 IU during the anhepatic phase, then 5000 IU every day for the first month, subsequently 5000 IU weekly for the second month and finally 5000 IU every 3-4 wk to maintain an anti-HBs titre above 250 IU/mL. This is the standard regimen of the transplant centers and it is applied even to HBV patients receiving an HBsAg-negative graft. Tacrolimus administration in the postoperative setting was adjusted to maintain a plasma concentration between 5 and 12 ng/mL. Steroids were started at a dose of 20 mg daily, then tapered down and discontinued within 6 mo.

Statistical analysis
We reported continuous data as mean ± SD, and then compared those data by using the 2-side Student's *t* test. The χ^2 test with Yates' correction, or Fisher's exact test when appropriate, was used to compare groups for categorial variables. Survival of grafts and patients were evaluated using the Kaplan-Meier method and compared with the log-rank test. The statistical significance was accepted for $P < 0.05$. All the statistical analysis were performed using SPSS© 19.0.

DISCUSSION

Recipient characteristics
Four out of 28 recipients were female (median age at liver transplantation: 57.6 years, range: 26-67). Data were collected from liver transplantation until the last follow-up visit and the average follow-up after liver transplantation was 63.7 mo (range: 0.1-119.4; SD ± 35.8). Recipient characteristics were reported on Tables 1 and 2.

HBV related cirrhosis, with or without HCC, was the indication for liver transplantation in 27 patients (Table 1), while 1 patient was transplanted due to secondary biliary cirrhosis.

The five HBsAg-negative patients showed serological evidence of past HBV infection. The MELD score (Model of End Stage Liver Disease) was applied to stage their liver disease status. In case of HCC, an extra score based on HCC stage was added, according to the centre (or regional) allocation policy.

Patients were transplanted after an average of 452 d on waiting list (range: 37-1962; SD ± 394) and at the time of liver transplantation presented an average MELD biochemical score of 15.6 (range: 7-33; SD ± 6.5) and an average MELD score correction (depending from other clinical variables) of 26.8 (range 11-39; SD ± 7.2).

The median body mass index (BMI) at the time LT was 25.3 (range: 19-34; SD ± 3.2).

Nineteen patients had hepatocellular carcinoma (67.9%) with 13 cases (68.4%) resulting within the Milan criteria, whereas 6 patients (31.6%) were outside Milan and inside UCSF criteria.

Table 1 describes different downstaging treatments for each patient.

The UNOS status was 2A in 5 patients (17.8%), 2B in 15 patients (53.6%), and 3 in 8 patients (28.6%).

Donor characteristics
Donor characteristics were reported on Table 3 and the overall serological state of the recipient/donor is shown in the Table 4.

The median age was 52.6 years (range: 13-79, SD ± 16.9). 13 donors were female (46.4%) while 15 donors were male (53.6%). The death causes are reported on the Table 3. The average body mass index (BMI) of donors was 25 (range: 19.5-29.4; SD ± 2.6) All the patients were HBsAg-positive. 21 donors (75%) were HBV-DNA positive while 7 (25%) were HBV-DNA-negative. 2 (7.1%) donors were anti-HCV positive but both were HCV-RNA negative.

None was HDV co-infected. Five patients (17.9%) were HBsAg negative, and 4 (14.3%) were HCV co-infected (Table 4).

Data on pre-perfusion histologic features of the biopsies are shown in Table 3. Most of the HBsAg positive grafts had a HAI inflammatory grade between 0-2 (71.4%), followed by an HAI inflammatory grade between 3-4 (28.6%). None of the grafts used had an HAI inflammatory grade score ≥ 5.

In particular, 6 donors (21.4%) had a grading score 0; 7 donors (25%) had a grading score 1; 7 donors (25%) had a grading score 2; 4 donors (14.3%) had a grading score 3; 4 donors (14.3%) had a Grading score 4. All the grafts had a fibrosis stage ≤ 1.

Table 1 Recipient characteristics at the time of liver transplantation

Case	Age	Gender	ABO	BMI	Indication	Real MELD	MELD correct	UNOS	Wating List(d)	Year LT	HCC criteria	Downstaging type (No.)
1	62	M	0	27	HCC/HBV	26	36	2A	37	2007	MILAN IN	LOC(1)[1]
2	65	M	B	21	HCC/HBV	16	39	3	522	2007	MILAN IN	LOC(1) + SUR(1)
3	54	M	A	22	HCC/HBV	10	33	3	513	2007	MILAN IN	LOC(1)
4	45	M	A	24	HCC/HBV	14	34	2B	419	2007	MILAN OUT	LOC (3)
5	62	M	0	29	HCC/HBV	12	33	3	445	2008	MILAN OUT	LOC (2)
6	53	M	0	28	HCC/HBV	12	35	2B	515	2008	MILAN IN	LOC (1)
7	45	M	A	23	HCV/HBV	24	24	2A	101	2005		
8	64	M	B	27	HBV/HCV	33	33	2A	478	2005		
9	26	M	A	23	HBV	23	23	2B	742	2005		
10	64	M	A	22	HCC/HBV	11	23	2B	144	2005	MILAN IN	LOC (2)
11	65	F	B	25	HCC/HBV	12	24	2A	195	2006	MILAN IN	LOC (2)
12	56	M	A	24	HCC/HBV	10	24	2B	217	2006	MILAN OUT	LOC (5)
13	61	F	0	23	HCC/HBV	14	30	2B	352	2006	MILAN IN	LOC (2)
14	59	M	A	24	HBV	30	30	2B	118	2007		
15	48	F	A	27	CBS	21	33	2A	82	2007		
16	65	M	A	24	HCC/HBV	8	25	2B	358	2009	MILAN IN	LOC (2)
17	57	M	A	30	HCC/HBV	14	39	2B	576	2009	MILAN OUT	LOC (7)
18	55	M	B	28	HCC/HBV	18	26	2B	38	2009	MILAN IN	LOC (3)
19	65	M	A	24	HCC/HBV	7	25	2B	371	2009	MILAN IN	LOC (3)
20	63	M	A	28	HCC/HBV	16	25	2B	1962	2010	MILAN IN	LOC (2)
21	60	M	A	34	HCC/HBV	10	27	2B	330	2010	MILAN OUT	LOC (1) + SUR (1)
22	61	M	A	24	HBV	11	11	3	855	2010		
23	67	M	0	19	HBV	16	16	2B	742	2004		
24	55	M	A	24	HBV	17	17	3	127	2004		
25	60	F	0	29	HBV/HCV	17	17	2B	961	2004		
26	55	M	A	26	HCC/HBV	10	20	3	748	2005	MILAN IN	LOC (1)
27	54	M	A	27	HCC/HBV	13	19	3	134	2006	MILAN OUT	LOC (2)
28	67	M	0	22	HCC/HBV	12	29	3	575	2009	MILAN IN	LOC (1) + SUR (1)

[1]LOC: Locoregional therapy [transcatheter arterial chemoembolization (TACE) and/or radiofrequency ablation (RITA)]. SUR: Surgery; MELD: Model for end-stage disease; BMI: Body mass index; LT: Liver transplantation; HCC: Hepatocellular carcinoma; CBS: Secondary biliary cirrhosis; HCV: Hepatitis C virus.

For 14 (50%) grafts the staging was 0. Macrosteatosis of the grafts are reported on the Table 3.

Operative factors

Cold ischemia time was in an average of 429 min (range: 255-632) and the warm ischemia time (WIT) was around 39.7 min (range: 30-55). The average hematic loss was 2307 mL (range: 300-13000). The mean length of stay in the Intensive Care Unit (ICU) was 5.5 d (range: 0-22), while the average Hospital stay was 21.4 d with a range from 6 to 143.

Clinical outcome

None primary non-function (PNF), re-LT, early or late hepatic artery thrombosis occurred after liver transplantation.

Two (7.1%) patients who received an HBsAg-positive donor liver had acute cellular rejection with a total of 1 event respectively for each patient.

Biliary complication occurred in seven patients (25%); in particular five biliary stenosis and two biliary leakages.

Five patients (17.9%) developed a major infection, 2 patients (7.1%) had an Hepatitis C recurrence.

Recurrence of HBV infection, confirmed histologically, occurred in 4 (14.3%) patients who received HBsAg positive grafts. The mean time of onset of HBV recurrence was 2.1 (± 1.4) mo.

The average follow-up was 63.6 mo (range: 0.1-119.4). The 6 deceased patients died not for the Hepatitis B recurrence but for different reasons. In particular, the cause and time of death were respectively: 1 patient for severe sepsis (0.4 mo), 1 patient for cardiac arrest (0.1 mo), 1 patient for HCV recurrence (11.8 mo), 2 patients for HCC recurrence (3.5 and 13 mo, respectively) and one patient for Merkel cell carcinoma (45 mo).

The 1-, 3- and 5-year graft and patient survival resulted of 85.7%, 82.1% and 78.4% (Figure 2).

Read-out

Liver transplantation is an established therapeutic modality for patients with end-stage liver disease or/and hepatocellular carcinoma. However, in recent years the number of patients needing a transplant increase overcoming the supply: as a result, the mean waiting time is now longer than before, with higher mortality rates of patients waiting for an organ. It is estimated that 15% to 20% of patients on the waiting list die each year without receiving a suitable organ.

Several strategies have been developed by transplant physicians to face this increased demand: innovative ways of expanding the donor pool are the use of split and live donor LT. Another approach is the use of organs from "less-than-perfect donors", also called "suboptimal donors". Non-heart-beating donors

Table 2 Recipient characteristics *n* (%)	
Recipient variables	***n* = 28**
Transplant center (No. of patients)	
Modena	6 (21.4)
Bologna	16 (57.1)
Padova	6 (21.4)
Gender	
Male	24 (85.7)
Female	4 (14.3)
AB0 blood group	
Isogroup	28 (100)
0	7 (25)
A	17 (60.7)
B	4 (14.3)
Age (yr), mean (range, SD)	57.6 (26-67, ± 8.7)
Body mass index, mean (range, SD)	25.3 (19-34, ± 3.1)
Real MELD score, mean (range, SD)	15.6 (7-33, ± 6.5)
Correct MELD score, mean (range, SD)	26.7 (11-39, ± 7.2)
UNOS status	
2A	5 (17.5)
2B	15 (53.6)
3	8 (28.6)
Time waiting list (d), mean (range, SD)	452 (37-1962, ± 393.5)
Associated hepatocellular carcinoma	19 (69.7)
Meeting Milan criteria	13 (46.4)
Meeting UCSF criteria	6 (21.4)
HBsAg status	
Positive	23 (82.1)
Negative	5 (17.9)
HBV DNA positive at LT	12 (52.1)
HCV co-infection	4 (14.3)
HDV co-infection	0

UCSF: University of California, San Francisco; LT: Liver transplantation; HCV: Hepatitis C virus; HDV: Hepatitis D virus; MELD: Model for end-stage disease.

and donors older than 65 years belong to such donors, as well as steatosic liver allografts and patients with previous exposure to HBV or HCV. Also the selection HBsAg positive donors represents a way to expand the pool of transplantable grafts.

On the other hand, living donor (LD) LT was adopted in Eastern countries to counterbalance the lack of deceased donors due to cultural reasons. Living donors and split liver transplantation have been used to contrast the donor shortage, but they have failed to significantly decrease the number of patients on the wait list. Those two approaches have ethical issues and technical complexities that make them less than ideal ways to expand the donor pool[13-15]. In addition, living donor programs have been activated in a small minority of transplant centers, and more institutions have been forced to resort to the use of other marginal organ donors.

As a matter of fact, wider acceptance criteria can assure more donors available for transplantation and several guidelines are available to classify donors as standard or ECD[16-20]. Two main categories of ECD can be identified: the first one includes grafts with risk of dysfunction due to direct or indirect liver injury, the second accounts for the risk of disease transmission between donor and recipient.
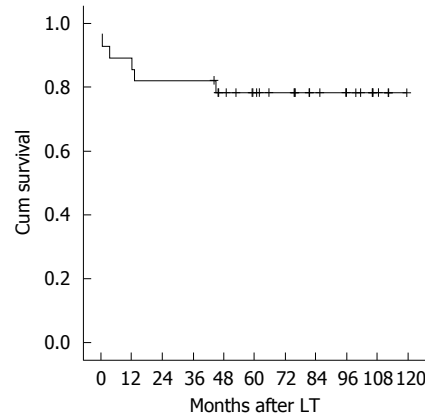
Figure 2 Patient and graft survival. LT: Liver transplantation.

In the first case should be taken into account that those grafts must be carefully evaluated and transplanted in recipients capable to overcome the increased physiologic stress.

The ECD liver disease transmission risk is broken into 2 separate categories: (1) viral transmission of HCV, HBV, HTLV-1, and HTLV-2; and (2) malignancy transmission. Our previously reported results are consistent with other studies showing that it is safe to allocate grafts from HCV positive donors into HCV positive recipients[21-25]. The HCV positive donor liver must have no evidence of cirrhosis or stage > 1 fibrosis. It is clear that HCV-positive livers should be declassified as ECDs.

HBV scenario: About 2 billion people have serological evidence of present or past HBV infection worldwide, and a prevalence of more than 350 million cases of chronic infection is estimated[26].

The selection criteria of the recipient of HBcAb-positive donors are currently debated, while it has been demonstrated that a lifelong antiviral therapy is needed after transplantation of those grafts[23,27,28]. The majority of chronic HBV infections is nowadays present in the Western Pacific region[29], while a recent survey from Korea showed an overall HBsAg prevalence of 3.7%. This group of ECD is currently underestimated due to the high risk of HBV reactivation and to the paucity of clinical data, and up-to-now they are not used in most of the transplant centers.

Because of the existing shortage of organs, the increased demand for LT, and given the possible implications in terms of extension of the donor pool, the use of HBsAg-positive grafts should be studied to assess safety policies. To date, only a few studies exist regarding the effect of donor HBsAg positivity on survival (Table 5). These available reports yield conflicting results and are limited by small sample sizes and short follow-up[30-38].

Gonzalez-Peralta *et al*[31] were the first to report a successful LT of an HBsAg-positive graft into HBV negative recipient, who shortly afterwards turns HBsAg positive. Several reports in literature attested

Table 3 Donor characteristics

Case	Age	Gender	ABO Gr.	BMI (kg/m^2)	Cause of death	Time ICU (d)	Sodium (mEq/mL)	Vasopressors	Histologic activity index		Graft steatosis macro
									Grading	Staging	
1	59	M	0	26	CH	2	165	No	1	0	20%
2	13	F	B	19	T	21	161	Yes	2	0	0%
3	69	M	A	24	CH	3	152	No	0	0	0%
4	72	F	A	27	CH	7	150	No	1	0	35%
5	66	M	0	22	CH	5	137	Yes	3	1	0%
6	60	M	0	26	CH	4	158	No	2	0	0%
7	73	M	A	26	CH	2	151	Yes	2	1	0%
8	51	M	B	23	CH	6	149	Yes	3	1	10%
9	54	M	A	24	T	13	160	Yes	2	1	10%
10	72	F	A	23	T	2	148	No	2	0	0%
11	60	F	B	29	CH	8	162	Yes	3	1	5%
12	65	M	A	29	CH	3	140	Yes	4	1	30%
13	50	M	0	24	CH	12	141	Yes	2	1	3%
14	48	M	A	23	T	2	143	No	4	1	5%
15	26	M	A	23	T	1	145	Yes	1	0	0%
16	52	F	A	28	CH	1	155	Yes	4	1	0%
17	79	F	A	24	CH	19	136	No	0	0	0%
18	46	F	B	29	CH	2	158	No	0	0	0%
19	61	M	A	29	CH	3	156	No	1	1	10%
20	53	F	A	25	CH	6	154	Yes	2	1	4%
21	44	F	A	24	CH	6	144	Yes	4	1	5%
22	23	F	A	21	other	7	149	Yes	1	1	0%
23	57	F	0	24	CH	4	147	Yes	0	0	0%
24	59	M	A	24	CH	1	160	Yes	0	0	5%
25	35	F	0	23	CH	1	140	Yes	1	0	0%
26	36	M	A	25	T	2	146	Yes	3	1	0%
27	23	M	A	29	T	2	147	Yes	0	0	0%
28	66	F	0	27	CH	3	151	Yes	1	0	10%

BMI: Body mass index; CH: Cerebral hemorrhage; T: Trauma; ICU: Intensive care unit.

the use of HBIg and antiviral drugs against HBV such as lamivudine, adefovir dipivoxil, and tenofovir in recipients with HBsAg-positive grafts[30,32-36,38]. Loggi *et al*[37] reported a series of 10 HBsAg-positive grafts with HBIg and nucleos(t)ide analogue prophylaxis. In their experience only one patient died due to HCV recurrence over a mean follow-up period of 36.8 mo. In a cohort with 8 patients out of 10 positive for HBsAg after LT, no patient ever had any signs of active HBV hepatitis.

However, there was no comparison of outcomes between HBsAg-positive graft recipients with and without HBIg prophylaxis.

Using comprehensive clinical data from the SRTR database, Li *et al*[39] failed to identify any significant association between the use of HBsAg-positive donors and post-transplant graft or patient survival, after adjusting for other predictors of post-transplant survival. Their results demonstrate that HBsAg-positive donors for liver transplantation are safe and comparable in terms of outcomes and long-term survival to the use of HBsAg-negative grafts. Furthermore, other studies clearly showed that using HBIg may improve post-transplant survival in recipients with HBsAg-positive grafts.

Several innovations have been introduced during the last two decades to improve the outcomes of patients receiving LT for HBV-related liver disease, such as the administration of HBIg since the early 1990s and

lamivudine in late 1990s[40-45]. Although there is now a consensus in favor of the use of HBIg in HBV-positive recipients, its application in HBV positive donors is still unclear.

Our study shows that the use of HBsAg positive grafts is a safe procedure when carried out in combination with appropriate antiviral therapy and when the graft fibrosis is ≤ 1 and the grading score is ≤ 4.

The 4 Hepatitis B recurrences that we have followed during the post-LT didn't influence the graft and patient survival. From our own experience, there were no cases of PNF and the infectious and biliary complications were similar to the cases of HBsAg negative graft recipients.

However, our research shows some relevant limitations: first, even if it represents the major European study, the number of patients is still too low and therefore it doesn't allow to establish ultimate conclusions. Then, we have chosen to focus only on a descriptive kind of analysis while for the future it will be necessary to perform comparative studies and matched analysis. Second, the lack of a common serial protocol hepatic biopsies has not allowed to examine the histological evolution of these grafts as well as a serial protocol for the dosage of the HBsAg quantification and the HBV-DNA level.

CONCLUSION

Despite the small number of cases, our results suggest

Table 4 Serological state of the recipient/donor

Case	HBsAg	HBsAb	HBcAb	HBeAg	HBeAb	HBV DNA	HDV	HDV RNA	HCV Ab	Therapy pre-LT	Mutation
R1	+	-	+	-	+	-	-	-	-	Lam	No
D1	+	-	+	-	+	+	-	-	-	No	-
R2	+	-	+	-	+	-	-	-	-	Lam	No
D2	+	-	+	-	+	+	-	-	-	No	-
R3	+	-	+	-	+	+	-	-	-	Lam + Adef	Yes
D3	+	-	+	-	+	+	-	-	-	No	-
R4	+	-	+	+	-	-	-	-	-	Lam	No
D4	+	-	+	-	+	-	-	-	-	No	-
R5	+	-	+	-	+	+	-	-	-	Lam + Adef	Yes
D5	+	-	+	-	+	-	-	-	-	No	-
R6	+	-	+	-	+	-	+	-	-	Lam	No
D6	+	-	+	-	+	+	-	-	-	No	-
R7	-	+	+	-	-	-	-	-	+	No	No
D7	+	-	+	+	-	+	-	-	+	No	-
R8	-	+	+	-	-	-	-	-	+	No	No
D8	+	-	+	-	+	+	-	-	-	No	-
R9	+	-	+	+	-	+	-	-	-	Lam	No
D9	+	-	+	+	-	+	-	-	-	No	-
R10	+	-	+	-	+	-	-	-	-	Lam	No
D10	+	-	+	+	-	+	-	-	-	No	-
R11	-	+	+	-	-	-	-	-	+	No	No
D11	+	-	+	+	-	+	-	-	+	No	-
R12	+	+	+	-	+	+	-	-	-	Lam	No
D12	+	-	+	-	+	+	-	-	-	No	-
R13	+	-	+	-	+	-	-	-	-	Lam	No
D13	+	-	+	+	-	+	-	-	-	No	-
R14	+	-	+	-	+	+	-	-	-	Lam	No
D14	+	-	+	-	+	-	-	-	-	No	-
R15	-	-	+	-	+	-	-	-	-	No	No
D15	+	-	+	-	+	+	-	-	-	No	-
R16	+	-	+	-	+	+	-	-	-	Lam	No
D16	+	-	+	-	+	+	-	-	-	No	-
R17	+	-	+	-	+	+	-	-	-	Lam + Adef	Yes
D17	+	-	+	+	-	+	-	-	-	No	-
R18	+	-	+	-	+	+	-	-	-	Lam	No
D18	+	-	+	-	+	-	-	-	-	No	-
R19	+	-	+	+	-	+	-	-	-	Adefovir	No
D19	+	-	+	+	-	+	-	-	-	No	-
R20	+	-	+	-	+	+	-	-	-	Lam + Adef	No
D20	+	-	+	+	-	+	-	-	-	No	-
R21	+	-	+	-	+	-	-	-	-	NA	No
D21	+	-	+	+	-	+	-	-	-	No	-
R22	+	-	+	-	+	-	-	-	-	Lam	No
D22	+	-	+	+	-	+	-	-	-	No	-
R23	+	-	+	-	+	-	-	-	-	Lam	No
D23	+	-	+	-	+	-	-	-	-	No	-
R24	+	-	+	-	+	+	-	-	-	Lam	Yes
D24	+	-	+	-	+	-	-	-	-	No	-
R25	-	+	+	-	+	-	-	-	+	No	No
D25	+	-	+	-	+	-	-	-	-	No	-
R26	+	-	+	-	+	-	-	-	-	Lam	No
D26	+	-	+	+	-	+	-	-	-	No	-
R27	+	-	+	-	+	-	-	-	-	Adef	Yes
D27	+	-	+	-	+	+	-	-	-	No	-
R28	+	-	+	-	+	-	+	-	-	Lam	No
D28	+	-	+	+	-	+	-	-	-	No	-

R: Recipient; D: Donor; LT: Liver transplant; Lam: Lamivudine; Adef: Adefovir.

that the utilization of grafts from deceased HBsAg positive donors, according to our allocation criteria, is feasible and HBV can be controlled with graft stability if selection of grafts and postoperative antiviral treatment are appropriately managed.

This way it could be possible to expand the donor pool, especially in the high-endemic areas where a large proportion of patients are highly viremic and HBeAg positive.

Long-term follow-up data and large-scale mul-

Table 5 Literature review

Ref.	Year	Cases
Franchello *et al*[46]	2005	3
Jiang *et al*[34]	2011	6
Loggi *et al*[37]	2012	10
Saidi *et al*[48]	2013	92
Choi *et al*[49]	2013	8
Li *et al*[39]	2013	78
Ju *et al*[50]	2013	23
Yu *et al*[51]	2014	42
Krishnamoorthi *et al*[47]	2014	28
Jeng *et al*[52]	2015	14

ticenter studies are required to confirm our findings.

REFERENCES

1 **Tejeda-Maldonado J**, García-Juárez I, Aguirre-Valadez J, González-Aguirre A, Vilatobá-Chapa M, Armengol-Alonso A, Escobar-Penagos F, Torre A, Sánchez-Ávila JF, Carrillo-Pérez DL. Diagnosis and treatment of hepatocellular carcinoma: An update. *World J Hepatol* 2015; **7**: 362-376 [PMID: 25848464 DOI: 10.4254/wjh.v7.i3.362]

2 **Dodson SF**, de Vera ME, Bonham CA, Geller DA, Rakela J, Fung JJ. Lamivudine after hepatitis B immune globulin is effective in preventing hepatitis B recurrence after liver transplantation. *Liver Transpl* 2000; **6**: 434-439 [PMID: 10915164 DOI: 10.1053/jlts.2000.6446]

3 **Schiff E**, Lai CL, Hadziyannis S, Neuhaus P, Terrault N, Colombo M, Tillmann H, Samuel D, Zeuzem S, Villeneuve JP, Arterburn S, Borroto-Esoda K, Brosgart C, Chuck S. Adefovir dipivoxil for wait-listed and post-liver transplantation patients with lamivudine-resistant hepatitis B: final long-term results. *Liver Transpl* 2007; **13**: 349-360 [PMID: 17326221 DOI: 10.1002/lt.20981]

4 **Ueda Y**, Marusawa H, Kaido T, Ogura Y, Ogawa K, Yoshizawa A, Hata K, Fujimoto Y, Nishijima N, Chiba T, Uemoto S. Efficacy and safety of prophylaxis with entecavir and hepatitis B immunoglobulin in preventing hepatitis B recurrence after living-donor liver transplantation. *Hepatol Res* 2013; **43**: 67-71 [PMID: 22548744 DOI: 10.1111/j.1872-034X.2012.01020.x]

5 **Kim WR**, Terrault NA, Pedersen RA, Therneau TM, Edwards E, Hindman AA, Brosgart CL. Trends in waiting list registration for liver transplantation for viral hepatitis in the United States. *Gastroenterology* 2009; **137**: 1680-1686 [PMID: 19632234 DOI: 10.1053/j.gastro.2009.07.047]

6 **Garg H**, Sarin SK, Kumar M, Garg V, Sharma BC, Kumar A. Tenofovir improves the outcome in patients with spontaneous reactivation of hepatitis B presenting as acute-on-chronic liver failure. *Hepatology* 2011; **53**: 774-780 [PMID: 21294143 DOI: 10.1002/hep.24109]

7 **Dao DY**, Seremba E, Ajmera V, Sanders C, Hynan LS, Lee WM. Use of nucleoside (tide) analogues in patients with hepatitis B-related acute liver failure. *Dig Dis Sci* 2012; **57**: 1349-1357 [PMID: 22198704 DOI: 10.1007/s10620-011-2013-3]

8 **Rosengard BR**, Feng S, Alfrey EJ, Zaroff JG, Emond JC, Henry ML, Garrity ER, Roberts JP, Wynn JJ, Metzger RA, Freeman RB, Port FK, Merion RM, Love RB, Busuttil RW, Delmonico FL. Report of the Crystal City meeting to maximize the use of organs recovered from the cadaver donor. *Am J Transplant* 2002; **2**: 701-711 [PMID: 12243491]

9 **Zaroff JG**, Rosengard BR, Armstrong WF, Babcock WD, D'Alessandro A, Dec GW, Edwards NM, Higgins RS, Jeevanandum V, Kauffman M, Kirklin JK, Large SR, Marelli D, Peterson TS, Ring WS, Robbins RC, Russell SD, Taylor DO, Van Bakel A, Wallwork J, Young JB. Consensus conference report: maximizing use of organs recovered from the cadaver donor: cardiac recommendations, March 28-29, 2001, Crystal City, Va. *Circulation* 2002; **106**: 836-841 [PMID: 12176957]

10 **Freeman RB**, Edwards EB. Liver transplant waiting time does not correlate with waiting list mortality: implications for liver allocation policy. *Liver Transpl* 2000; **6**: 543-552 [PMID: 10980052 DOI: 10.1053/jlts.2000.9744]

11 **Brandsaeter B**, Höckerstedt K, Friman S, Ericzon BG, Kirkegaard P, Isoniemi H, Olausson M, Broome U, Schmidt L, Foss A, Bjøro K. Fulminant hepatic failure: outcome after listing for highly urgent liver transplantation-12 years experience in the nordic countries. *Liver Transpl* 2002; **8**: 1055-1062 [PMID: 12424720 DOI: 10.1053/jlts.2002.35556]

12 **Venettoni S**, Grigioni W, Grossi P, Gianelli Castiglione A, Nanni Costa A. Criteria and terms for certified suitability of organ donors: assumptions and operational strategies in Italy. *Ann Ist Super Sanita* 2007; **43**: 279-286 [PMID: 17938459]

13 **Busuttil RW**, Goss JA. Split liver transplantation. *Ann Surg* 1999; **229**: 313-321 [PMID: 10077042]

14 **Miller C**, Florman S, Kim-Schluger L, Lento P, De La Garza J, Wu J, Xie B, Zhang W, Bottone E, Zhang D, Schwartz M. Fulminant and fatal gas gangrene of the stomach in a healthy live liver donor. *Liver Transpl* 2004; **10**: 1315-1319 [PMID: 15376309 DOI: 10.1002/lt.20227]

15 **Miller CM**. Regulation and oversight of adult living donor liver transplantation. *Liver Transpl* 2003; **9**: S69-S72 [PMID: 14528433 DOI: 10.1053/jlts.2003.50220]

16 **Bernat JL**, D'Alessandro AM, Port FK, Bleck TP, Heard SO, Medina J, Rosenbaum SH, Devita MA, Gaston RS, Merion RM, Barr ML, Marks WH, Nathan H, O'connor K, Rudow DL, Leichtman AB, Schwab P, Ascher NL, Metzger RA, Mc Bride V, Graham W, Wagner D, Warren J, Delmonico FL. Report of a National Conference on Donation after cardiac death. *Am J Transplant* 2006; **6**: 281-291 [PMID: 16426312 DOI: 10.1111/j.1600-6143.2005.01194.x]

17 **Busuttil RW**, Tanaka K. The utility of marginal donors in liver transplantation. *Liver Transpl* 2003; **9**: 651-663 [PMID: 12827549 DOI: 10.1053/jlts.2003.50105]

18 **Renz JF**, Kin C, Kinkhabwala M, Jan D, Varadarajan R, Goldstein M, Brown R, Emond JC. Utilization of extended donor criteria liver allografts maximizes donor use and patient access to liver transplantation. *Ann Surg* 2005; **242**: 556-563; discussion 563-565 [PMID: 16192816]

19 **Mor E**, Klintmalm GB, Gonwa TA, Solomon H, Holman MJ, Gibbs JF, Watemberg I, Goldstein RM, Husberg BS. The use of marginal donors for liver transplantation. A retrospective study of 365 liver donors. *Transplantation* 1992; **53**: 383-386 [PMID: 1738933]

20 **Wall WJ**, Mimeault R, Grant DR, Bloch M. The use of older donor livers for hepatic transplantation. *Transplantation* 1990; **49**: 377-381 [PMID: 2305468]

21 **Ballarin R**, Cucchetti A, Spaggiari M, Montalti R, Di Benedetto F, Nadalin S, Troisi RI, Valmasoni M, Longo C, De Ruvo N, Cautero N, Cillo U, Pinna AD, Burra P, Gerunda GE. Long-term follow-up and outcome of liver transplantation from anti-hepatitis C virus-positive donors: a European multicentric case-control study. *Transplantation* 2011; **91**: 1265-1272 [PMID: 21478815 DOI: 10.1097/TP.0b013e318219eb8f]

22 **Marroquin CE**, Marino G, Kuo PC, Plotkin JS, Rustgi VK, Lu AD, Edwards E, Taranto S, Johnson LB. Transplantation of hepatitis C-positive livers in hepatitis C-positive patients is equivalent to transplanting hepatitis C-negative livers. *Liver Transpl* 2001; **7**: 762-768 [PMID: 11552208 DOI: 10.1053/jlts.2001.27088]

23 **Saab S**, Chang AJ, Comulada S, Geevarghese SK, Anselmo RD, Durazo F, Han S, Farmer DG, Yersiz H, Goldstein LI, Ghobrial RM, Busuttil RW. Outcomes of hepatitis C- and hepatitis B core antibody-positive grafts in orthotopic liver transplantation. *Liver Transpl* 2003; **9**: 1053-1061 [PMID: 14526400 DOI: 10.1053/jlts.2003.50208]

24 **Saab S**, Ghobrial RM, Ibrahim AB, Kunder G, Durazo F, Han S, Farmer DG, Yersiz H, Goldstein LI, Busuttil RW. Hepatitis C positive grafts may be used in orthotopic liver transplantation: a matched analysis. *Am J Transplant* 2003; **3**: 1167-1172 [PMID: 12919097]

25 **Vargas HE**, Laskus T, Wang LF, Lee R, Radkowski M, Dodson F, Fung JJ, Rakela J. Outcome of liver transplantation in hepatitis C virus-infected patients who received hepatitis C virus-infected grafts.

Gastroenterology 1999; **117**: 149-153 [PMID: 10381921]

26   **Lavanchy D**. Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. *J Viral Hepat* 2004; **11**: 97-107 [PMID: 14996343]

27   **Donataccio D**, Roggen F, De Reyck C, Verbaandert C, Bodeus M, Lerut J. Use of anti-HBc positive allografts in adult liver transplantation: toward a safer way to expand the donor pool. *Transpl Int* 2006; **19**: 38-43 [PMID: 16359375 DOI: 10.1111/j.1432-2277.2005.00225.x]

28   **Jain A**, Orloff M, Abt P, Kashyap R, Mohanka R, Lansing K, Kelley M, Bozorgzadeh A. Use of hepatitis B core antibody-positive liver allograft in hepatitis C virus-positive and -negative recipients with use of short course of hepatitis B immunoglobulin and Lamivudine. *Transplant Proc* 2005; **37**: 3187-3189 [PMID: 16213345 DOI: 10.1016/j.transproceed.2005.07.049]

29   **Clements CJ**, Baoping Y, Crouch A, Hipgrave D, Mansoor O, Nelson CB, Treleaven S, van Konkelenberg R, Wiersma S. Progress in the control of hepatitis B infection in the Western Pacific Region. *Vaccine* 2006; **24**: 1975-1982 [PMID: 16361001 DOI: 10.1016/j.vaccine.2005.11.035]

30   **Bahde R**, Hölzen JP, Wolters HH, Schmidt HH, Bock CT, Lügering A, Spieker T, Senninger N, Brockmann JG. Course of a HBsAg positive liver transplantation in a hepatitis B and D virus coinfected recipient. *Ann Hepatol* 2011; **10**: 355-360 [PMID: 21677340]

31   **González-Peralta RP**, Andres JM, Tung FY, Fang JW, Brunson ME, Davis GL, Lau JY. Transplantation of a hepatitis B surface antigen-positive donor liver into a hepatitis B virus-negative recipient. *Transplantation* 1994; **58**: 114-116 [PMID: 8036699]

32   **Ho JK**, Harrigan PR, Sherlock CH, Steinbrecher UP, Erb SR, Mo T, Chung SW, Buczkowski AK, Intaraprasong P, Scudamore CH, Yoshida EM. Utilization of a liver allograft from a hepatitis B surface antigen positive donor. *Transplantation* 2006; **81**: 129-131 [PMID: 16421489]

33   **Hwang S**, Lee SG, Park KM, Kim KH, Ahn CS, Oh HB, Moon DB, Ha TY, Lim YS, Jung DH. Five-year follow-up of a hepatitis B virus-positive recipient of hepatitis B surface antigen-positive living donor liver graft. *Liver Transpl* 2006; **12**: 993-997 [PMID: 16721765 DOI: 10.1002/lt.20799]

34   **Jiang L**, Yan L, Li B, Wen T, Zhao J, Jiang L, Yang J, Xu M, Wang W. Successful use of hepatitis B surface antigen-positive liver grafts in recipients with hepatitis B virus-related liver diseases. *Liver Transpl* 2011; **17**: 1236-1238 [PMID: 21748846 DOI: 10.1002/lt.22379]

35   **Jiao Z**, Zhang Y, Han L, Zeng Y, Yan L. Four-year follow-up of two chronic hepatitis B recipients of hepatitis B surface antigen-positive cadaveric liver grafts from asymptomatic carriers. *Hepatol Res* 2011; **41**: 846-852 [PMID: 21883736 DOI: 10.1111/j.1872-034X.2011.00840.x]

36   **Loggi E**, Bihl F, Chisholm JV, Biselli M, Bontadini A, Vitale G, Ercolani G, Grazi GL, Pinna AD, Bernardi M, Brander C, Andreone P. Anti-HBs re-seroconversion after liver transplantation in a patient with past HBV infection receiving a HBsAg positive graft. *J Hepatol* 2009; **50**: 625-630 [PMID: 19157623 DOI: 10.1016/j.jhep.2008.08.026]

37   **Loggi E**, Micco L, Ercolani G, Cucchetti A, Bihl FK, Grazi GL, Gitto S, Bontadini A, Bernardi M, Grossi P, Costa AN, Pinna AD, Brander C, Andreone P. Liver transplantation from hepatitis B surface antigen positive donors: a safe way to expand the donor pool. *J Hepatol* 2012; **56**: 579-585 [PMID: 22027583 DOI: 10.1016/j.jhep.2011.09.016]

38   **Soejima Y**, Shimada M, Taketomi A, Yoshizumi T, Uchiyama H, Ikegami T, Nakamuta M, Maehara Y. Successful living donor liver transplantation using a graft from a hepatitis B surface antigen-positive donor. *Liver Int* 2007; **27**: 1282-1286 [PMID: 17919241 DOI: 10.1111/j.1478-3231.2007.01528.x]

39   **Li Z**, Hu Z, Xiang J, Zhou J, Yan S, Wu J, Zhou L, Zheng S. Use of hepatitis B surface antigen-positive grafts in liver transplantation: a matched analysis of the US National database. *Liver Transpl* 2014; **20**: 35-45 [PMID: 24142889 DOI: 10.1002/lt.23774]

40   **Samuel D**, Muller R, Alexander G, Fassati L, Ducot B, Benhamou JP, Bismuth H. Liver transplantation in European patients with the hepatitis B surface antigen. *N Engl J Med* 1993; **329**: 1842-1847 [PMID: 8247035 DOI: 10.1056/NEJM199312163292503]

41   **Terrault NA**, Zhou S, Combs C, Hahn JA, Lake JR, Roberts JP, Ascher NL, Wright TL. Prophylaxis in liver transplant recipients using a fixed dosing schedule of hepatitis B immunoglobulin. *Hepatology* 1996; **24**: 1327-1333 [PMID: 8938155 DOI: 10.1002/hep.510240601]

42   **Dienstag JL**, Perrillo RP, Schiff ER, Bartholomew M, Vicary C, Rubin M. A preliminary trial of lamivudine for chronic hepatitis B infection. *N Engl J Med* 1995; **333**: 1657-1661 [PMID: 7477217 DOI: 10.1056/NEJM199512213332501]

43   **Han SH**, Ofman J, Holt C, King K, Kunder G, Chen P, Dawson S, Goldstein L, Yersiz H, Farmer DG, Ghobrial RM, Busuttil RW, Martin P. An efficacy and cost-effectiveness analysis of combination hepatitis B immune globulin and lamivudine to prevent recurrent hepatitis B after orthotopic liver transplantation compared with hepatitis B immune globulin monotherapy. *Liver Transpl* 2000; **6**: 741-748 [PMID: 11084061 DOI: 10.1053/jlts.2000.18702]

44   **Marzano A**, Salizzoni M, Debernardi-Venon W, Smedile A, Franchello A, Ciancio A, Gentilcore E, Piantino P, Barbui AM, David E, Negro F, Rizzetto M. Prevention of hepatitis B virus recurrence after liver transplantation in cirrhotic patients treated with lamivudine and passive immunoprophylaxis. *J Hepatol* 2001; **34**: 903-910 [PMID: 11451175]

45   **Zheng S**, Chen Y, Liang T, Lu A, Wang W, Shen Y, Zhang M. Prevention of hepatitis B recurrence after liver transplantation using lamivudine or lamivudine combined with hepatitis B Immunoglobulin prophylaxis. *Liver Transpl* 2006; **12**: 253-258 [PMID: 16447195 DOI: 10.1002/lt.20701]

46   **Franchello A**, Ghisetti V, Marzano A, Romagnoli R, Salizzoni M. Transplantation of hepatitis B surface antigen-positive livers into hepatitis B virus-positive recipients and the role of hepatitis delta coinfection. *Liver Transpl* 2005; **11**: 922-928 [PMID: 16035057 DOI: 10.1002/lt.20471]

47   **Krishnamoorthi R**, Manickam P, Cappell MS. Liver transplantation of hepatitis B surface antigen positive donors to hepatitis B core antibody recipients: analysis of 27 patients. *Minerva Gastroenterol Dietol* 2014; **60**: 113-118 [PMID: 24780945]

48   **Saidi RF**, Jabbour N, Shah SA, Li YF, Bozorgzadeh A. Liver transplantation from hepatitis B surface antigen-positive donors. *Transplant Proc* 2013; **45**: 279-280 [PMID: 23267801 DOI: 10.1016/j.transproceed.2012.05.077]

49   **Choi Y**, Choi JY, Yi NJ, Lee K, Mori S, Hong G, Kim H, Park MS, Yoo T, Suh SW, Lee HW, Lee KW, Suh KS. Liver transplantation for HBsAg-positive recipients using grafts from HBsAg-positive deceased donors. *Transpl Int* 2013; **26**: 1173-1183 [PMID: 24131436 DOI: 10.1111/tri.12177]

50   **Ju W**, Chen M, Guo Z, Wang D, Zhu X, Huang J, He X. Allografts positive for hepatitis B surface antigen in liver transplant for disease related to hepatitis B virus. *Exp Clin Transplant* 2013; **11**: 245-249 [PMID: 23176583 DOI: 10.6002/ect.2012.0095]

51   **Yu S**, Yu J, Zhang W, Cheng L, Ye Y, Geng L, Yu Z, Yan S, Wu L, Wang W, Zheng S. Safe use of liver grafts from hepatitis B surface antigen positive donors in liver transplantation. *J Hepatol* 2014; **61**: 809-815 [PMID: 24824283 DOI: 10.1016/j.jhep.2014.05.003]

52   **Jeng LB**, Thorat A, Yang HR, Yeh CC, Chen TH, Hsu CH, Hsu SC, Poon KS, Li PC, Lai HC, Su WP, Peng CY. Successful use of hepatitis B surface antigen-positive liver grafts - an effective source for donor organs in endemic areas: a single-center experience. *Ann Transplant* 2015; **20**: 103-111 [PMID: 25703063 DOI: 10.12659/AOT.893032]

REVIEW

Regulation of intestinal permeability: The role of proteases

Hanne Van Spaendonk, Hannah Ceuleers, Leonie Witters, Eveline Patteet, Jurgen Joossens, Koen Augustyns, Anne-Marie Lambeir, Ingrid De Meester, Joris G De Man, Benedicte Y De Winter

Hanne Van Spaendonk, Hannah Ceuleers, Leonie Witters, Eveline Patteet, Joris G De Man, Benedicte Y De Winter, Laboratory of Experimental Medicine and Pediatrics, Division of Gastroenterology, University of Antwerp, 2610 Antwerp, Belgium

Jurgen Joossens, Koen Augustyns, Laboratory of Medicinal Chemistry and Antwerp Drug Discovery Network, University of Antwerp, 2610 Antwerp, Belgium

Anne-Marie Lambeir, Ingrid De Meester, Laboratory of Medical Biochemistry, University of Antwerp, 2610 Antwerp, Belgium

Abstract

The gastrointestinal barrier is - with approximately 400 m^2 - the human body's largest surface separating the external environment from the internal milieu. This barrier serves a dual function: permitting the absorption of nutrients, water and electrolytes on the one hand, while limiting host contact with noxious luminal antigens on the other hand. To maintain this selective barrier, junction protein complexes seal the intercellular space between adjacent epithelial cells and regulate the paracellular transport. Increased intestinal permeability is associated with and suggested as a player in the pathophysiology of various gastrointestinal and extra-intestinal diseases such as inflammatory bowel disease, celiac disease and type 1 diabetes. The gastrointestinal tract is exposed to high levels of endogenous and exogenous proteases, both in the lumen and in the mucosa. There is increasing evidence to suggest that a dysregulation of the protease/antiprotease balance in the gut contributes to epithelial damage and increased permeability. Excessive proteolysis leads to direct cleavage of intercellular junction proteins, or to opening of the junction proteins *via* activation of protease activated receptors. In addition, proteases regulate the activity and availability of cytokines and growth factors, which are also known modulators of intestinal permeability. This review aims at outlining the mechanisms by which proteases alter the intestinal permeability. More knowledge on the role of proteases in mucosal homeostasis and gastrointestinal barrier function will definitely contribute to the identification

of new therapeutic targets for permeability-related diseases.

Key words: Intestinal permeability; Intestinal barrier; Tight junction; Paracellular permeability; Proteases; Proteinase-activated receptor; Protease inhibitor; Antiproteases

Core tip: Increased intestinal permeability is a novel player in the pathophysiology of various intestinal and extra-intestinal diseases such as inflammatory bowel disease, celiac disease and type 1 diabetes. A dysregulated protease/antiproteases balance is suggested as a cause of intestinal barrier dysfunction, with a subsequent increase in permeability. Immune cells infiltrating in the lamina propria during inflammatory conditions provide a pro-inflammatory environment by the production of cytokines and proteases. Protease inhibition has therapeutic potential but more research is needed to elucidate the exact involvement of specific proteases in gut physiology and intestinal barrier function.

INTRODUCTION

The intestinal barrier represents the largest interface between the external environment and the internal milieu. Given the enormous intraluminal load of essential and noxious molecules, a selectively per-meable barrier is indispensable for maintaining mucosal homeostasis[1]. The intestinal barrier serves a dual function: on the one hand limiting host contact with pathogens and antigens and on the other hand at the same time allowing the absorption of nutrients and water. Physical, biochemical and immune elements make up the heterogeneous intestinal barrier and collaborate to exert these functions. Firstly, the mucus layer covers the entire epithelial cell surface and consists of gel-forming mucins, produced by the goblet cells. This chemical barrier also contains defensins or antimicrobial peptides that are secreted by Paneth cells within the epithelial cell layer[2]. Secondly, the epithelial cell layer itself is a physical barrier that consists for 80% of enterocytes, regulating nutrient absorption *via* specific transporters, channels and receptors (transcellular transport)[3]. Finally, the immunological barrier consists of microfold (M) cells in the epithelial

cell layer and patrolling antigen presenting cells (APC) in the lamina propria. The M cells constantly sample luminal antigens and deliver them to APC such as dendritic cells and macrophages. Innocuous antigens drive the APCs to create a tolerogenic environment with the production of immunosuppressive factors such as IL-10, TGF-β and nitric oxide. Further tolerance is thereby created through the induction of regulatory T cells[4]. Noxious antigens are also recognized by the APCs and trigger the activation of the inflammatory cascade, starting with T cell activation[5,6].

The movement of molecules, solutes and ions across the intestinal epithelial cell layer can take place by the trans- or paracellular pathway. Transcellular transport is the main route for nutrient absorption and is facilitated through size- and charge- selective channels and transporters. The paracellular pathway is less selective since it occurs through the intercellular space between neighboring intestinal epithelial cells. The capacity of this paracellular pathway is however low as cells are bound tightly together by junction proteins, with particularly the tight junctions (TJ) regulating transport in response to numerous stimuli[7].

In the last decade, a barrier defect is suggested as a common factor in the onset of various local and systemic diseases of an inflammatory, autoimmune or functional nature such as inflammatory bowel disease (IBD), celiac disease, irritable bowel syndrome (IBS), type 1 diabetes mellitus and multiple sclerosis[8-13]. For more detailed information on the relation between intestinal barrier function and these disease pathologies, we refer the readers to Odenwald and Turner who nicely reviewed this topic in 2013[14] and very recently updated their overview in 2016[15]. Although the literature data are rather scarce, proteases are believed to regulate the intestinal permeability. They can intervene directly by their proteolytic action on the junction proteins, both intra- and extracellularly, and indirectly through activation of proteinase-activated receptors (PARs). This review provides an overview of the proteases (Table 1) putting emphasis on their role as regulators of the intestinal paracellular permeability.

INTESTINAL TIGHT JUNCTIONS REGULATE PARACELLULAR PERMEABILITY

The intercellular spaces between neighboring intestinal epithelial cells are sealed by the apical junction complex, which contains TJs and adherens junctions, and by the subjacent desmosomes (Figure 1). The selective paracellular permeability is mediated by the TJs, which encircle the apical end of the intercellular spaces[7]. Various proteins make up TJs, including the adhesive transmembrane proteins occludin, claudins and junctional adhesion molecules as well as cytoplasmic proteins such as zonula occludens

Table 1 Overview of proteases affecting intestinal permeability

Protease	Effect	Model	Mechanism of action	Ref.
Serine proteases				
Matriptase	Protective	ST14 hypomorphic mice	Genetic depletion of matriptase induces an increase in intestinal permeability (decreased TER and increased FITC-dextran flux)	[65, 66]
		Epithelial cell monolayer	Inhibition of matriptase with silencing RNA and the synthetic inhibitor MI-432 increased the intestinal permeability (decreased TER and increased FITC-dextran flux)	[65, 68]
Granzyme M	Protective	GrzM$^{-/-}$ mice	GrzM$^{-/-}$ mice display a permeability increase (FITC-dexran method)	[73]
Zonulin, Zonula occludens toxin (Zot)	Harmful	Human epithelial cell monolayer	↑ Permeability after exposure to gliadin (triggers zonulin release; disruption of occludin and ZO-1)	[76]
		Ileal tissue of diabetes prone rats	Zonulin-dependent permeability increase in diabetic rats was abolished after oral treatment with zonulin inhibitor FZI/0 (AT1001/Larazotide)	[82]
PAR$_2$ activation				
Trypsin, tryptase, chymase, synthetic SLIGRL	Harmful	WT mice, WT rats	↑ Permeability due to PAR$_2$ activation (confirmed by selective PAR$_2$ agonist SLIGRL; increased ^{51}Cr-EDTA flux)	[47, 48, 51]
PAR$_4$ activation				
Cathepsin G	Harmful	Colonic biopsies from UC and healthy patients	↑ Permeability in response to UC fecal supernatant was abolished by cathepsin G inhibition	[58]
PAR$_1$ activation				
Thrombin, synthetic TFLLR-NH2	Harmful	WT mice, epithelial cell monolayer	↑ Permeability after PAR$_1$ activation (caspase-3 mediated; disruption of ZO-1)	[62]
Endogenous inhibitors				
Elafin	Protective	Gluten sensitive mice	↓ Permeability after elafin delivery by recombinant *Lactococcus lactis* (^{51}Cr-EDTA flux)	[87]
		Human epithelial cell monolayer	Treatment with elafin normalized the TNF-α-induced increase in paracellular permeability (FITC-dextran method)	[88]
Synthetic inhibitors				
Camostat mesilate	Protective	Rat IBS model	Treatment with camostat mesilate normalized the elevated permeability in the rats (^{51}Cr-EDTA flux and ZO-1 expression)	[89]
Nafamostat mesilate	Protective	Rectal biopsies from IBS and healthy patients	Nafamostat abolished the trypsin-induced hyperpermeability (macromolecular flux in Ussing chambers)	[94]
		Human epithelial cell monolayer	Treatment with nafamostat normalized the tryptase-induced permeability increase (TER and FITC-dextran method)	[95]
SPI	Protective	IBD mouse model	Treatment with SPI normalized the increased permeability in the T-cell transfer colitis model (FITC-dextran method)	[96]
Metalloproteases				
Meprin β	Protective	Mep1b$^{-/-}$ mice	Meprin β cleaves MUC2 and alters mucus composition	[128, 129]
Matrix metalloproteinases				
MMP-2	Protective	MMP-2$^{-/-}$ mice	↑ permeability in MMP-2$^{-/-}$ mice (FITC-dextran method)	[111]
MMP-9	Harmful	MMP-9$^{-/-}$ mice	= Permeability in MMP-9$^{-/-}$ mice after DSS (FITC-dextran method; no increase in MLCK expression)	[114]
		MMP-9$^{-/-}$ mice	↑ Goblet cells and MUC2 expression in MMP-9$^{-/-}$ mice	[113]
		MMP-9 transgenic mice	↑ Permeability in mice overexpressing MMP-9 (FITC-dextran method)	[112]

MMP-3, MMP-7	Harmful	Epithelial cell culture	MMP-7 cleaves E-cadherin	[121]
ADAM				
TACE/ADAM17	Harmful	Human and mouse colon samples	↑ TACE activity in IBD; ↑ TNF-α release; ↑ TNF-α-induced permeability increase	[131, 134, 135]
		Caco-2	↓ Permeability after TACE inhibition (by TAPI-2 and GM6001)	[136]
Cysteine proteases				
Caspase-3, caspase-8	Harmful	Human epithelial cell monolayer	↓ Cell-cell adhesion (epithelial cell apoptosis; disruption of TJ proteins occludin and claudin-4)	[144]
Endogenous inhibitor				
Cystatin	No effect	WT mice	No effect on colonic paracellular permeability (^{51}Cr-EDTA flux)	[51]
Luminal proteases				
Bacteroides fragilis				
Fragilysin	Harmful	Human epithelial cell monolayer	↑ Permeability (decreased TER and increase in mannitol flux)	[149, 150]
Entamoeba histolytica				
Cysteine protease	Harmful	Mice transfected with E. histolytica trophozoites	↑ Permeability (FITC-dextran method)	[151]
Enterococcus faecalis				
Gelatinases	Harmful	IL10$^{-/-}$ mice	↑ Permeability (E-cadherin splicing)	[156]
		Epithelial cell monolayers	↑ Permeability (PAR2 signaling)	[155]
Dermatophagoides pteronyssinus				
Der p 1	Harmful	Human colonic biopsies	↑ Permeability (decreased TER in Ussing chambers; disruption of TJ proteins occludin and ZO-1	[158]
Kiwifruit cysteine protease				
Act d1	Harmful	Epithelial cell monolayer	↑ Permeability (disruption of TJ proteins occludin and ZO-1)	[162]
		WT mice	↑ Permeability (FITC-dextran method)	[161]
Aspergillus				
Amano SD	Protective	WT rat	Improved mucosal homeostasis through alteration of the microbiome composition and SCFA induction	[163]

TJ: Tight junction; PARs: Proteinase-activated receptors; MLC: Myosin light chain; MLCK: Myosin light chain kinase; PKC: Protein kinase C; ROCK: Rho-associated protein kinase; ZO-1: Zonula occludens 1.

(ZO) proteins (Figure 2). The latter act as scaffolding proteins that connect the transmembrane proteins at their cytoplasmic C-terminal strands with F-actin, a filamentous cytoskeleton component[16]. The adherens junction transmembrane protein, epithelial-cadherin (E-cadherin), is connected to F-actin *via* intracellular proteins of the catenin-family[17].

The opening of the intercellular spaces is achieved by contraction of the actomyosin microfilaments. Myosin is a motor protein that co-localizes with F-actin and converts chemical energy from adenosine triphosphate into mechanical energy. A crucial step in the induction of this mechanochemical contractile machinery is the phosphorylation of myosin light chain (MLC), the regulatory component of myosin (Figure 2). Myosin light chain kinase (MLCK) mediates the phosphorylation of MLC upon activation in response to Ca^{2+}/calmodulin binding. However, there is evidence for other intracellular signaling pathways besides the calmodulin pathway to activate MLCK. The extracellular signal-regulated kinases (ERK1/2) have shown to induce MLCK activation[18]. Protein kinase C (PKC) on the other hand favors the phosphorylation of MLC by the inhibition of myosin light chain phosphatase (MLCP), the enzyme that dephosphorylates MLC[19].

Rho-associated protein kinase (ROCK) can increase contractility both by activating MLCK and inactivating MLCP, favoring MLC phosphorylation[20]. The phosphorylation status of myosin light chain induces a change in myosin tertiary structure causing myosin to "walk" along the actin filaments, increasing the tension in the cytoskeleton resulting in the disruption and cytosolic migration of TJ proteins[21,22]. This results in an impaired barrier function which is also referred to as a "leaky" barrier. Potentially noxious luminal proteins can now migrate to the underlying mucosal tissue and provoke a pro-inflammatory response. Even whole bacteria can cross the epithelial cells unrestricted at sites of epithelial damage caused by erosions and ulcers in GI disease. A "leaky" gut and epithelial damage often co-exist in disease state[14].

MUCOSAL IMMUNOLOGY AND BARRIER FUNCTION

In both physiological and pathological conditions, various mediators are able to affect the TJ conformation in order to control the paracellular permeability in epi- and endothelial cell layers throughout the body. Growth factors, cytokines, intestinal bacteria, dietary
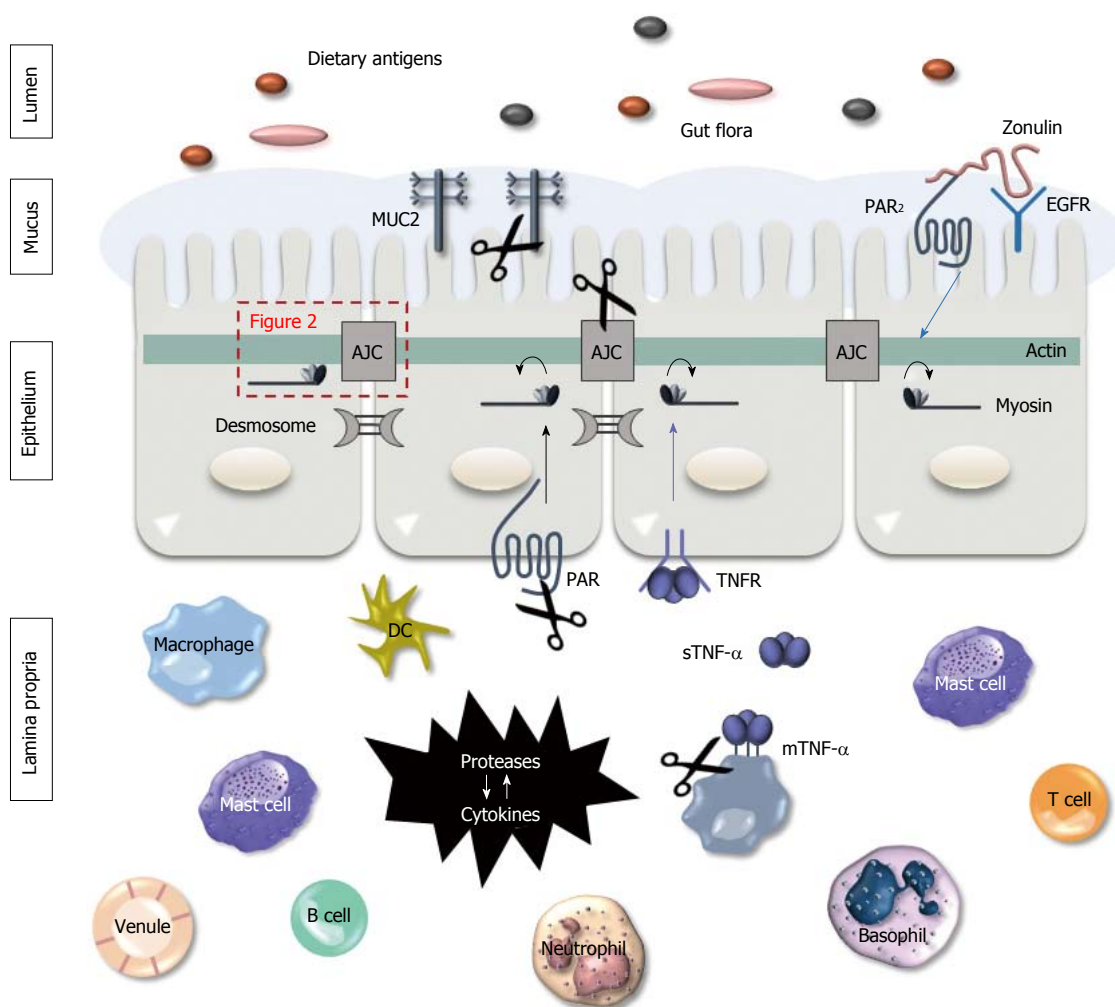
Figure 1 Proteases mediate gut barrier function. Intestinal epithelial cells are constantly exposed to proteases, both on their apical and basolateral side. Luminal proteases can be endogenous (*e.g.*, pancreatic proteases) or can originate form bacteria or food particles present in the lumen. Their proteolytic activity can cause damage to the mucus layer and the junction proteins, affecting the barrier function. In the lamina propria, proteases are produced by various inflammatory cells and by the intestinal epithelial cells. In inflammatory conditions such as inflammatory bowel disease (IBD), immune cells infiltrate in the lamina propria where they produce various cytokines and proteases, contributing to the pro-inflammatory environment. Proteases stimulate immune cells to produce cytokines and vice versa. Besides, they alter the paracellular permeability by direct proteolytic cleaving of the junction proteins and by activation of the proteinase-activated receptors (PARs) on the epithelial cell surface, that induces a contraction of the actomyosin complex and subsequent opening of the apical junction complex (AJC; more in detail in Figure 2).

components and proteases are known to regulate the intestinal TJ opening[3,23-25]. Though the barrier-regulating capacity of pro-inflammatory cytokines is well studied, the effect of other mediators has received far less attention[24,26].

An inflamed mucosa -as seen in IBD patients- is characterized by the presence of cytokines amongst which TNF-α and IFN-γ, which are produced by a variety of cells including macrophages, T-cells and natural killer (NK) cells. The binding of these cytokines to specific receptors on the surface of infiltrating immune cells initiates a cascade of events starting with the activation of cell signaling pathways leading to the production of more inflammatory mediators (NFκB) or apoptosis maintaining on their turn the inflammatory process. Extensive reviews have been published on the regulation of TJs by cytokines[24,27]. It has been shown in cell culture experiments that

TNF-α and IFN-γ regulate the paracellular permeability through the activation of MLCK, resulting in MLC hyperphosphorylation and opening of the TJs[28-30], while IL-4 and IL-13 increase paracellular permeability through the induction of the pore-forming claudin-2 and apoptotic pathways[31-33].

Next to cytokines also proteases are released into the mucosa by inflammatory cells such as macrophages, neutrophils and mast cells to regulate inflammation. On the one hand these proteases degrade the extracellular matrix, mucosal proteins and even live bacteria[34]. On the other hand, proteases act as signaling molecules *via* specific receptors, which will be discussed in the next section.

Intestinal epithelial cells also express receptors for cytokine and protease signaling. Since the apical and basolateral membranes of the intestinal epithelial cells are constantly exposed to large amounts of bacterial
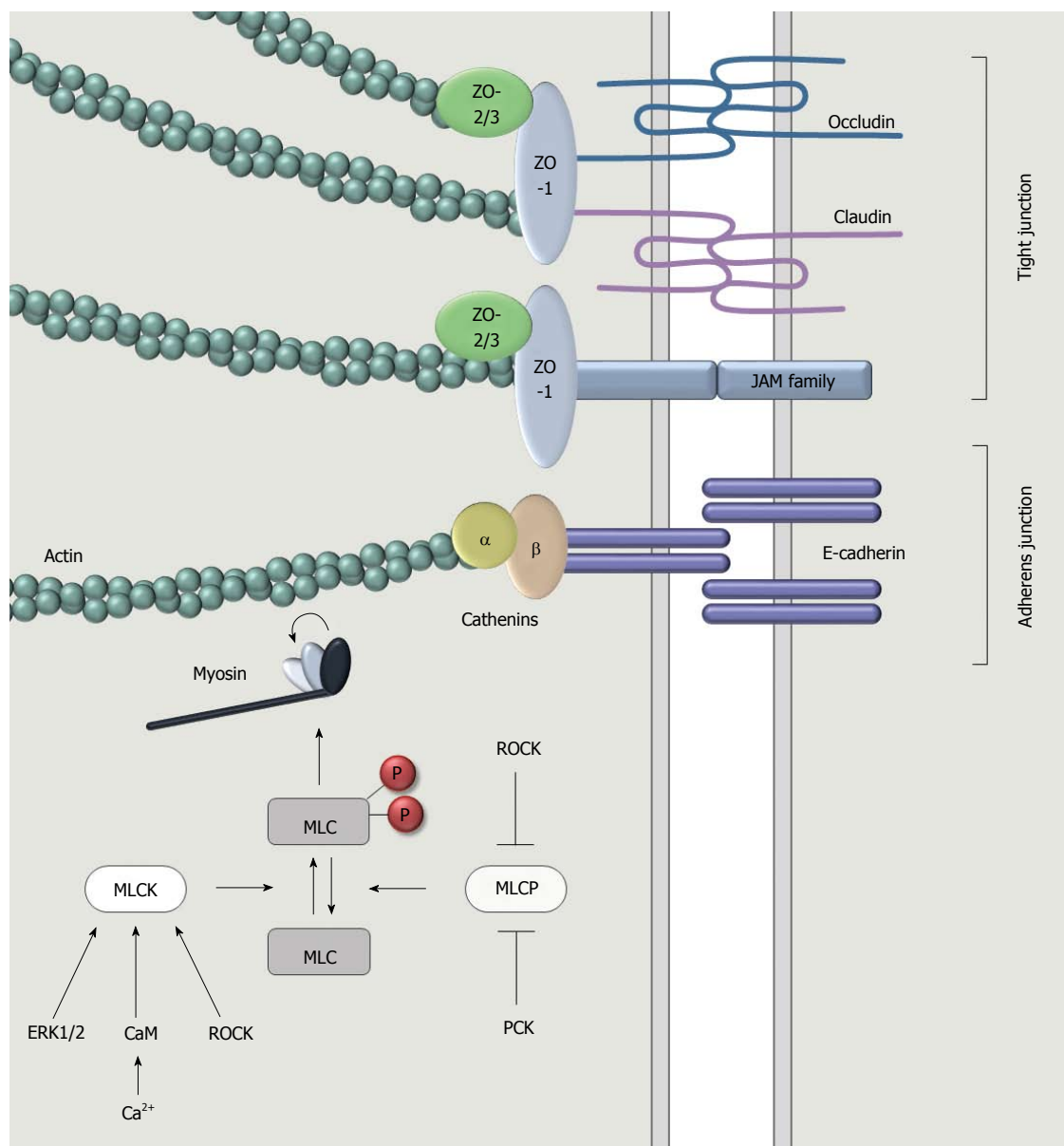
Figure 2 A more detailed representation of the apical junction complex at the intercellular surface between adjacent intestinal epithelial cells. Tight junctions are comprised of three types of transmembrane proteins: occludin, claudins and junctional adhesion molecules (JAMs). Adaptor proteins such as zonula occludens 1 (ZO-1), ZO-2 and ZO-3 connect the transmembrane proteins to filamentous actin. This cytoskeleton component interacts with myosin to induce a contraction, followed by the opening of the intercellular space. Myosin light chain (MLC) is the main regulator of this contractile machinery. Contraction occurs when MLC is phosphorylated. This is regulated through the activity of myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP), which is on their turn regulated by intracellular signaling pathways involving for instance the extracellular signal-regulated kinases (ERK1/2), calcium, calmodulin, protein kinase C (PKC) or Rho-associated protein kinase (ROCK).

and endogenous proteases, the function of these proteolytic enzymes in intestinal barrier homeostasis should be further elucidated.

PROTEASES AND PROTEINASE-ACTIVATED RECEPTORS

Defined in general manner, proteases are enzymes that hydrolyze a peptide bond and in this respect they are best known for their digestive properties *e.g.,* pancreatic proteases. However, also bacteria, epithelial cells, resident and infiltrating inflamma-tory cells produce luminal and mucosal proteases exerting various biological functions, both intra- and extracellularly. For instance, proteases are vital in inflammation, apoptosis, coagulation and cell growth and migration[35,36]. Since excessive proteolysis can cause tissue damage, a tight regulation of protease activity in order to prevent pathology is necessary. There are multiple mechanisms that control the protease activity such as the synthesis as inactive zymogens that require proteolytic cleaving for activation and on the other hand the termination of protease activity by endogenous inhibitors or

antiproteases. A dysregulation in the protease balance with an increased protease activity has been observed in gastrointestinal diseases such as IBD and IBS, making protease inhibition by endogenous or synthetic inhibitors a potential therapeutic intervention[37]. Proteases are classified based on their mechanism of hydrolysis of the target peptide bond. This implicates that all proteases that belong to the same clan, share the same nucleophilic amino acid in their active site and are more likely to react with the same inhibitors. In mammals, five classes of proteases have been identified: serine-, metallo-, cysteine-, aspartate- and threonine proteases.

Proteases are not merely degrading enzymes. They can also act as signaling molecules by the proteolytic activation of the PARs. Four receptors have been identified in this family (PAR$_1$, PAR$_2$, PAR$_3$, PAR$_4$)[38]. Activation of these G-protein coupled receptors occurs after the proteolytic truncation of the N-terminal extracellular tail, releasing a new N-terminus that functions as a tethered ligand. This specific domain binds the second of three extracellular loops on the receptor and thereby generates an intracellular signal. PARs are expressed ubiquitously among tissues and cell types. In the gut, they are present on epithelial cells, endothelial cells, neurons, inflammatory cells, mast cells, smooth muscle cells and fibroblasts. Depending on the cell type, different signaling pathways have been described[39]. This also implicates that proteases mediate different GI physiological processes such as motility, cell proliferation and apoptosis, immune response, cytokine production, neurogenic inflammation, pain and epithelial barrier function through PAR activation[40]. For a more detailed description of this topic, we refer the reader to the companion review in (WJG Dec. 2016) from our group, illustrating this topic in detail[41].

SERINE PROTEASES

Of all proteolytic enzymes, serine proteases are by far the most abundant group[42,43]. Their successful mechanism of hydrolysis of peptide bonds occurs throughout the entire body in functionally diverse processes including digestion, immune response, blood coagulation, fibrinolysis, apoptosis and pro-hormone processing[42]. Serine proteases act both directly and indirectly as paracrine signaling effectors on PARs, provoking intracellular signals in order to mediate these vital processes. During the inflammatory cascade for example, proteases are released by infiltrating inflammatory cells and modulate the bioactivity of cytokines and chemokines by proteolytic cleavage[37]. For instance, the N-terminal truncation of CXCL-8 and CXCL-5 respectively by proteinase 3 and cathepsin G provides an increased chemotactic activity towards neutrophils[44,45]. Most proteases involved in PAR

activation on the other hand belong to the serine clan of proteases[8]. In inflammatory cells, the activation of the G-protein coupled pathway leads to downstream activation and nuclear translocation of NFκB. In response, the cell synthesizes pro-inflammatory cytokines boosting inflammation[46]. In epithelial cells, paracrine signaling of proteases through PARs induces changes in paracellular permeability which will be discussed in the next paragraph.

The majority of the research in the field of intestinal permeability involves PAR$_2$. In 2002, it was shown for the first time by Coelho *et al*[47] and Cenac *et al*[48] that PAR$_2$ activation by the serine proteases trypsin, tryptase and chymase induced an increase in the colonic permeability of Cr^{51}-EDTA, a marker of paracellular permeability[49,50]. The selective synthetic PAR$_2$ agonist SLIGRL (H-serine-leucine-isoleucine-glycine-arginine-leucine-OH) mimicked the effect of endogenous serine proteases, thereby increasing intestinal permeability[47,51]. Although PAR$_2$ receptors are expressed on both apical and basolateral membranes of epithelial cells, some authors suggest that only apical administration of PAR$_2$ agonists - and not intraperitoneal (i.p.) administration- alters the intestinal barrier function[51]. Other authors however provided proof of direct basolateral PAR$_2$ activation and a subsequent increase in paracellular permeability[18,52]. Further investigation into the mechanism of action revealed the involvement of calmodulin and MLCK in the PAR$_2$-mediated alterations of paracellular permeability as intracolonic injection of SLIGRL increased the MLC phosphorylation on western blot. Pretreatment with ML-7, an MLCK inhibitor, abolished the elevated mucosal permeability caused by contraction of the epithelial cell cytoskeleton after phosphorylation of MLC[53]. In addition, MLCK was activated by the Ca^{2+}-binding messenger protein calmodulin since precipitation of MLCK revealed an increased binding of calmodulin and the inhibition of calmodulin by chlorpromazine reduced the SLIGRL-induced increase in paracellular permeability[54,55]. Besides the calmodulin pathway, also ERK1/2 can activate MLCK which directly leads to the disruption of TJ composition and function[18]. The increased permeability induced by tryptase in cultured colonocytes was not only abolished by a tryptase inhibitor but also by the ERK1/2 inhibitor UO126. Finally, incubation of a human intestinal epithelial cell line (SCBN) with SLIGRL induced the disruption and migration into the cytoplasm of the TJ protein ZO-1[55].

The latest discovered member of the PAR-family, PAR$_4$, is receiving increasing attention[56]. This is mainly due to the discovery that cathepsin G is a PAR$_4$-selective neutrophil serine protease. As cathepsin G is a neutrophil protease (alongside with proteinase 3 and neutrophil elastase) it might represent an inflammatory mediator in conditions such as IBD, where neutrophil accumulation within the submucosa is considered a hallmark[57]. And indeed, ulcerative colitis (UC) patients

express higher colonic levels of cathepsin G and PAR4, both involved in the increased paracellular permeability in UC patients[58]. This was proven by using the PAR4 inhibitor P4pal-10, a pepducin that selectively blocks PAR4 signaling[59]. In Ussing chamber experiments with mice colonic strips, fecal supernatant of UC and Crohn's disease (CD) patients triggered a significant increase in FITC dextran permeability abolished by the pre-treatment with P4pal-10 in strips triggered with UC supernatant but interestingly not for the strips triggered with the supernatant of CD patients[58]. In addition, the PAR4 activating peptide AYPGKF-NH2 was able to induce an increased mucosal permeability with a similar effect as the UC fecal supernatant[60]. Finally, pre-incubation of UC fecal supernatant with the specific cathepsin G inhibitor completely normalized the elevated permeability effect[58]. These data suggest that cathepsin G plays a predominant role in the pathophysiology of UC by activating PAR4, while this is not shown in CD. However there is a lack of studies investigating the expression of cathepsin G and comparing this expression between UC *vs* CD patients. Other proteases are likely to play a key role in barrier function in CD.

Also PAR1-signaling induces epithelial barrier dysfunction, but in this case apoptosis seems to mediate the phenomenon. Treatment with PAR1 agonists (thrombin and selective PAR1 activating peptide TFLLR-NH2) increased the paracellular per-meability *in vitro* in an epithelial cell line (SCBN) as well as *in vivo* in mice. This permeability increase depends on the disruption of ZO-1. Chromatin condensation and nuclear fragmentation, hallmarks for apoptosis, were induced in a caspase-3-de-pendent manner. Interestingly, pretreatment with a caspase-3 inhibitor (Z-DEVD-FMK), which irreversibly inhibits apoptosis[61], completely abolished the effect of the PAR1 agonists on permeability and apoptosis. Also the MLCK inhibitor ML-9 abolished the abnormalities[62]. Apart from PARs, also specific serine proteases were studied in the field of intestinal permeability.

A serine protease that is important for the intestinal barrier homeostasis is the transmembrane protein matriptase, or membrane type serine protease-1. A critical role in the epithelial barrier formation and the apical junction complex assembly is attributed to this trypsin-like serine protease[63,64]. Suppressor of tumorigenicity-14 (ST14) hypomorphic mice, which express less than 1% of the matriptase mRNA levels present in the intestine of control littermates, were found to have an impaired intestinal barrier as measured by transepithelial electrical resistance and FITC-dextran permeability[65,66]. This could explain the increased susceptibility of ST14 hypomorphic mice to DSS colitis, with a 30% survival rate after 7 d DSS *vs* 100% in control littermates[67]. Not only genetic depletion but also pharmacological inhibition

(with MI-432) and RNAi silencing of matriptase modulate the TJ assembly, causing the opening of the paracellular gate[65,68]. In physiological conditions, matriptase regulates the expression pattern of the "pore-forming" TJ protein claudin-2. Since there is no evidence of direct proteolytic processing by matriptase, it is likely that matriptase enhances the claudin-2 protein turnover *via* activation of protein kinase C-zeta (PKC-ζ)[65]. Other studies also reported an association of matriptase with other TJ proteins. For instance in ST14 hypomorphic mice, multiple grades of TJ disruption were identified with expression patterns of occludin, ZO-1 and claudin-1 ranging from a decreased protein expression to areas of complete absence, whereas claudin-2 was upregulated[66,67]. E-cadherin levels however remained unaltered despite co-localisation of matriptase with E-cadherin[65]. In addition, matriptase expression in inflamed colonic tissues from CD and UC patients is significantly downregulated, making matriptase induction a potential therapeutic strategy[68]. Recently, Pászti-Gere *et al*[69] showed that reinforcement of the intestinal barrier is in fact possible by the induction of matriptase. Incubation of an intestinal epithelial cell line with the matriptase activator sphingosine-1-phosphate increased the TER and resulted in an upregulation of occludin at the apical junction. Also *in vivo* it was shown that matriptase restoration recovers the barrier integrity by decreasing permeability-associated claudin-2 protein levels and thereby protecting against DSS colitis[67]. Although it should be noted that a tight regulation of matriptase is necessary, since an overexpression could result in malignancies due to its involvement in epithelial proliferation[70-72].

Recently, serine protease granzyme M was also shown to be essential for normal barrier function[73]. In this study, mice deficient of granzyme M were more susceptible to DSS colitis and showed an elevated paracellular permeability compared to wild type (WT) mice. Furthermore, granzyme M expression was upregulated in the inflamed colon tissue samples from UC patients, suggesting that granzyme M acts to induce colonic protection during active disease[73].

In 2000, Fasano *et al*[74] discovered the serine protease analogue zonulin, the first known endogenous physiologic modulator of TJ proteins regulating the paracellular permeability. The human protein zonulin is similar to Zonula occludens toxin (Zot) that was discovered earlier in Vibrio cholerae. It increased intestinal paracellular permeability in a similar fashion[75]. Luminal exposure to bacteria and the gluten component gliadin are identified as the two most powerful triggers for zonulin release in the gut[76,77]. In addition, gliadin can cause celiac disease in genetically susceptible individuals, which is associated with increased paracellular permeability. The expression level of zonulin was shown to be increased in the intestinal submucosa of celiac disease patients[74]. Also in CD

patients, serum zonulin levels are higher compared to their relatives and to healthy control subjects. Interestingly, 50% of the first degree relatives had serum zonulin levels that were increased tremendously (more than two times the standard deviation above the mean) whereas this large increase could only be observed in 4.9% of controls[78]. For both zonulin and Zot, the mechanism of opening intercellular TJs is likely to resemble the effect of certain serine proteases (*cfr. supra*) although it is not fully elucidated yet. It is suggested that zonulin and Zot cause TJ disassembly by activating the epidermal growth factor receptor (EGFR) on the epithelial cell through transactivation of PAR$_2$ (Figure 1)[78,79]. The involvement of PAR$_2$ in EGFR activation was confirmed by the findings that zonulin could not reduce the TER in ileal strips from PAR$_2^{-/-}$ mice in contrast to strips of WT mice. Subsequently, intracellular signaling involves protein kinase C-α (PKC-α)-activation that leads to polymerization of F-actin, TJ disassembly and opening of the paracellular space[80]. Meanwhile, a synthetic octapeptide resembling the receptor-binding domain of zonulin was developed. This molecule, named Larazotide acetate or AT-1001, prevents the opening of TJs in response to zonulin by competitively antagonizing the zonulin receptors (EGFR and PAR$_2$)[81,82]. Clinical trials are currently ongoing to assess its efficacy as a therapy for celiac disease, but there also is evidence for therapeutic potential in other pathologies such as IBD[83,84].

Serine protease inhibitors

Two families of endogenous protease inhibitors, the Serpins and Chelonianins, tightly regulate the activity of the serine proteases by binding to the target protease and largely adjusting its confirmation leading to an irreversible disruption of the active site[85]. A defect in the protease/antiprotease balance leads to tissue damage due to excessive proteolytic capacity causing gastrointestinal diseases.

Serpins are most studied in their role in controlling the coagulation cascade. However, in the gastrointestinal tract, secretory leucocyte protease inhibitor (SLPI) and elafin are of importance. Both are produced by intestinal epithelial cells or leucocytes and inhibit neutrophil elastase and proteinase-3. Besides, SLPI is also a potent inhibitor of trypsin, tryptase, chymotrypsin, chymase and cathepsin G[37]. Although elafin, or skin-derived antileukoproteinase, and SLPI are poorly investigated when it comes to barrier function disturbances, an elevated expression of these antiproteases was reported in "leaky gut"-related gastrointestinal diseases. For instance in CD and UC patients it was shown that the levels of elafin and SLPI are significantly higher in inflamed colonic tissue *vs* noninflamed and control tissues. Interestingly, the upregulation was less pronounced in inflamed CD samples *vs* inflamed UC samples[86]. In contrast, the elafin expression in small intestinal samples was lower in patients with active celiac disease compared to control patients. Local treatment of gluten-sensitive mice with elafin using a recombinant *Lactococcus lactis* vector, restored the intestinal barrier function and normalized the ZO-1 expression, which was disrupted in a mouse model of celiac disease[87]. *In vitro* experiments in a Caco-2 cell line confirm these barrier-protective effects of elafin. Cells treated with TNF-α to induce a barrier defect, showed a complete restoration in paracellular permeability after simultaneous treatment with elafin[88].

Restoring an impaired barrier by pharmacological protease inhibition has been proposed as a promising therapeutic treatment option for IBD, functional GI disorders such as IBS and for colorectal cancer[37] because epithelial barrier dysfunction is a common factor in these diverse pathologies. Studies with the synthetic broad specificity serine protease inhibitors nafamostat mesilate and camostat mesilate revealed positive effects on paracellular permeability. In an animal model for IBS, where the colonic permeability towards ^{51}Cr-EDTA was elevated and ZO-1 disrupted, treatment with camostat mesilate not only normalized the fecal protease activity but also the colonic permeability significantly improved and the ZO-1 protein levels were restored[89]. Nafamostat mesilate, that has shown beneficial effects on disease outcome in animal models of colitis[90], IBS[91], acute pancreatitis[92] and colorectal cancer[93], also seems to restore the intestinal barrier function. The addition of tryptase to the basolateral side of human colonic strips mounted in Ussing chambers increased the permeability proportional to the tryptase concentration. This was abolished after simultaneous addition of nafamostat mesilate[94]. In an *in vitro* co-culture model, Caco-2 cells responded to mast cell degranulation with a disruption of epithelial integrity shown by a decrease in TER, an increase of FITC-dextran flux and a decrease in the expression of the TJ proteins claudin-1 and ZO-1. These effects were prevented by tryptase inhibition using nafamostat mesilate[95]. These positive findings of nafamostat on intestinal permeability could however not be confirmed *in vivo* in a chronic animal model of T-cell transfer colitis (unpublished results). However, in our own lab, a beneficial effect of a novel serine protease inhibitor (Di-(4-acetamidophenyl) 1-(benzyloxycarbonylamino)-2-[(4-guanidino)phenyl]ethanephosphonate trifluoroacetate; abbreviated as SPI in Table 1) was shown in a T-cell transfer colitis model. A curative i.p. treatment with this novel protease inhibitor abolished the elevated intestinal permeability that was seen in the vehicle-treated colitis animals while also exerting anti-inflammatory effects whereas nafamostat only showed the anti-inflammatory effects[96].

METALLOPROTEASES

Matrix metalloproteases

Matrix metalloproteases (MMPs) are generally

known for their ability to degrade and remodel the extracellular matrix (ECM). But in addition to their role in ECM turnover, MMPs degrade or proteolytically activate a wide range of molecules such as che-mokines, cytokines, growth factors, membrane receptors, cytoskeleton proteins and junctional proteins[97,98]. Under normal physiological conditions, their activity is tightly regulated by the tissue inhibitors of metalloproteases (TIMP-1-4). A dysregulation of the balance between MMPs and TIMPs is associated with inflammation and tissue damage[99,100]. Indeed, various studies have reported an upregulation of MMPs in inflamed IBD epithelium, suggesting that the inhibition of MMPs could be an interesting therapeutic intervention in IBD[99,101-104]. However, the failure of MMP inhibitors in cancer trials has led to rethink the clinical potential of these compounds[105,106]. Indirect inhibition of the effects of MMPs or intervening in the signal transduction pathways influenced by MMPs seems more likely to be successful. In this respect, the *new* physiological and pathological roles of MMPs in specific diseases are being further investigated, such as their effect on the epithelial barrier integrity, discussed below.

Previous studies have demonstrated the upre-gulation of the gelatinases MMP-2 and MMP-9 in IBD patients[103,107,108] as well as in animal models of colitis[109-111]. It was found that a specific overexpression of MMP-9 in the intestinal epithelium is associated with a defective barrier function and mucin production due to a decrease in goblet cell differentiation[112]. Supporting their role in barrier function, MMP-9$^{-/-}$ mice have an increased number of goblet cells and MUC-2 expression compared to WT mice[113]. Recently, Nighot *et al*[114] showed an attenuation of the DSS-induced increase in colonic permeability in MMP-9$^{-/-}$ mice. The protein levels of tight junctional occludin were elevated in MMP-9$^{-/-}$ mice, both DSS- and vehicle-treated, *vs* WT mice. It was also found that the protein expression of MLCK was upregulated in DSS colitis in WT mice but not in MMP-9$^{-/-}$ mice. Interestingly, MLCK is a key regulator of TJ permeability as described above[115], suggesting that MMP-9 is a key regulator of TJ permeability *via* MLC phosphorylation. The other gelatinase, MMP-2, plays a barrier protective role in contrast to MMP-9. Mice deficient from MMP-2 have an impaired intestinal barrier function, making them more susceptible to develop experimental colitis compared to their WT counterparts[111]. The authors suggest that MMP-2 exerts its barrier protective function by associating with TJ proteins, since multiple studies have shown close interaction between MMP-2 and claudins[116,117].

Besides MMP-2 and MMP-9, transcript and protein levels of MMP-7 (matrilysin) and MMP-3 (stromelysin-1) are shown to be upregulated in the mucosal tissues of active IBD patients *vs* healthy control tissue[118-120]. These MMPs are linked to the ectodomain shedding of E-cadherin and thereby releasing soluble E-cadherin in the interstitium and loss of E-cadherin function in cell-cell adhesio[121]. Although the majority of the research revolves around cancer, it is relevant to include the proteolytic splicing of E-cad in this review since loss of E-cadherin is associated with disturbances in barrier function during intestinal inflammatory diseases[122,123]. In cancerous cell lines the ability of matrilysin and stromelysin-1 to release soluble E-cadherin was proven with a loss of E-cad function in the cell-cell adhesion and a facilitation of tumor cell invasion as a consequence[121,124,125]. The released sE-cadherin works as a paracrine/autocrine signaling molecule, promoting MMP production and thereby worsening the disease progression[126,127].

Not all metalloproteases are however harmful. The transmembrane endopeptidase meprin β is found to be essential in mucus homeostasis. A loosely organized, nonattached mucus layer covers the epithelial cells on the luminal side, protecting them from digestive proteases thereby forming an important part of the physical barrier against infiltration of harmful substances. The mucin MUC2 is produced by goblet cells and detached by meprin β splicing. Mice lacking the gene encoding for meprin β (Mep1b$^{-/-}$) display a more dense mucus fenotype[128]. In addition, the detached MUC2 is an important luminal factor promoting oral tolerance. The outer mucus layer inhabits commensal bacteria which bind MUC2 and are picked up by patrolling dendritic cells in the intestinal mucosa. After binding, MUC2 induces immunoregulatory signals such as IL-10 and retinoic acid, which are important co-stimulatory factors helping CD103^{+} dendritic cells to induce regulatory T cells, and thus oral tolerance[129].

A disintegrin and metalloproteinase
Another family of metalloproteases are A Disintegrin and Metalloproteinase (ADAM) involved in the release of the ectodomain from transmembrane proteins, a process that is referred to as "shedding".

TNF-α converting enzyme (TACE), or ADAM17, generates the biologically active, soluble form of TNF-α by cleaving the transmembrane bound precursor at the cell surface[28,130,131]. Since TNF-α is a major contributor of the increased intestinal permeability in inflammatory conditions[28,131,132], TACE is a potential key player in the regulation of paracellular permeability. In the past, TACE inhibition (by the endogenous inhibitor TIMP-3 or synthetic inhibitors) has been investigated in diseases that benefit from anti-TNF-α therapy, such as IBD, showing promising results[133-135]. Recently, Al-Sadi *et al*[132] investigated the signaling pathways that mediate TNF-α-induced modulation of intestinal paracellular permeability showing MAP kinase ERK1/2 activation, on their turn phosphorylating and activating Elk-1 which subsequently leads to an increase in MLCK and opening of TJs.

The effect on intestinal permeability of pharmacological TACE-inhibition was investigated on a Caco-2 monolayer. Pretreatment with two synthetic inhibitors, the broad MMP-TACE inhibitor GM6001 or the TACE-specific inhibitor TAPI-2, suppressed the permeability increase induced by TACE[136]. In contrast, Fréour *et al*[137] demonstrated that TACE inhibition, by TIMP-3 or by TAPI-2, amplified the TNF-α-mediated increase in paracellular permeability *in vitro*. The authors suggest that this might be due to an autocrine effect of TIMP-3, triggering the release of pro-inflammatory cytokines that contribute to a hyperpermeable intestinal barrier. It should be noted however that no hard evidence to prove this statement was provided.

CYSTEINE PROTEASES

The vast majority of cysteine proteases reside intracellularly, where they mediate distinct signaling pathways affecting programmed cell death and inflammation[37,138]. Particularly the caspase family of cysteine proteases is well known in the field of cell death. For instance, caspase 1 and 5 take part in inflammasome activation, promoting IL1-β and IL-18 maturation. Caspase 8 plays a central role in both apoptotic and inflammatory pathways, activating respectively pro-apoptotic proteins and NF-κB[138]. Hence, cysteine proteases affect the epithelial barrier integrity for the most part indirectly *via* their effect on inflammation and cell death.

Intestinal epithelial cells undergo apoptosis in a tightly regulated fashion in order to renew the entire cell population every 72 to 96 h[139]. Under inflammatory conditions, such as during IBD, the apoptosis rate increases significantly, inducing morphologic changes in the intestinal barrier[140,141]. During apoptosis of the intestinal epithelial cells, TJ proteins undergo proteolytic cleavage and dislocate from the lateral cell surface[142]. In human colonic HT-29/B6 cells, induction of apoptosis resulted in a significant decrease of TER and an increase in macromolecular tracer permeability[143]. Caspases cleave the transmembrane protein occludin (at the cytoplasmic tail) and adaptor proteins ZO-1 and ZO-2. Although the chronologic sequence of events may not always be clear, there is evidence that a disruption of TJ proteins, caused by for instance bacterial infection or inflammation, activates caspase-8 and -3 and thus initiating cell death[144]. In an *in vivo* permeability assay however, no effect on Cr51-EDTA flux over the colonic barrier was measured after intraluminal administration of cystatin, a cysteine protease inhibitor, whereas serine- and metalloprotease inhibitors lowered the flux significantly[51].

LUMINAL PROTEASES

When it comes to research into the pathogenesis of IBD and IBS, both the gut microbiome and proteases are receiving increasing attention[37,145,146]. Surprisingly, the current knowledge on proteases focusses mostly on host-derived proteases while bacteria-derived proteases have been largely ignored[147]. As previously mentioned, IBD and IBS patients have elevated fecal protease levels. In IBS-D patients it was shown that the majority of the fecal protease activity is most likely due to human pancreatic enzymes and not bacteria. However, an oral antibiotic treatment in mice resulted in decreased fecal activity, supporting the hypothesis that bacteria contribute to the luminal protease content[148].

In the 1990s it was discovered that the metalloproteinase fragilysin, which is produced by the colonic commensal *bacteroides fragilis*, alters the intestinal permeability. Experiments with the purified fragilysin on a human colon cell line (HT-29) revealed the increase in permeability towards ions (shown by a decrease in TER) as well as towards macromolecules (increase in mannitol passage across the cell monolayer)[149,150]. Later, the role of *Entamoeba histolytica* (*Eh*)-derived cysteine proteases in the pathogenesis of amoebic colitis was investigated. The amoebic cysteine proteases induce inflammation by activating IL-1β in a way similar to the human caspases (*cfr. supra*), and damage to the intestinal epithelial barrier resulting in an increased paracellular permeability[151,152]. Two specific proteases mediating TJ disruption were identified; *Eh* cysteine protease A5 (EhCPA5) and EhCP112[152,153]. Recently, bacterial-derived gelatinases were investigated. Pruteanu *et al*[154] screened bacterial colonies in fecal samples of healthy controls and IBD patients for gelatinolytic activity. The researchers could link *Clostridium perfringens* to the majority of the proteolytic activity in the fecal samples. In addition, *C. perfringens* culture supernatant reduced the TER in Ussing chamber experiments performed on rat distal colon. Gelatinase (GelE) produced by the enteric microbe *Enterococcus faecalis* also has shown to induce a barrier defect in epithelial cell monolayers and *in vivo* in mice[155,156]. Originally, the degradation of E-cadherin by proteolytic activity of GelE was considered to be the cause of the permeability increase[156]. Later on, Maharshak *et al*[155] discovered the involvement of PAR2 activation in the GelE-induced permeability increase. Purified *E. faecalis* GelE failed to induce a permeability increase in PAR2-/- mice as well as in epithelial monolayers treated with a PAR2 antagonist.

The feces of the house dust mite (HDM; *Dermatophagoides pteronyssinus*) contains a cysteine protease, Der p 1, which is known to disrupt the lung epithelial TJ proteins occludin and ZO-1 and thereby facilitating allergen delivery and eventually provoking asthma[157]. Recently, Der p 1 was shown to be present in the human gut, affecting barrier function. Exposure of colonic biopsies to a HDM extract reduced the

expression of TJ proteins occludin and ZO-1, reduced the mucus layer thickness, increased TNF-α and increased the paracellular permeability. Pre-incubation of the HDM-extract with the cysteine protease inhibitor E64 abolished the HDM-induced damage to the intestinal barrier[158].

Furthermore, there is an established link between food allergens and the degradation of the epithelial barrier[159,160]. In the digestion process, food particles are broken down by pancreatic and brush border proteases into tripeptides, dipeptides and single amino acids. When the intestinal barrier function is not impaired, oral tolerance is induced against these soluble peptides that can cross the epithelium in a selective and regulated manner. But in other -possibly genetically susceptible- individuals, partially or undigested proteins can still reach the mucosa where they provoke an inflammatory signal instead of tolerance. Plasma cells produce allergen-specific IgE which causes mast cell degranulation at contact. The secreted mast cell proteases and cytokines both contribute to the increased barrier dysfunction *via* their effect on the TJ configuration, leading to an opening of the paracellular route. The difficulty lies however in the "chicken and egg" paradigm. Until today there is evidence for only one food protease to affect the intestinal barrier directly. The kiwifruit cysteine protease actinidin (Act d1) has shown to induce a loss of barrier function in epithelial cell monolayers by the disruption of the occludin and ZO-1 network[161,162]. This effect could be confirmed *in vivo*. Mice gavaged with actinidin exerted an elevated permeability towards FITC-dextran compared to mice gavaged with the vehicle[161].

Dietary proteases can also contribute to intestinal health. Addition of *Aspergillus*-derived proteases (Amano SD) to the diet of rats improved intestinal health *via* the expansion of commensal colonic bacteria of the *Bifidobacterium* and *Lactobacillus* species. The altered microbiota composition enhanced the formation of short chain fatty (SCFA) acids such as butyrate, propionate and lactate[163]. SCFA with butyrate in particular promote mucosal homeostasis among other things by enhancing the intestinal barrier function through the upregulation of tight junction proteins[164].

CONCLUSION

The epithelial cell layer lining the gastrointestinal tract is the body's largest surface area in contact with environmental antigens. The role of these intestinal epithelial cells in the continuous maintenance of intestinal homeostasis is indispensable, providing a physical and biochemical barrier against noxious luminal antigens as well as allowing nutrient absorption. The selective opening of the intercellular spaces, allowing paracellular transport of macromolecules, is regulated by the interplay between TJ proteins and the actomyosin contraction upon activation of intracellular signaling pathways. An increased intestinal permeability is suggested as an important player in the pathophysiology of various intestinal and extra-intestinal pathologies. Targeting the epithelial barrier is a tempting therapeutic approach, but so far no therapies have succeeded. Larazotide acetate showed promising results in preclinical trials, restoring the intestinal barrier function. But clinical trials failed to mirror these effects. This shows that more research is needed to define epithelial barrier function and dysfunction, underlying different pathologies and diseases. Proteases are important signaling molecules in this regard. With their proteolytic capacity they can cleave proteinase-activated receptors on the cell surface of intestinal epithelial cells, influencing the cytoskeleton contractile machinery and paracellular permeability. Also extracellular proteolytic cleavage of the junction proteins occurs, leading directly to epithelial damage and increased intestinal per-meability. In homeostasis, the proteolytic activity is tightly regulated by antiproteases, but this balance is dysregulated in organic and functional GI disorders. As a result, protease inhibition has become a "hot topic" in a therapeutic point of view, mainly focusing on inflammation and hypersensitivity, ignoring the effect on permeability. But since a large array of proteases is involved and for many proteases no specific inhibitors are available yet, more research is needed to elucidate the exact involvement of specific proteases in gut physiology in general and intestinal permeability in particular, in order to become a therapeutic target.

REFERENCES

1   **Groschwitz KR**, Hogan SP. Intestinal barrier function: molecular regulation and disease pathogenesis. *J Allergy Clin Immunol* 2009; **124**: 3-20; quiz 21-22 [PMID: 19560575 DOI: 10.1016/j.jaci.2009.05.038]

2   **Ayabe T**, Satchell DP, Wilson CL, Parks WC, Selsted ME, Ouellette AJ. Secretion of microbicidal alpha-defensins by intestinal Paneth cells in response to bacteria. *Nat Immunol* 2000; **1**: 113-118 [PMID: 11248802 DOI: 10.1038/77783]

3   **Ulluwishewa D**, Anderson RC, McNabb WC, Moughan PJ, Wells JM, Roy NC. Regulation of tight junction permeability by intestinal bacteria and dietary components. *J Nutr* 2011; **141**: 769-776 [PMID: 21430248 DOI: 10.3945/jn.110.135657]

4   **Li H**, Shi B. Tolerogenic dendritic cells and their applications in transplantation. *Cell Mol Immunol* 2015; **12**: 24-30 [PMID: 25109681 DOI: 10.1038/cmi.2014.52]

5   **Rautava S**, Walker WA. Commensal bacteria and epithelial cross talk in the developing intestine. *Curr Gastroenterol Rep* 2007; **9**: 385-392 [PMID: 17991339 DOI: 10.1007/s11894-007-0047-7]

6   **Pabst O**, Mowat AM. Oral tolerance to food protein. *Mucosal Immunol* 2012; **5**: 232-239 [PMID: 22318493 DOI: 10.1038/mi.2012.4]

7   **Suzuki T**. Regulation of intestinal epithelial permeability by tight junctions. *Cell Mol Life Sci* 2013; **70**: 631-659 [PMID: 22782113 DOI: 10.1007/s00018-012-1070-x]

8   **Vogelsang H**, Schwarzenhofer M, Oberhuber G. Changes in gastrointestinal permeability in celiac disease. *Dig Dis* 1998; **16**: 333-336 [PMID: 10207217 DOI: 10.1159/000016886]

9 **Gerova VA**, Stoynov SG, Katsarov DS, Svinarov DA. Increased intestinal permeability in inflammatory bowel diseases assessed by iohexol test. *World J Gastroenterol* 2011; **17**: 2211-2215 [PMID: 21633531 DOI: 10.3748/wjg.v17.i17]

10 **Michielan A**, D'Incà R. Intestinal Permeability in Inflammatory Bowel Disease: Pathogenesis, Clinical Evaluation, and Therapy of Leaky Gut. *Mediators Inflamm* 2015; **2015**: 628157 [PMID: 26582965 DOI: 10.1155/2015/628157]

11 **Vaarala O**. Leaking gut in type 1 diabetes. *Curr Opin Gastroenterol* 2008; **24**: 701-706 [PMID: 19122519 DOI: 10.1097/MOG.0b013e32830e6d98]

12 **Camilleri M**, Gorman H. Intestinal permeability and irritable bowel syndrome. *Neurogastroenterol Motil* 2007; **19**: 545-552 [PMID: 17593135 DOI: 10.1111/j.1365-2982.2007.00925.x]

13 **Pastorelli L**, De Salvo C, Mercado JR, Vecchi M, Pizarro TT. Central role of the gut epithelial barrier in the pathogenesis of chronic intestinal inflammation: lessons learned from animal models and human genetics. *Front Immunol* 2013; **4**: 280 [PMID: 24062746 DOI: 10.3389/fimmu.2013.00280]

14 **Odenwald MA**, Turner JR. The intestinal epithelial barrier: a therapeutic target? *Nat Rev Gastroenterol Hepatol* 2017; **14**: 9-21 [PMID: 27848962 DOI: 10.1038/nrgastro.2016.169]

15 **Odenwald MA**, Turner JR. Intestinal permeability defects: is it time to treat? *Clin Gastroenterol Hepatol* 2013; **11**: 1075-1083 [PMID: 23851019 DOI: 10.1016/j.cgh.2013.07.001]

16 **Anderson JM**, Van Itallie CM. Physiology and function of the tight junction. *Cold Spring Harb Perspect Biol* 2009; **1**: a002584 [PMID: 20066090 DOI: 10.1101/cshperspect.a002584]

17 **Hartsock A**, Nelson WJ. Adherens and tight junctions: structure, function and connections to the actin cytoskeleton. *Biochim Biophys Acta* 2008; **1778**: 660-669 [PMID: 17854762 DOI: 10.1016/j.bbamem.2007.07.012]

18 **Jacob C**, Yang PC, Darmoul D, Amadesi S, Saito T, Cottrell GS, Coelho AM, Singh P, Grady EF, Perdue M, Bunnett NW. Mast cell tryptase controls paracellular permeability of the intestine. Role of protease-activated receptor 2 and beta-arrestins. *J Biol Chem* 2005; **280**: 31936-31948 [PMID: 16027150 DOI: 10.1074/jbc.M506338200]

19 **Weber LP**, Seto M, Sasaki Y, Swärd K, Walsh MP. The involvement of protein kinase C in myosin phosphorylation and force development in rat tail arterial smooth muscle. *Biochem J* 2000; **352 Pt 2**: 573-582 [PMID: 11085953 DOI: 10.1042/bj3520573]

20 **Riento K**, Ridley AJ. Rocks: multifunctional kinases in cell behaviour. *Nat Rev Mol Cell Biol* 2003; **4**: 446-456 [PMID: 12778124 DOI: 10.1038/nrm1128]

21 **Shen Q**, Rigor RR, Pivetti CD, Wu MH, Yuan SY. Myosin light chain kinase in microvascular endothelial barrier function. *Cardiovasc Res* 2010; **87**: 272-280 [PMID: 20479130 DOI: 10.1093/cvr/cvq144]

22 **Rigor RR**, Shen Q, Pivetti CD, Wu MH, Yuan SY. Myosin light chain kinase signaling in endothelial barrier dysfunction. *Med Res Rev* 2013; **33**: 911-933 [PMID: 22886693 DOI: 10.1002/med.21270]

23 **Harhaj NS**, Antonetti DA. Regulation of tight junctions and loss of barrier function in pathophysiology. *Int J Biochem Cell Biol* 2004; **36**: 1206-1237 [PMID: 15109567 DOI: 10.1016/j.biocel.2003.08.007]

24 **Capaldo CT**, Nusrat A. Cytokine regulation of tight junctions. *Biochim Biophys Acta* 2009; **1788**: 864-871 [PMID: 18952050 DOI: 10.1016/j.bbamem.2008.08.027]

25 **Nava P**, Kamekura R, Nusrat A. Cleavage of transmembrane junction proteins and their role in regulating epithelial homeostasis. *Tissue Barriers* 2013; **1**: e24783 [PMID: 24665393 DOI: 10.4161/tisb.24783]

26 **Al-Sadi R**, Boivin M, Ma T. Mechanism of cytokine modulation of epithelial tight junction barrier. *Front Biosci* (Landmark Ed) 2009; **14**: 2765-2778 [PMID: 19273235 DOI: 10.2741/3413]

27 **Lee SH**. Intestinal permeability regulation by tight junction: implication on inflammatory bowel diseases. *Intest Res* 2015; **13**: 11-18 [PMID: 25691839 DOI: 10.5217/ir.2015.13.1.11]

28 **Ye D**, Ma I, Ma TY. Molecular mechanism of tumor necrosis factor-alpha modulation of intestinal epithelial tight junction barrier. *Am J Physiol Gastrointest Liver Physiol* 2006; **290**: G496-G504 [PMID: 16474009 DOI: 10.1152/ajpgi.00318.2005]

29 **Zolotarevsky Y**, Hecht G, Koutsouris A, Gonzalez DE, Quan C, Tom J, Mrsny RJ, Turner JR. A membrane-permeant peptide that inhibits MLC kinase restores barrier function in in vitro models of intestinal disease. *Gastroenterology* 2002; **123**: 163-172 [PMID: 12105845 DOI: 10.1053/gast.2002.34235]

30 **Madara JL**, Stafford J. Interferon-gamma directly affects barrier function of cultured intestinal epithelial monolayers. *J Clin Invest* 1989; **83**: 724-727 [PMID: 2492310 DOI: 10.1172/JCI113938]

31 **Ceponis PJ**, Botelho F, Richards CD, McKay DM. Interleukins 4 and 13 increase intestinal epithelial permeability by a phosphatidylinositol 3-kinase pathway. Lack of evidence for STAT 6 involvement. *J Biol Chem* 2000; **275**: 29132-29137 [PMID: 10871612 DOI: 10.1074/jbc.M003516200]

32 **Zünd G**, Madara JL, Dzus AL, Awtrey CS, Colgan SP. Interleukin-4 and interleukin-13 differentially regulate epithelial chloride secretion. *J Biol Chem* 1996; **271**: 7460-7464 [PMID: 8631774 DOI: 10.1074/jbc.271.13.7460]

33 **Wisner DM**, Harris LR, Green CL, Poritz LS. Opposing regulation of the tight junction protein claudin-2 by interferon-gamma and interleukin-4. *J Surg Res* 2008; **144**: 1-7 [PMID: 17640674 DOI: 10.1016/j.jss.2007.03.059]

34 **Pham CT**. Neutrophil serine proteases: specific regulators of inflammation. *Nat Rev Immunol* 2006; **6**: 541-550 [PMID: 16799473 DOI: 10.1038/nri1841]

35 **Rao MB**, Tanksale AM, Ghatge MS, Deshpande VV. Molecular and biotechnological aspects of microbial proteases. *Microbiol Mol Biol Rev* 1998; **62**: 597-635 [PMID: 9729602]

36 **Antalis TM**, Shea-Donohue T, Vogel SN, Sears C, Fasano A. Mechanisms of disease: protease functions in intestinal mucosal pathobiology. *Nat Clin Pract Gastroenterol Hepatol* 2007; **4**: 393-402 [PMID: 17607295 DOI: 10.1038/ncpgasthep0846]

37 **Vergnolle N**. Protease inhibition as new therapeutic strategy for GI diseases. *Gut* 2016; **65**: 1215-1224 [PMID: 27196587 DOI: 10.1136/gutjnl-2015-309147]

38 **Vergnolle N**. Protease-activated receptors as drug targets in inflammation and pain. *Pharmacol Ther* 2009; **123**: 292-309 [PMID: 19481569 DOI: 10.1016/j.pharmthera.2009.05.004]

39 **Macfarlane SR**, Seatter MJ, Kanke T, Hunter GD, Plevin R. Proteinase-activated receptors. *Pharmacol Rev* 2001; **53**: 245-282 [PMID: 11356985]

40 **Amadesi S**, Bunnett N. Protease-activated receptors: protease signaling in the gastrointestinal tract. *Curr Opin Pharmacol* 2004; **4**: 551-556 [PMID: 15525542 DOI: 10.1016/j.coph.2004.08.004]

41 **Ceuleers H**, Van Spaendonk H, Hanning N, Heirbaut J, Lambeir AM, Joossens J, Augustyns K, De Man JG, De Meester I, De Winter BY. Visceral hypersensitivity in inflammatory bowel diseases and irritable bowel syndrome: The role of proteases. *World J Gastroenterol* 2016; **22**: 10275-10286 [PMID: 28058009 DOI: 10.3748/wjg.v22.i47.10275]

42 **Di Cera E**. Serine proteases. *IUBMB Life* 2009; **61**: 510-515 [PMID: 19180666 DOI: 10.1002/iub.186]

43 **Page MJ**, Di Cera E. Serine peptidases: classification, structure and function. *Cell Mol Life Sci* 2008; **65**: 1220-1236 [PMID: 18259688 DOI: 10.1007/s00018-008-7565-9]

44 **Padrines M**, Wolf M, Walz A, Baggiolini M. Interleukin-8 processing by neutrophil elastase, cathepsin G and proteinase-3. *FEBS Lett* 1994; **352**: 231-235 [PMID: 7925979 DOI: 10.1016/0014-5793(94)00952-X]

45 **Nufer O**, Corbett M, Walz A. Amino-terminal processing of chemokine ENA-78 regulates biological activity. *Biochemistry* 1999; **38**: 636-642 [PMID: 9888803 DOI: 10.1021/bi981294s]

46 **Rothmeier AS**, Ruf W. Protease-activated receptor 2 signaling in inflammation. *Semin Immunopathol* 2012; **34**: 133-149 [PMID: 21971685 DOI: 10.1007/s00281-011-0289-1]

47 **Coelho AM**, Vergnolle N, Guiard B, Fioramonti J, Bueno L. Proteinases and proteinase-activated receptor 2: a possible role to

promote visceral hyperalgesia in rats. *Gastroenterology* 2002; **122**: 1035-1047 [PMID: 11910355 DOI: 10.1053/gast.2002.32387]

48   Cenac N, Coelho AM, Nguyen C, Compton S, Andrade-Gordon P, MacNaughton WK, Wallace JL, Hollenberg MD, Bunnett NW, Garcia-Villar R, Bueno L, Vergnolle N. Induction of intestinal inflammation in mouse by activation of proteinase-activated receptor-2. *Am J Pathol* 2002; **161**: 1903-1915 [PMID: 12414536 DOI: 10.1016/S0002-9440(10)64466-5]

49   Bjarnason I, MacPherson A, Hollander D. Intestinal permeability: an overview. *Gastroenterology* 1995; **108**: 1566-1581 [PMID: 7729650 DOI: 10.1016/0016-5085(95)90708-4]

50   Groschwitz KR, Wu D, Osterfeld H, Ahrens R, Hogan SP. Chymase-mediated intestinal epithelial permeability is regulated by a protease-activating receptor/matrix metalloproteinase-2-dependent mechanism. *Am J Physiol Gastrointest Liver Physiol* 2013; **304**: G479-G489 [PMID: 23306080 DOI: 10.1152/ajpgi.00186.2012]

51   Róka R, Demaude J, Cenac N, Ferrier L, Salvador-Cartier C, Garcia-Villar R, Fioramonti J, Bueno L. Colonic luminal proteases activate colonocyte proteinase-activated receptor-2 and regulate paracellular permeability in mice. *Neurogastroenterol Motil* 2007; **19**: 57-65 [PMID: 17187589 DOI: 10.1111/j.1365-2982.2006.00851.x]

52   Chin AC, Lee WY, Nusrat A, Vergnolle N, Parkos CA. Neutrophil-mediated activation of epithelial protease-activated receptors-1 and -2 regulates barrier function and transepithelial migration. *J Immunol* 2008; **181**: 5702-5710 [PMID: 18832729 DOI: 10.4049/jimmunol.181.8.5702]

53   Turner JR, Rill BK, Carlson SL, Carnes D, Kerner R, Mrsny RJ, Madara JL. Physiological regulation of epithelial tight junctions is associated with myosin light-chain phosphorylation. *Am J Physiol* 1997; **273**: C1378-C1385 [PMID: 9357784]

54   Cenac N, Garcia-Villar R, Ferrier L, Larauche M, Vergnolle N, Bunnett NW, Coelho AM, Fioramonti J, Bueno L. Proteinase-activated receptor-2-induced colonic inflammation in mice: possible involvement of afferent neurons, nitric oxide, and paracellular permeability. *J Immunol* 2003; **170**: 4296-4300 [PMID: 12682265 DOI: 10.4049/jimmunol.170.8.4296]

55   Cenac N, Chin AC, Garcia-Villar R, Salvador-Cartier C, Ferrier L, Vergnolle N, Buret AG, Fioramonti J, Bueno L. PAR2 activation alters colonic paracellular permeability in mice via IFN-gamma-dependent and -independent pathways. *J Physiol* 2004; **558**: 913-925 [PMID: 15194744 DOI: 10.1113/jphysiol.2004.061721]

56   Kahn ML, Zheng YW, Huang W, Bigornia V, Zeng D, Moff S, Farese RV, Tam C, Coughlin SR. A dual thrombin receptor system for platelet activation. *Nature* 1998; **394**: 690-694 [PMID: 9716134 DOI: 10.1038/29325]

57   Shea-Donohue T, Thomas K, Cody MJ, Aiping Zhao LJ, Kopydlowski KM, Fukata M, Lira SA, Vogel SN. Mice deficient in the CXCR2 ligand, CXCL1 (KC/GRO-alpha), exhibit increased susceptibility to dextran sodium sulfate (DSS)-induced colitis. *Innate Immun* 2008; **14**: 117-124 [PMID: 18713728 DOI: 10.1177/1753425908088724]

58   Dabek M, Ferrier L, Roka R, Gecse K, Annahazi A, Moreau J, Escourrou J, Cartier C, Chaumaz G, Leveque M, Ait-Belgnaoui A, Wittmann T, Theodorou V, Bueno L. Luminal cathepsin g and protease-activated receptor 4: a duet involved in alterations of the colonic epithelial barrier in ulcerative colitis. *Am J Pathol* 2009; **175**: 207-214 [PMID: 19528350 DOI: 10.2353/ajpath.2009.080986]

59   Covic L, Misra M, Badar J, Singh C, Kuliopulos A. Pepducin-based intervention of thrombin-receptor signaling and systemic platelet activation. *Nat Med* 2002; **8**: 1161-1165 [PMID: 12357249 DOI: 10.1038/nm760]

60   Houle S, Papez MD, Ferazzini M, Hollenberg MD, Vergnolle N. Neutrophils and the kallikrein-kinin system in proteinase-activated receptor 4-mediated inflammation in rodents. *Br J Pharmacol* 2005; **146**: 670-678 [PMID: 16100525 DOI: 10.1038/sj.bjp.0706371]

61   Nicholson DW, Ali A, Thornberry NA, Vaillancourt JP, Ding CK, Gallant M, Gareau Y, Griffin PR, Labelle M, Lazebnik YA. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* 1995; **376**: 37-43 [PMID: 7596430 DOI: 10.1038/376037a0]

62   Chin AC, Vergnolle N, MacNaughton WK, Wallace JL, Hollenberg MD, Buret AG. Proteinase-activated receptor 1 activation induces epithelial apoptosis and increases intestinal permeability. *Proc Natl Acad Sci USA* 2003; **100**: 11104-11109 [PMID: 12960392 DOI: 10.1073/pnas.1831452100]

63   Bugge TH, List K, Szabo R. Matriptase-dependent cell surface proteolysis in epithelial development and pathogenesis. *Front Biosci* 2007; **12**: 5060-5070 [PMID: 17569630 DOI: 10.2741/2448]

64   List K, Haudenschild CC, Szabo R, Chen W, Wahl SM, Swaim W, Engelholm LH, Behrendt N, Bugge TH. Matriptase/MT-SP1 is required for postnatal survival, epidermal barrier function, hair follicle development, and thymic homeostasis. *Oncogene* 2002; **21**: 3765-3779 [PMID: 12032844 DOI: 10.1038/sj.onc.1205502]

65   Buzza MS, Netzel-Arnett S, Shea-Donohue T, Zhao A, Lin CY, List K, Szabo R, Fasano A, Bugge TH, Antalis TM. Membrane-anchored serine protease matriptase regulates epithelial barrier formation and permeability in the intestine. *Proc Natl Acad Sci USA* 2010; **107**: 4200-4205 [PMID: 20142489 DOI: 10.1073/pnas.0903923107]

66   List K, Kosa P, Szabo R, Bey AL, Wang CB, Molinolo A, Bugge TH. Epithelial integrity is maintained by a matriptase-dependent proteolytic pathway. *Am J Pathol* 2009; **175**: 1453-1463 [PMID: 19717635 DOI: 10.2353/ajpath.2009.090240]

67   Netzel-Arnett S, Buzza MS, Shea-Donohue T, Désilets A, Leduc R, Fasano A, Bugge TH, Antalis TM. Matriptase protects against experimental colitis and promotes intestinal barrier recovery. *Inflamm Bowel Dis* 2012; **18**: 1303-1314 [PMID: 22081509 DOI: 10.1002/ibd.21930]

68   Pászti-Gere E, McManus S, Meggyeshazi N, Balla P, Gálfi P, Steinmetzer T. Inhibition of Matriptase Activity Results in Decreased Intestinal Epithelial Monolayer Integrity In Vitro. *PLoS One* 2015; **10**: e0141077 [PMID: 26488575 DOI: 10.1371/journal.pone.0141077]

69   Pászti-Gere E, Jerzsele Á, Balla P, Ujhelyi G, Székács A. Reinforced Epithelial Barrier Integrity via Matriptase Induction with Sphingosine-1-Phosphate Did Not Result in Disturbances in Physiological Redox Status. *Oxid Med Cell Longev* 2016; **2016**: 9674272 [PMID: 26823955 DOI: 10.1155/2016/9674272]

70   Förbs D, Thiel S, Stella MC, Stürzebecher A, Schweinitz A, Steinmetzer T, Stürzebecher J, Uhland K. In vitro inhibition of matriptase prevents invasive growth of cell lines of prostate and colon carcinoma. *Int J Oncol* 2005; **27**: 1061-1070 [PMID: 16142324 DOI: 10.3892/ijo.27.4.1061]

71   Zoratti GL, Tanabe LM, Varela FA, Murray AS, Bergum C, Colombo É, Lang JE, Molinolo AA, Leduc R, Marsault E, Boerner J, List K. Targeting matriptase in breast cancer abrogates tumour progression via impairment of stromal-epithelial growth factor signalling. *Nat Commun* 2015; **6**: 6776 [PMID: 25873032 DOI: 10.1038/ncomms7776]

72   Wadhawan V, Kolhe YA, Sangith N, Gautam AK, Venkatraman P. From prediction to experimental validation: desmoglein 2 is a functionally relevant substrate of matriptase in epithelial cells and their reciprocal relationship is important for cell adhesion. *Biochem J* 2012; **447**: 61-70 [PMID: 22783993 DOI: 10.1042/BJ20111432]

73   Souza-Fonseca-Guimaraes F, Krasnova Y, Putoczki T, Miles K, MacDonald KP, Town L, Shi W, Gobe GC, McDade L, Mielke LA, Tye H, Masters SL, Belz GT, Huntington ND, Radford-Smith G, Smyth MJ. Granzyme M has a critical role in providing innate immune protection in ulcerative colitis. *Cell Death Dis* 2016; **7**: e2302 [PMID: 27441655 DOI: 10.1038/cddis.2016.215]

74   Fasano A, Not T, Wang W, Uzzau S, Berti I, Tommasini A, Goldblum SE. Zonulin, a newly discovered modulator of intestinal permeability, and its expression in coeliac disease. *Lancet* 2000; **355**: 1518-1519 [PMID: 10801176 DOI: 10.1016/S0140-6736(00)02169-3]

75   Fasano A, Fiorentini C, Donelli G, Uzzau S, Kaper JB,

Margaretten K, Ding X, Guandalini S, Comstock L, Goldblum SE. Zonula occludens toxin modulates tight junctions through protein kinase C-dependent actin reorganization, in vitro. *J Clin Invest* 1995; **96**: 710-720 [PMID: 7635964 DOI: 10.1172/JCI118114]

76 **Drago S**, El Asmar R, Di Pierro M, Grazia Clemente M, Tripathi A, Sapone A, Thakar M, Iacono G, Carroccio A, D'Agate C, Not T, Zampini L, Catassi C, Fasano A. Gliadin, zonulin and gut permeability: Effects on celiac and non-celiac intestinal mucosa and intestinal cell lines. *Scand J Gastroenterol* 2006; **41**: 408-419 [PMID: 16635908 DOI: 10.1080/00365520500235334]

77 **El Asmar R**, Panigrahi P, Bamford P, Berti I, Not T, Coppa GV, Catassi C, Fasano A. Host-dependent zonulin secretion causes the impairment of the small intestine barrier function after bacterial exposure. *Gastroenterology* 2002; **123**: 1607-1615 [PMID: 12404235 DOI: 10.1053/gast.2002.36578]

78 **Fasano A**. Zonulin, regulation of tight junctions, and autoimmune diseases. *Ann N Y Acad Sci* 2012; **1258**: 25-33 [PMID: 22731712 DOI: 10.1111/j.1749-6632.2012.06538.x]

79 **Soh UJ**, Dores MR, Chen B, Trejo J. Signal transduction by protease-activated receptors. *Br J Pharmacol* 2010; **160**: 191-203 [PMID: 20423334 DOI: 10.1111/j.1476-5381.2010.00705.x]

80 **Fasano A**. Zonulin and its regulation of intestinal barrier function: the biological door to inflammation, autoimmunity, and cancer. *Physiol Rev* 2011; **91**: 151-175 [PMID: 21248165 DOI: 10.1152/physrev.00003.2008]

81 **Di Pierro M**, Lu R, Uzzau S, Wang W, Margaretten K, Pazzani C, Maimone F, Fasano A. Zonula occludens toxin structure-function analysis. Identification of the fragment biologically active on tight junctions and of the zonulin receptor binding domain. *J Biol Chem* 2001; **276**: 19160-19165 [PMID: 11278543 DOI: 10.1074/jbc.M009674200]

82 **Watts T**, Berti I, Sapone A, Gerarduzzi T, Not T, Zielke R, Fasano A. Role of the intestinal tight junction modulator zonulin in the pathogenesis of type I diabetes in BB diabetic-prone rats. *Proc Natl Acad Sci USA* 2005; **102**: 2916-2921 [PMID: 15710870 DOI: 10.1073/pnas.0500178102]

83 **Arrieta MC**, Madsen K, Doyle J, Meddings J. Reducing small intestinal permeability attenuates colitis in the IL10 gene-deficient mouse. *Gut* 2009; **58**: 41-48 [PMID: 18829978 DOI: 10.1136/gut.2008.150888]

84 **Paterson BM**, Lammers KM, Arrieta MC, Fasano A, Meddings JB. The safety, tolerance, pharmacokinetic and pharmacodynamic effects of single doses of AT-1001 in coeliac disease subjects: a proof of concept study. *Aliment Pharmacol Ther* 2007; **26**: 757-766 [PMID: 17697209 DOI: 10.1111/j.1365-2036.2007.03413.x]

85 **Gettins PG**. Serpin structure, mechanism, and function. *Chem Rev* 2002; **102**: 4751-4804 [PMID: 12475206 DOI: 10.1021/cr010170]

86 **Schmid M**, Fellermann K, Fritz P, Wiedow O, Stange EF, Wehkamp J. Attenuated induction of epithelial and leukocyte serine antiproteases elafin and secretory leukocyte protease inhibitor in Crohn's disease. *J Leukoc Biol* 2007; **81**: 907-915 [PMID: 17200145 DOI: 10.1189/jlb.0906581]

87 **Galipeau HJ**, Wiepjes M, Motta JP, Schulz JD, Jury J, Natividad JM, Pinto-Sanchez I, Sinclair D, Rousset P, Martin-Rosique R, Bermudez-Humaran L, Leroux JC, Murray JA, Smecuol E, Bai JC, Vergnolle N, Langella P, Verdu EF. Novel role of the serine protease inhibitor elafin in gluten-related disorders. *Am J Gastroenterol* 2014; **109**: 748-756 [PMID: 24710505 DOI: 10.1038/ajg.2014.48]

88 **Motta JP**, Magne L, Descamps D, Rolland C, Squarzoni-Dale C, Rousset P, Martin L, Cenac N, Balloy V, Huerre M, Fröhlich LF, Jenne D, Wartelle J, Belaaouaj A, Mas E, Vinel JP, Alric L, Chignard M, Vergnolle N, Sallenave JM. Modifying the protease, antiprotease pattern by elafin overexpression protects mice from colitis. *Gastroenterology* 2011; **140**: 1272-1282 [PMID: 21199654 DOI: 10.1053/j.gastro.2010.12.050]

89 **Zhao J**, Wang J, Dong L, Shi H, Wang Z, Ding H, Shi H, Lu X. A protease inhibitor against acute stress-induced visceral hypersensitivity and paracellular permeability in rats. *Eur J Pharmacol* 2011; **654**: 289-294 [PMID: 21237151 DOI: 10.1016/j.ejphar.2010.12.032]

90 **Isozaki Y**, Yoshida N, Kuroda M, Handa O, Takagi T, Kokura S, Ichikawa H, Naito Y, Okanoue T, Yoshikawa T. Anti-tryptase treatment using nafamostat mesilate has a therapeutic effect on experimental colitis. *Scand J Gastroenterol* 2006; **41**: 944-953 [PMID: 16803693 DOI: 10.1080/00365520500529470]

91 **Ceuleers H**, Segaert E, Heirbaut J, Hanning N, Francque SM, Joossens J, De Man JG, De Winter BY. Su 1937 Two Serine Protease Inhibitors, Nafamostat Mesylate and the Newly Developed SPIx, Decrease Post-Inflammatory Visceral Hypersensitivity in Rats. *Gastroenterology* 2016; **150**: S593-S594

92 **Chen CC**, Wang SS, Lee FY. Action of antiproteases on the inflammatory response in acute pancreatitis. *JOP* 2007; **8**: 488-494 [PMID: 17625305]

93 **Liu W**, Hu D, Huo H, Zhang W, Adiliaghdam F, Morrison S, Ramirez JM, Gul SS, Hamarneh SR, Hodin RA. Intestinal Alkaline Phosphatase Regulates Tight Junction Protein Levels. *J Am Coll Surg* 2016; **222**: 1009-1017 [PMID: 27106638 DOI: 10.1016/j.jamcollsurg.2015.12.006]

94 **Lee JW**, Park JH, Park DI, Park JH, Kim HJ, Cho YK, Sohn CI, Jeon WK, Kim BI. Subjects with diarrhea-predominant IBS have increased rectal permeability responsive to tryptase. *Dig Dis Sci* 2010; **55**: 2922-2928 [PMID: 20087660 DOI: 10.1007/s10620-009-1094-8]

95 **Wilcz-Villega EM**, McClean S, O'Sullivan MA. Mast cell tryptase reduces junctional adhesion molecule-A (JAM-A) expression in intestinal epithelial cells: implications for the mechanisms of barrier dysfunction in irritable bowel syndrome. *Am J Gastroenterol* 2013; **108**: 1140-1151 [PMID: 23588236 DOI: 10.1038/ajg.2013.92]

96 **Van Spaendonk H**, Nullens S, Ceuleers H, Schrijvers D, Francque SM, De Man J, De Winter BY. Tu1883 The Effect of a Protease Inhibitor in a Chronic Colitis Transfer Model. *Gastroenterology* 2016; **150**: S967

97 **Rodríguez D**, Morrison CJ, Overall CM. Matrix metalloproteinases: what do they not do? New substrates and biological roles identified by murine models and proteomics. *Biochim Biophys Acta* 2010; **1803**: 39-54 [PMID: 19800373 DOI: 10.1016/j.bbamcr.2009.09.015]

98 **Cauwe B**, Van den Steen PE, Opdenakker G. The biochemical, biological, and pathological kaleidoscope of cell surface substrates processed by matrix metalloproteinases. *Crit Rev Biochem Mol Biol* 2007; **42**: 113-185 [PMID: 17562450 DOI: 10.1080/10409230701340019]

99 **Mäkitalo L**, Kolho KL, Karikoski R, Anthoni H, Saarialho-Kere U. Expression profiles of matrix metalloproteinases and their inhibitors in colonic inflammation related to pediatric inflammatory bowel disease. *Scand J Gastroenterol* 2010; **45**: 862-871 [PMID: 20367198 DOI: 10.3109/00365520903583863]

100 **Louis E**, Ribbens C, Godon A, Franchimont D, De Groote D, Hardy N, Boniver J, Belaiche J, Malaise M. Increased production of matrix metalloproteinase-3 and tissue inhibitor of metalloproteinase-1 by inflamed mucosa in inflammatory bowel disease. *Clin Exp Immunol* 2000; **120**: 241-246 [PMID: 10792371 DOI: 10.1046/j.1365-2249.2000.01227.x]

101 **Lawrance IC**, Fiocchi C, Chakravarti S. Ulcerative colitis and Crohn's disease: distinctive gene expression profiles and novel susceptibility candidate genes. *Hum Mol Genet* 2001; **10**: 445-456 [PMID: 11181568 DOI: 10.1093/hmg/10.5.445]

102 **Rath T**, Roderfeld M, Halwe JM, Tschuschner A, Roeb E, Graf J. Cellular sources of MMP-7, MMP-13 and MMP-28 in ulcerative colitis. *Scand J Gastroenterol* 2010; **45**: 1186-1196 [PMID: 20568971 DOI: 10.3109/00365521.2010.499961]

103 **von Lampe B**, Barthel B, Coupland SE, Riecken EO, Rosewicz S. Differential expression of matrix metalloproteinases and their tissue inhibitors in colon mucosa of patients with inflammatory bowel disease. *Gut* 2000; **47**: 63-73 [PMID: 10861266 DOI: 10.1136/gut.47.1.63]

104 **Pedersen G**, Saermark T, Kirkegaard T, Brynskov J. Spontaneous and cytokine induced expression and activity of matrix

metalloproteinases in human colonic epithelium. *Clin Exp Immunol* 2009; **155**: 257-265 [PMID: 19137636 DOI: 10.1111/j.1365-2249.2008.03836.x]

105 **Dufour A**, Overall CM. Missing the target: matrix metalloproteinase antitargets in inflammation and cancer. *Trends Pharmacol Sci* 2013; **34**: 233-242 [PMID: 23541335 DOI: 10.1016/j.tips.2013.02.004]

106 **Hu J**, Van den Steen PE, Sang QX, Opdenakker G. Matrix metalloproteinase inhibitors as therapy for inflammatory and vascular diseases. *Nat Rev Drug Discov* 2007; **6**: 480-498 [PMID: 17541420 DOI: 10.1038/nrd2308]

107 **Stallmach A**, Chan CC, Ecker KW, Feifel G, Herbst H, Schuppan D, Zeitz M. Comparable expression of matrix metalloproteinases 1 and 2 in pouchitis and ulcerative colitis. *Gut* 2000; **47**: 415-422 [PMID: 10940281 DOI: 10.1136/gut.47.3.415]

108 **Bailey CJ**, Hembry RM, Alexander A, Irving MH, Grant ME, Shuttleworth CA. Distribution of the matrix metalloproteinases stromelysin, gelatinases A and B, and collagenase in Crohn's disease and normal intestine. *J Clin Pathol* 1994; **47**: 113-116 [PMID: 8132824 DOI: 10.1136/jcp.47.2.113]

109 **Ishida K**, Takai S, Murano M, Nishikawa T, Inoue T, Murano N, Inoue N, Jin D, Umegaki E, Higuchi K, Miyazaki M. Role of chymase-dependent matrix metalloproteinase-9 activation in mice with dextran sodium sulfate-induced colitis. *J Pharmacol Exp Ther* 2008; **324**: 422-426 [PMID: 18024785 DOI: 10.1124/jpet.107.131946]

110 **Tarlton JF**, Whiting CV, Tunmore D, Bregenholt S, Reimann J, Claesson MH, Bland PW. The role of up-regulated serine proteases and matrix metalloproteinases in the pathogenesis of a murine model of colitis. *Am J Pathol* 2000; **157**: 1927-1935 [PMID: 11106565 DOI: 10.1016/S0002-9440(10)64831-6]

111 **Garg P**, Rojas M, Ravi A, Bockbrader K, Epstein S, Vijay-Kumar M, Gewirtz AT, Merlin D, Sitaraman SV. Selective ablation of matrix metalloproteinase-2 exacerbates experimental colitis: contrasting role of gelatinases in the pathogenesis of colitis. *J Immunol* 2006; **177**: 4103-4112 [PMID: 16951375 DOI: 10.4049/jimmunol.177.6.4103]

112 **Liu H**, Patel NR, Walter L, Ingersoll S, Sitaraman SV, Garg P. Constitutive expression of MMP9 in intestinal epithelium worsens murine acute colitis and is associated with increased levels of proinflammatory cytokine Kc. *Am J Physiol Gastrointest Liver Physiol* 2013; **304**: G793-G803 [PMID: 23471340 DOI: 10.1152/ajpgi.00249.2012]

113 **Garg P**, Ravi A, Patel NR, Roman J, Gewirtz AT, Merlin D, Sitaraman SV. Matrix metalloproteinase-9 regulates MUC-2 expression through its effect on goblet cell differentiation. *Gastroenterology* 2007; **132**: 1877-1889 [PMID: 17484881 DOI: 10.1053/j.gastro.2007.02.048]

114 **Nighot P**, Al-Sadi R, Rawat M, Guo S, Watterson DM, Ma T. Matrix metalloproteinase 9-induced increase in intestinal epithelial tight junction permeability contributes to the severity of experimental DSS colitis. *Am J Physiol Gastrointest Liver Physiol* 2015; **309**: G988-G997 [PMID: 26514773 DOI: 10.1152/ajpgi.00256.2015]

115 **Cunningham KE**, Turner JR. Myosin light chain kinase: pulling the strings of epithelial tight junction function. *Ann N Y Acad Sci* 2012; **1258**: 34-42 [PMID: 22731713 DOI: 10.1111/j.1749-6632.2012.06526.x]

116 **Agarwal R**, D'Souza T, Morin PJ. Claudin-3 and claudin-4 expression in ovarian epithelial cells enhances invasion and is associated with increased matrix metalloproteinase-2 activity. *Cancer Res* 2005; **65**: 7378-7385 [PMID: 16103090 DOI: 10.1158/0008-5472.CAN-05-1036]

117 **Miyamori H**, Takino T, Kobayashi Y, Tokai H, Itoh Y, Seiki M, Sato H. Claudin promotes activation of pro-matrix metalloproteinase-2 mediated by membrane-type matrix metalloproteinases. *J Biol Chem* 2001; **276**: 28204-28211 [PMID: 11382769 DOI: 10.1074/jbc.M103083200]

118 **Meijer MJ**, Mieremet-Ooms MA, van der Zon AM, van Duijn W, van Hogezand RA, Sier CF, Hommes DW, Lamers CB, Verspaget HW. Increased mucosal matrix metalloproteinase-1, -2, -3 and -9 activity in patients with inflammatory bowel disease and the relation with Crohn's disease phenotype. *Dig Liver Dis* 2007; **39**: 733-739 [PMID: 17602907 DOI: 10.1016/j.dld.2007.05.010]

119 **O'Sullivan S**, Gilmer JF, Medina C. Matrix metalloproteinases in inflammatory bowel disease: an update. *Mediators Inflamm* 2015; **2015**: 964131 [PMID: 25948887 DOI: 10.1155/2015/964131]

120 **Rath T**, Roderfeld M, Graf J, Wagner S, Vehr AK, Dietrich C, Geier A, Roeb E. Enhanced expression of MMP-7 and MMP-13 in inflammatory bowel disease: a precancerous potential? *Inflamm Bowel Dis* 2006; **12**: 1025-1035 [PMID: 17075343 DOI: 10.1097/01.mib.0000234133.97594.04]

121 **Noë V**, Fingleton B, Jacobs K, Crawford HC, Vermeulen S, Steelant W, Bruyneel E, Matrisian LM, Mareel M. Release of an invasion promoter E-cadherin fragment by matrilysin and stromelysin-1. *J Cell Sci* 2001; **114**: 111-118 [PMID: 11112695]

122 **Schneider MR**, Dahlhoff M, Horst D, Hirschi B, Trülzsch K, Müller-Höcker J, Vogelmann R, Allgäuer M, Gerhard M, Steininger S, Wolf E, Kolligs FT. A key role for E-cadherin in intestinal homeostasis and Paneth cell maturation. *PLoS One* 2010; **5**: e14325 [PMID: 21179475 DOI: 10.1371/journal.pone.0014325]

123 **Muise AM**, Walters TD, Glowacka WK, Griffiths AM, Ngan BY, Lan H, Xu W, Silverberg MS, Rotin D. Polymorphisms in E-cadherin (CDH1) result in a mis-localised cytoplasmic protein that is associated with Crohn's disease. *Gut* 2009; **58**: 1121-1127 [PMID: 19398441 DOI: 10.1136/gut.2008.175117]

124 **Davies G**, Jiang WG, Mason MD. Matrilysin mediates extracellular cleavage of E-cadherin from prostate cancer cells: a key mechanism in hepatocyte growth factor/scatter factor-induced cell-cell dissociation and in vitro invasion. *Clin Cancer Res* 2001; **7**: 3289-3297 [PMID: 11595727]

125 **Wheelock MJ**, Buck CA, Bechtol KB, Damsky CH. Soluble 80-kd fragment of cell-CAM 120/80 disrupts cell-cell adhesion. *J Cell Biochem* 1987; **34**: 187-202 [PMID: 3611200 DOI: 10.1002/jcb.240340305]

126 **Hu QP**, Kuang JY, Yang QK, Bian XW, Yu SC. Beyond a tumor suppressor: Soluble E-cadherin promotes the progression of cancer. *Int J Cancer* 2016; **138**: 2804-2812 [PMID: 26704932 DOI: 10.1002/ijc.29982]

127 **Nawrocki-Raby B**, Gilles C, Polette M, Bruyneel E, Laronze JY, Bonnet N, Foidart JM, Mareel M, Birembaut P. Upregulation of MMPs by soluble E-cadherin in human lung tumor cells. *Int J Cancer* 2003; **105**: 790-795 [PMID: 12767064 DOI: 10.1002/ijc.11168]

128 **Schütte A**, Ermund A, Becker-Pauly C, Johansson ME, Rodriguez-Pineiro AM, Bäckhed F, Müller S, Lottaz D, Bond JS, Hansson GC. Microbial-induced meprin β cleavage in MUC2 mucin and a functional CFTR channel are required to release anchored small intestinal mucus. *Proc Natl Acad Sci USA* 2014; **111**: 12396-12401 [PMID: 25114233 DOI: 10.1073/pnas.1407597111]

129 **Shan M**, Gentile M, Yeiser JR, Walland AC, Bornstein VU, Chen K, He B, Cassis L, Bigas A, Cols M, Comerma L, Huang B, Blander JM, Xiong H, Mayer L, Berin C, Augenlicht LH, Velcich A, Cerutti A. Mucus enhances gut homeostasis and oral tolerance by delivering immunoregulatory signals. *Science* 2013; **342**: 447-453 [PMID: 24072822 DOI: 10.1126/science.1237910]

130 **Moss ML**, Jin SL, Milla ME, Bickett DM, Burkhart W, Carter HL, Chen WJ, Clay WC, Didsbury JR, Hassler D, Hoffman CR, Kost TA, Lambert MH, Leesnitzer MA, McCauley P, McGeehan G, Mitchell J, Moyer M, Pahel G, Rocque W, Overton LK, Schoenen F, Seaton T, Su JL, Becherer JD. Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor-alpha. *Nature* 1997; **385**: 733-736 [PMID: 9034191 DOI: 10.1038/385733a0]

131 **Ma TY**, Iwamoto GK, Hoa NT, Akotia V, Pedram A, Boivin MA, Said HM. TNF-alpha-induced increase in intestinal epithelial tight junction permeability requires NF-kappa B activation. *Am J Physiol Gastrointest Liver Physiol* 2004; **286**: G367-G376 [PMID: 14766535 DOI: 10.1152/ajpgi.00173.2003]

132 **Al-Sadi R**, Guo S, Ye D, Ma TY. TNF-α modulation of intestinal

epithelial tight junction barrier is regulated by ERK1/2 activation of Elk-1. *Am J Pathol* 2013; **183**: 1871-1884 [PMID: 24121020 DOI: 10.1016/j.ajpath.2013.09.001]

133 **Moss ML**, White JM, Lambert MH, Andrews RC. TACE and other ADAM proteases as targets for drug discovery. *Drug Discov Today* 2001; **6**: 417-426 [DOI: 10.1016/S1359-6446(01)01738-X]

134 **Colón AL**, Menchén LA, Hurtado O, De Cristóbal J, Lizasoain I, Leza JC, Lorenzo P, Moro MA. Implication of TNF-alpha convertase (TACE/ADAM17) in inducible nitric oxide synthase expression and inflammation in an experimental model of colitis. *Cytokine* 2001; **16**: 220-226 [PMID: 11884025 DOI: 10.1006/cyto.2001.0969]

135 **Brynskov J**, Foegh P, Pedersen G, Ellervik C, Kirkegaard T, Bingham A, Saermark T. Tumour necrosis factor alpha converting enzyme (TACE) activity in the colonic mucosa of patients with inflammatory bowel disease. *Gut* 2002; **51**: 37-43 [PMID: 12077089 DOI: 10.1136/gut.51.1.37]

136 **Forsyth CB**, Banan A, Farhadi A, Fields JZ, Tang Y, Shaikh M, Zhang LJ, Engen PA, Keshavarzian A. Regulation of oxidant-induced intestinal permeability by metalloprotease-dependent epidermal growth factor receptor signaling. *J Pharmacol Exp Ther* 2007; **321**: 84-97 [PMID: 17220428 DOI: 10.1124/jpet.106.113019]

137 **Fréour T**, Jarry A, Bach-Ngohou K, Dejoie T, Bou-Hanna C, Denis MG, Mosnier JF, Laboisse CL, Masson D. TACE inhibition amplifies TNF-alpha-mediated colonic epithelial barrier disruption. *Int J Mol Med* 2009; **23**: 41-48 [PMID: 19082505]

138 **Man SM**, Kanneganti TD. Converging roles of caspases in inflammasome activation, cell death and innate immunity. *Nat Rev Immunol* 2016; **16**: 7-21 [PMID: 26655628 DOI: 10.1038/nri.2015.7]

139 **Leblond CP**. The life history of cells in renewing systems. *Am J Anat* 1981; **160**: 114-158 [PMID: 6168194 DOI: 10.1002/aja.1001600202]

140 **Fiocchi C**. Inflammatory bowel disease: etiology and pathogenesis. *Gastroenterology* 1998; **115**: 182-205 [PMID: 9649475 DOI: 10.1016/S0016-5085(98)70381-6]

141 **Ruemmele FM**, Seidman EG, Lentze MJ. Regulation of intestinal epithelial cell apoptosis and the pathogenesis of inflammatory bowel disorders. *J Pediatr Gastroenterol Nutr* 2002; **34**: 254-260 [PMID: 11964947 DOI: 10.1097/00005176-200203000-00005]

142 **Bojarski C**, Weiske J, Schöneberg T, Schröder W, Mankertz J, Schulzke JD, Florian P, Fromm M, Tauber R, Huber O. The specific fates of tight junction proteins in apoptotic epithelial cells. *J Cell Sci* 2004; **117**: 2097-2107 [PMID: 15054114 DOI: 10.1242/jcs.01071]

143 **Bojarski C**, Gitter AH, Bendfeldt K, Mankertz J, Schmitz H, Wagner S, Fromm M, Schulzke JD. Permeability of human HT-29/B6 colonic epithelium as a function of apoptosis. *J Physiol* 2001; **535**: 541-552 [PMID: 11533143 DOI: 10.1111/j.1469-7793.2001.00541.x]

144 **Beeman N**, Webb PG, Baumgartner HK. Occludin is required for apoptosis when claudin-claudin interactions are disrupted. *Cell Death Dis* 2012; **3**: e273 [PMID: 22361748 DOI: 10.1038/cddis.2012.14]

145 **Kostic AD**, Xavier RJ, Gevers D. The microbiome in inflammatory bowel disease: current status and the future ahead. *Gastroenterology* 2014; **146**: 1489-1499 [PMID: 24560869 DOI: 10.1053/j.gastro.2014.02.009]

146 **Motta JP**, Martin L, Vergnolle N. Proteases/Antiproteases in Inflammatory Bowel Disease. In: Vergnolle N, Chignard M, SpringerLink (Online service), editors. Proteases and Their Receptors in Inflammation. Basel: Springer Basel AG, 2011: 1 online resource

147 **Steck N**, Mueller K, Schemann M, Haller D. Bacterial proteases in IBD and IBS. *Gut* 2012; **61**: 1610-1618 [PMID: 21900548 DOI: 10.1136/gutjnl-2011-300775]

148 **Róka R**, Rosztóczy A, Leveque M, Izbéki F, Nagy F, Molnár T, Lonovics J, Garcia-Villar R, Fioramonti J, Wittmann T, Bueno L. A pilot study of fecal serine-protease activity: a pathophysiologic

factor in diarrhea-predominant irritable bowel syndrome. *Clin Gastroenterol Hepatol* 2007; **5**: 550-555 [PMID: 17336590 DOI: 10.1016/j.cgh.2006.12.004]

149 **Obiso RJ**, Azghani AO, Wilkins TD. The Bacteroides fragilis toxin fragilysin disrupts the paracellular barrier of epithelial cells. *Infect Immun* 1997; **65**: 1431-1439 [PMID: 9119484]

150 **Wells CL**, van de Westerlo EM, Jechorek RP, Feltis BA, Wilkins TD, Erlandsen SL. Bacteroides fragilis enterotoxin modulates epithelial permeability and bacterial internalization by HT-29 enterocytes. *Gastroenterology* 1996; **110**: 1429-1437 [PMID: 8613048 DOI: 10.1053/gast.1996.v110.pm8613048]

151 **Zhang Z**, Wang L, Seydel KB, Li E, Ankri S, Mirelman D, Stanley SL. Entamoeba histolytica cysteine proteinases with interleukin-1 beta converting enzyme (ICE) activity cause intestinal inflammation and tissue damage in amoebiasis. *Mol Microbiol* 2000; **37**: 542-548 [PMID: 10931347 DOI: 10.1046/j.1365-2958.2000.02037.x]

152 **Kissoon-Singh V**, Moreau F, Trusevych E, Chadee K. Entamoeba histolytica exacerbates epithelial tight junction permeability and proinflammatory responses in Muc2(-/-) mice. *Am J Pathol* 2013; **182**: 852-865 [PMID: 23357502 DOI: 10.1016/j.ajpath.2012.11.035]

153 **Betanzos A**, Javier-Reyna R, García-Rivera G, Bañuelos C, González-Mariscal L, Schnoor M, Orozco E. The EhCPADH112 complex of Entamoeba histolytica interacts with tight junction proteins occludin and claudin-1 to produce epithelial damage. *PLoS One* 2013; **8**: e65100 [PMID: 23762290 DOI: 10.1371/journal.pone.0065100]

154 **Pruteanu M**, Hyland NP, Clarke DJ, Kiely B, Shanahan F. Degradation of the extracellular matrix components by bacterial-derived metalloproteases: implications for inflammatory bowel diseases. *Inflamm Bowel Dis* 2011; **17**: 1189-1200 [PMID: 20853433 DOI: 10.1002/ibd.21475]

155 **Maharshak N**, Huh EY, Paiboonrungruang C, Shanahan M, Thurlow L, Herzog J, Djukic Z, Orlando R, Pawlinski R, Ellermann M, Borst L, Patel S, Dotan I, Sartor RB, Carroll IM. Enterococcus faecalis Gelatinase Mediates Intestinal Permeability via Protease-Activated Receptor 2. *Infect Immun* 2015; **83**: 2762-2770 [PMID: 25916983 DOI: 10.1128/IAI.00425-15]

156 **Steck N**, Hoffmann M, Sava IG, Kim SC, Hahne H, Tonkonogy SL, Mair K, Krueger D, Pruteanu M, Shanahan F, Vogelmann R, Schemann M, Kuster B, Sartor RB, Haller D. Enterococcus faecalis metalloprotease compromises epithelial barrier and contributes to intestinal inflammation. *Gastroenterology* 2011; **141**: 959-971 [PMID: 21699778 DOI: 10.1053/j.gastro.2011.05.035]

157 **Wan H**, Winton HL, Soeller C, Tovey ER, Gruenert DC, Thompson PJ, Stewart GA, Taylor GW, Garrod DR, Cannell MB, Robinson C. Der p 1 facilitates transepithelial allergen delivery by disruption of tight junctions. *J Clin Invest* 1999; **104**: 123-133 [PMID: 10393706 DOI: 10.1172/JCI5844]

158 **Tulic MK**, Vivinus-Nébot M, Rekima A, Rabelo Medeiros S, Bonnart C, Shi H, Walker A, Dainese R, Boyer J, Vergnolle N, Piche T, Verhasselt V. Presence of commensal house dust mite allergen in human gastrointestinal tract: a potential contributor to intestinal barrier dysfunction. *Gut* 2016; **65**: 757-766 [PMID: 26646935 DOI: 10.1136/gutjnl-2015-310523]

159 **Yu LC**. Intestinal epithelial barrier dysfunction in food hypersensitivity. *J Allergy* (Cairo) 2012; **2012**: 596081 [PMID: 21912563]

160 **Perrier C**, Corthésy B. Gut permeability and food allergies. *Clin Exp Allergy* 2011; **41**: 20-28 [PMID: 21070397 DOI: 10.1111/j.1365-2222.2010.03639.x]

161 **Grozdanovic MM**, Čavić M, Nešić A, Andjelković U, Akbari P, Smit JJ, Gavrović-Jankulović M. Kiwifruit cysteine protease actinidin compromises the intestinal barrier by disrupting tight junctions. *Biochim Biophys Acta* 2016; **1860**: 516-526 [PMID: 26701113 DOI: 10.1016/j.bbagen.2015.12.005]

162 **Cavic M**, Grozdanovic MM, Bajic A, Jankovic R, Andjus PR, Gavrovic-Jankulovic M. The effect of kiwifruit (Actinidia deliciosa) cysteine protease actinidin on the occludin tight junction network in T84 intestinal epithelial cells. *Food Chem Toxicol* 2014;

72: 61-68 [PMID: 25042511 DOI: 10.1016/j.fct.2014.07.012]

163 **Yang Y**, Sitanggang NV, Kato N, Inoue J, Murakami T, Watanabe T, Takafumi Iguchi, Yukako Okazaki. Beneficial effects of protease preparations derived from Aspergillus on the colonic luminal environment in rats consuming a high-fat diet. *Biomed Rep* 2015; **3**:

715-720 [DOI: 10.3892/br.2015.490]

164 **Morrison DJ**, Preston T. Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. *Gut Microbes* 2016; **7**: 189-200 [PMID: 26963409 DOI: 10.1080/1949 0976.2015.1134082]

Diet and microbiota in inflammatory bowel disease: The gut in disharmony

Davy CM Rapozo, Claudio Bernardazzi, Heitor Siffert Pereira de Souza

Davy CM Rapozo, Coordenação de Pesquisa, Instituto Nacional de Câncer, Rio de Janeiro, RJ 20230-130, Brazil

Claudio Bernardazzi, Heitor Siffert Pereira de Souza, Serviço de Gastroenterologia e Laboratório Multidisciplinar de Pesquisa, Departamento de Clínica Médica, Hospital Universitário Clementino Fraga Filho, Universidade Federal do Rio de Janeiro, RJ 21941-913, Brazil

Heitor Siffert Pereira de Souza, D'Or Institute for Research and Education, Rua Diniz Cordeiro 30, Botafogo, Rio de Janeiro, RJ 22281-100, Brazil

Abstract

Bacterial colonization of the gut shapes both the local and the systemic immune response and is implicated in the modulation of immunity in both healthy and disease states. Recently, quantitative and qualitative changes in the composition of the gut microbiota have been detected in Crohn's disease and ulcerative colitis, reinforcing the hypothesis of dysbiosis as a relevant mechanism underlying inflammatory bowel disease (IBD) pathogenesis. Humans and microbes have co-existed and co-evolved for a long time in a mutually beneficial symbiotic association essential for maintaining homeostasis. However, the microbiome is dynamic, changing with age and in response to environmental modifications. Among such environmental factors, food and alimentary habits, progressively altered in modern societies, appear to be critical modulators of the microbiota, contributing to or co-participating in dysbiosis. In addition, food constituents such as micronutrients are important regulators of mucosal immunity, with direct or indirect effects on the gut microbiota. Moreover, food constituents have recently been shown to modulate epigenetic mechanisms, which can result in increased risk for the development and progression of IBD. Therefore, it is likely that a better understanding of the role of different food components in intestinal homeostasis and the resident microbiota will be essential for unravelling the complex molecular basis of the epigenetic, genetic and environment interactions underlying IBD pathogenesis as well as for offering dietary interventions with minimal side effects.

Core tip: The gut microbiota has a recognized role in immunity, and changes in its composition, or dysbiosis, may be the basis for the worldwide increased incidence of inflammatory bowel disease (IBD). Dietary constituents have been shown to affect the immune response and the inflammatory status, in great part mediated through the modulation of the microbiota. Environmental compounds, including nutrients, can induce alterations in the epigenome interface, resulting in long lasting phenotypic or even tissue structure and function modifications. Unravelling the complex molecular basis of the epigenetic, genetic and environmental interactions underlying IBD pathogenesis will have implications for the development of novel therapies.

INTRODUCTION

The gastrointestinal tract is relentlessly challenged by the luminal contents harbouring innumerable microorganisms and food antigens. To maintain the normal homeostatic equilibrium, it is critical for the system to be capable of identifying whether a stimulus is pathogenic or not and of mounting an appropriate response, resulting in either inflammation or tolerance[1]. Particularly in the context of the gut, defence mechanisms and tolerance should act in concert, allowing the organism to control the inflammatory response and tissue injury that may occur following exposure to a given pathogen[2]. While immune deficiency inevitably culminates in recurrent infections, defective tolerance may result in uncontrolled inflammation and immunopathology[3]. In fact, an abnormal relationship between host and microbiota is believed to result in intestinal immune imbalance[4], leading to the development of conditions such as inflammatory bowel disease (IBD), which consists of two major forms, Crohn's disease (CD) and ulcerative colitis (UC)[5,6]. In this paper, we discuss basic mechanisms and potential connections between microbiota, diet, and the development of IBD.

INTESTINAL MICROBIOTA

A mutually beneficial association between humans and microbes is essential for maintaining homeostasis. Such co-existence highlights the predominantly symbiotic nature of the interaction between humans and microorganisms despite the remarkable variation that occurs over time at diverse body sites[7]. As a consequence, abnormalities of the intestinal microbiota have been implicated in the pathogenesis of several health conditions, including gastrointestinal diseases.

The development and adaptation of the intestinal microbiota represents a continuous process that occurs throughout the lifetime. In this regard, several environmental factors contribute to the microbial colonization of the gastrointestinal tract. The composition of the intestinal microbiota is affected very early in life, beginning with the route of delivery[8]. Shortly after birth, breast-feeding, exposure to food and other environmental factors play a pivotal role in the development of the intestinal microbiota. The microbial composition of the gut, in turn, also shapes the development of both the innate and the adaptive immune system[9]. The commensal microbiota is universally distributed throughout the gastrointestinal tract, with a characteristic progressive increase in both diversity and density from the upper to the lower segments. Studies of the human microbiome have identified more than three million unique genes within the gut, widely outnumbering the human genome and containing more than a thousand bacterial species, most of them of the *Bacteroidetes* and *Firmicutes* phyla[10]. In fact, several different groups around the world are currently investigating the composition of the human microbiome. Recently, the phylogenetic composition of faecal samples from different nationalities was investigated in a metagenomic analysis, which demonstrated the presence of robust bacterial clusters, defined as enterotypes. These enterotypes, mostly defined by species composition, were not nation- or continent-specific, supporting the idea of a relatively limited number of established host-microbe symbiotic conditions, which may behave distinctly upon exposure to food or drugs[11].

The complexity of the human gut microbiome is further evidenced by the spatial distribution and alternation of microorganisms throughout the length of the gastrointestinal tract and across the radial axis. It has been demonstrated, for example, that different bacteria inhabit distinct segments of the intestine and are found in different layers of the gut, such as the central lumen, associated with mucosal folds, or embedded in the mucus layer[12]. Together, these findings support the hypothesis that the resident or autochthonous microbiota has been modified to adapt to new functional specializations, therefore playing a distinct role compared to the transient microbiota present in the faecal stream. In this sense, each intestinal niche is thought to shelter the microbes that would be the most convenient to preserve local tissue homeostasis and exhibiting clear beneficial mutualism with the host[12].

EFFECTS OF THE INTESTINAL MICROBIOTA ON IMMUNITY

Currently, it is well accepted that one of the key functions of the gut microbiota, in addition to nutrition, metabolism and energy production, consists of the development and maturation of the immune system[13]. In fact, bacterial colonization of the gut is believed to shape not only the local but also the systemic immune response, being implicated in the modulation of immunity in both healthy and disease states[14]. Under normal conditions, gastrointestinal microorganisms are recognized by NOD-like and Toll-like receptors, specialized molecules of the innate immune system predominantly localized in epithelial and immune cells, and this recognition process results in activation of the immune response, which is indispensable to intestinal homeostasis[15].

To maintain homeostasis, the microbiota is regulated by several mechanisms involving epithelial and immune cell molecules, including IgA, RegⅢγ, and defensins, whereas the immune response is reciprocally regulated by the microbiota, with particular microorganisms promoting the growth of distinct T cell subsets[16]. For example, commensal segmented filamentous bacteria were shown to induce Th17 cells[17,18] capable of identifying extraintestinal autoimmune inflammation in experimental models[19,20]. On the other hand, *Clostridia* and *Bacteroides fragilis* were shown to favour the induction of Treg cells and type 1 T helper (Th1) cells, respectively[16]. Of note, *Clostridia* were demonstrated to induce Tregs within the gut with a concomitant down-regulation of Th1 and Th17 responses[21]. Although the exact mechanism by which Tregs are induced by the intestinal microbiota are yet to be determined, there is evidence suggesting a role for microbe-derived short-chain fatty acids[22]. Alimentary fibres are not digested by the human gastrointestinal tract but, instead, they are fermented in the gut by bacteria, which in turn modifies the gut microbiota. The microbial processing of fibres results in the formation of short-chain fatty acids (SCFAs), such as acetate, propionate and butyrate, which are used by colonocytes as crucial energy sources, with important anti-inflammatory activities *in vitro* and *in vivo*[23,24]. In particular, butyrate, produced by commensal bacteria, was also shown to participate in Treg differentiation and suppression of pro-inflammatory cytokines from macrophages and dendritic cells[25,26], while its *in vivo* administration was shown to ameliorate experimental colitis[27], suggesting the importance of specific luminal nutrients in the homeostasis of the colon.

In addition to the effects of the gut microbiota on immunity, dietary factors have also been implicated in gut microbial regulation of intestinal immunity. Therefore, diet has emerged as another critical element that interacts with the microbiota and immunity to actively affect homeostatic control[28].

DIET: INFLUENCE ON THE INTESTINAL MICROBIOTA

The influence of food in shaping the intestinal microbiota has been hypothesized for a long time. However, only in recent years have consistent data on this subject been obtained, due in particular to the advent of technologies such as next-generation DNA sequencing and metabolic profiling[29]. As a result, interesting new data have been reported, consequently shaping conceptual changes in the field. For example, the role of early nutrition in moulding the gut microbiota appears to impact the risk of diseases development even late in life[30,31]. Furthermore, it is now clear that the microbiota composition is dynamic, changing with age and oscillating according to environmental modifications, including food intake patterns, among other factors[32].

Network-based studies of microbial communities performed with faecal samples of several mammalian species have confirmed that diet does determine bacterial diversity, which increases from carnivore to omnivore to herbivore, whereas microbial communities diversify concomitantly with their hosts, supporting the hypothesis of the co-evolution of gut microbiotas and their hosts[33]. Although there is a general assumption that the typical modern human intestinal microbiota tends to be one of omnivorous habits, considerable heterogeneity still exists in the world, with some remarkable discrepancies. An interesting study, for example, demonstrated substantial differences in the intestinal microbiota of children living in African rural communities compared with children living in Europe. The guts of African children were rich in *Bacteroidetes* and poor in *Firmicutes* and *Enterobacteriaceae,* while the results obtained from European children were quite the opposite[34]. The investigators suggested that the findings were mostly attributable to radically different dietary patterns (Table 1).

Following the same line of evidence, several other studies have raised the issue of diet potentially affecting the gut microbiota. Of note, animal fat-based diets and carbohydrate-based diets lead to a specific enrichment of *Bacteroides* and *Prevotella* in adult individuals. Moreover, it is important to highlight that the gut microbiome composition undergoes relatively rapid changes upon exposure to a low-fat/high-fibre or high-fat/low-fibre diet, for example[35]. In another short-term dietary intervention in humans, in contrast to the effects of a plant-based diet, consumption of strictly animal-based products increased the abundance of bile-tolerant microorganisms and decreased the levels of Firmicutes that metabolize dietary plant polysaccharides. These results reflect the differences between herbivorous and carnivorous habits, depicting specific adjustments between carbohydrate and protein fermentation. In particular, the identification of increases in the abundance

Table 1 Gut microbiota taxonomic classification and alterations associated with dietary patterns or the presence of inflammatory bowel disease

Phylum	Class	Order	Family	Genus	Species	Characteristics	Action in the GI tract	Ref.
Bacteroidetes	Bacteroidetes	Bacteroidales	Prevotellaceae	Prevotella	*P. sp*	Gram-negative Anaerobic Commensal bacteria	Diets rich in carbohydrates and fat Involved in colitis	[10, 16, 22-24, 34, 35, 37, 38, 43, 44, 80, 82, 83]
				Bacteroides	*B. fragilis* *B. uniformis*			
Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium	*C. lavalense* *C. perfringens*	Gram-positive Anaerobic	Play a role in the clinical course of IBD	[10, 16, 21, 34, 37, 38, 43, 44, 80-84, 86, 87, 129, 159, 160, 181-185]
			Ruminococcaceae	Ruminococcus	*R. torques*	Gram-positive Anaerobic	Fermentation of dietary fibre	
				Faecalibacterium/ Fusobacterium	*F. prausnitzii*	Anaerobic Commensal bacteria		
			Lachnospiraceae	Roseburia	*R. faecis* *R. hominis* *R. cecicola* *R. intestinalis* *R. inulinivorans*	Gram-positive Anaerobic	Fermentation of dietary fibre	
				Fusicatenibacter	*F. saccharivorans*			
				Blautia	*B. faecis*		Present in the intestine	
	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	*S. spp*	Gram-positive	Part of the normal animal microbiota	
			Lactobacillaceae	Lactobacillus	*L. acidophilus*		Induces remission in UC patients	
	Negativicutes	Veillonellales	Veillonellaceae	Veillonella	*V. spp*	Gram-negative Anaerobic	Present in the intestine and oral mucosa	
	Erysipelotrichia	Erysipelotrichales	Erysipelotrichaceae	Turicibacter	*T. sp*	Gram-positive	Present in mammal intestines	
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Escherichia	*E. coli*	Gram-negative Anaerobic	Involved in colitis	[34, 36, 80, 83, 186]
		Desulfovibrionales	Desulfovibrionaceae	Bilophila	*B. wadsworthia*			
		Pasteurellales	Pasteurellaceae	Pasteurella	*P. sp*	Gram-negative Commensal bacteria Facultative anaerobes	Present in the nose and mouth	
Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	Bifidobacterium	*B. breve* *B. bifidum*	Gram-positive Anaerobic	Induces remission in UC patients	[159, 187]
Fusobacteria	Fusobacteria	Fusobacteriales	Fusobacteriaceae	Fusobacterium	*F. spp*	Gram-negative Anaerobic	Involved in colitis and colon cancer	[83]

The five major bacterial phyla of the human GI microbiota and the potential relationship with diet and inflammatory bowel disease.

and activity of *Bilophila wadsworthia* as a result of an animal-based diet was interpreted as a probable link between dietary fat, bile acids, and the prominence of microorganisms potentially involved in the development of IBD[36].

Several studies have provided additional information on dietary fibre supplementation and the effect of SCFAs on the intestinal microbiota. In regards to the type of SCFA generated in the intestine, both the type of fibre ingested, usually composed of non-digestible complex carbohydrates, and the metabolizing microbiota are determining factors. While resistant starch promotes the production of relatively more butyrate, pectin leads to more acetate and propionate production. Regarding the gut microbiota, bacteria of the Bacteroidetes phylum predominantly produce more acetate and propionate, whereas bacteria of the Firmicutes phylum predominantly produce butyrate[37,38]. In animal models of colitis, for example, dietary fibres, including fermentable fibres and starches, are metabolized by colonic bacteria into SCFAs, with relevant anti-inflammatory effects[39-41]. In addition to the high fat content of Western diets in general, it is also important to call attention to the high levels of dietary omega-6 fatty acids, due to the use of vegetable oils, resulting in a high omega-6 to omega-3 ratio. Omega-6 fatty acids, especially arachidonic acid, are potentially

pro-inflammatory, whereas omega-3 fatty acids, such as a-linolenic acid from plants and eicosapentaenoic acid and docosahexaenoic acid from fish, are anti-inflammatory[42].

High caloric intake with a large consumption of carbohydrates, typical of Western diets, has been associated with less microbiome diversity, in contrast to the Mediterranean diet based on fruits, vegetables, and red wine[43]. Nevertheless, recently, exclusion diets such as the specific carbohydrate diet (SCD, which restricts all carbohydrates except monosaccharides) and a diet low in fermentable oligo-, di-, and monosaccharides and polyols (FODMAPs) have produced promising results in IBD[44]. In uncontrolled trials of restriction diets for IBD, SCD-like diets were shown to reduce symptoms and intestinal inflammation[45,46]. These observations support the notion that dietary manipulations might modify the intestinal microbiota despite the presence of resident enterotypes settled by long-term dietary patterns.

The effects of specific nutritional changes on the mammalian system have been increasingly investigated, including the impact of micronutrients on the gut microbiota. For example, in weaned-mouse models of zinc or protein deficiency, considerable changes in the gut microbiota were observed, in addition to reductions in microbial proteolysis and increases in microbial dietary choline processing[47]. Processed foods are usually low in micronutrients and have been associated with a greater risk of developing several diseases. In this sense, zinc and other nutrients such as n-3 fatty acids and vitamins D and E are thought to protect from preclinical and/or clinical type 1 diabetes, for example[48].

INTESTINAL MICROBIOTA-HOST INTERACTIONS AND THE DEVELOPMENT OF IBD

In the last decade, the intestinal microbiota-host interaction has gained progressively increasing attention, as it has been associated, directly or indirectly, with a variety of immune, inflammatory, and metabolic disorders[49]. Furthermore, in recent years, the increase in the incidence of autoimmune and chronic inflammatory disorders has been attributed to alterations in the microbial composition and the role of the intestinal microbiota in immune regulation[50]. Modifications in human habits have been implicated in the rise of IBD worldwide[51]. This thought is supported by the evidence showing a consistent increase in the incidence and prevalence of IBD in Western countries and, more recently, in the Asia Pacific area[52].

The idea of "Western lifestyle factors" triggering intestinal inflammation appears to be reinforced by the dramatic increase in the incidence of IBD in last half century, which is likely not paralleled by changes in the human genome[53,54]. In this regard, several factors such as the improvement of general sanitary conditions and antibiotic usage, resulting in a decreased incidence of infectious diseases, coincide with the increase in autoimmune diseases and chronic inflammatory conditions, constituting the basis for the hygiene hypothesis[55,56]. In fact, some events likely related to changes in the gut microbiota appear to be associated with the development of IBD. For example, the risk of IBD has been shown to increase after an episode of acute gastroenteritis[57] and in children repeatedly treated with antibiotics[58]. IBD-associated genetic findings have also provided important evidence for the role of microorganisms in disease pathogenesis. Several sources of information, including genome-wide association studies, have identified more than 200 genetic risk loci as predisposing factors for IBD. Of note, several of the genetic risk alleles for IBD are directly associated with pathways that regulate the adaptive immune system, while many others are involved in innate immune responses or epithelial barrier regulation, crucial mechanisms in the defence against microbial invasion[59,60] (Figure 1).

Intestinal microbiota in IBD

Interestingly, abnormalities of the gut microbiota are present in common intestinal conditions, including irritable bowel syndrome, chronic idiopathic diarrhoea, and IBD[61-63]. In addition, recent evidence has suggested that the impact of the intestinal microbiota in disease pathogenesis can extend to other immune-mediated conditions beyond the gut including, for example, type 1 diabetes, cardiovascular disease, and autoimmune demyelination[64-66].

In IBD, distinct abnormalities of the intestinal microbiota have been reported, including changes in the microbial composition, an inappropriate immune response towards commensal microorganisms, or even both[67]. In CD, for example, immune reactivity against microbial-derived antigens has long been reported, characterized by several different circulating serum antibodies[68-71]. Another clinically relevant observation to support a role for the gut microbiota in the inflammatory process of CD comes from postsurgical relapses triggered by agents present in the faecal stream[72]. Recently, longitudinal studies have provided evidence implicating dietary patterns as risk factors for IBD. In general, a lower risk of IBD has been associated with habits of consuming more vegetables and fruits, in contrast to a higher risk among people whose diet is based more on animal fats and sugar[73-76]. In particular, the association between the ingestion of fats and the development of UC has been most prominently related to the long-term high intake of trans-unsaturated fats[76], likely due to dietary linoleic acid, an n-6 polyunsaturated fatty acid[75]. Of note, dietary-fat-induced taurocholic acid, secondary to the intake of saturated fats from milk, was shown
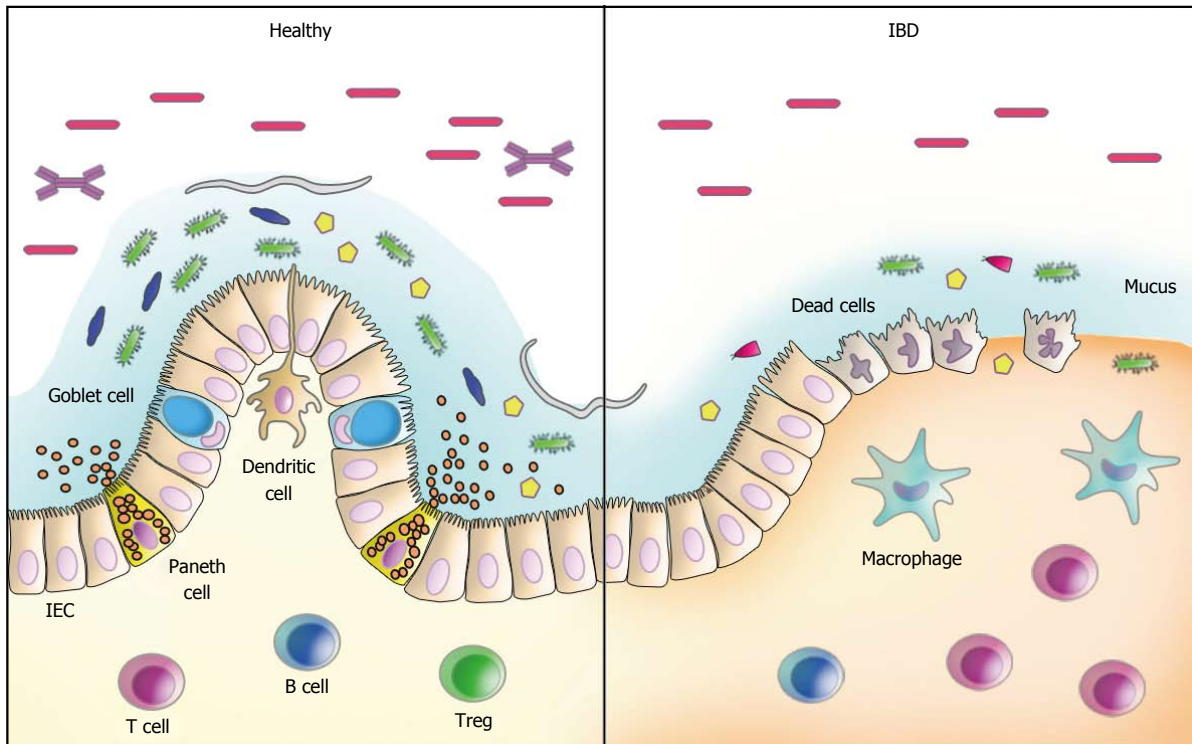
Figure 1 Schematic model of host-microbiota interactions in the intestine. The interaction between the resident (autochthonous) microbiota and the mucosal immune system is highly complex and, in normal conditions, results in a tolerogenic response. In genetically predisposed individuals, a dysbiotic microbiota, fuelled by environmental factors, particularly dietary constituents, induces pathogenic immune recognition and responses, further compromising the epithelial barrier and defence mechanisms, leading to chronic inflammation, as observed in inflammatory bowel disease.

to boost pathobiont expansion, triggering colitis in IL-10-deficient mice, with the induction of a pro-inflammatory Th1 immune response[77].

Quantitative and qualitative changes in the composition of the gut microbiota have been detected in CD and in UC, reinforcing the hypothesis of dysbiosis as a relevant mechanism underlying IBD pathogenesis[78]. Changes in the composition of the intestinal microbiota have been reported in CD, for example, including an overall decreased diversity[79] but also an increase in Bacteroidetes and Proteobacteria paralleled by a decrease in Firmicutes abundance[80]. Additional evidence corroborating the role of bacteria in intestinal inflammation was the finding of a lower proportion of *Faecalibacterium prausnitzii*, a member of the phylum Firmicutes with anti-inflammatory properties, in patients with CD with increased risk of postoperative recurrence after resection for ileal disease[81]. At the species level, in addition to *Faecalibacterium prausnitzii*, several other butyrate-producing bacterial species, such as *Blautia faecis, Roseburia inulinivorans, Ruminococcus torques, Clostridium lavalense*, and *Bacteroides uniformis*, were also shown to be significantly reduced in CD patients[82]. Also interesting is the fact that exposure to antibiotics may amplify the microbial dysbiosis associated with CD. In particular, in a large paediatric cohort of new-onset CD, an increased abundance of bacteria including Enterobacteriaceae, Pasteurellaceae,

Veillonellaceae, and Fusobacteriaceae and a decreased abundance of Erysipelotrichales, Bacteroidales, and Clostridiales were consistently correlated with disease severity[83]. The changes in microbial composition in CD have been further corroborated by a recent systematic review confirming a relative increase in Bacteroidetes and decrease in Firmicutes abundance. In particular, Enterobacteriaceae were increased, while *Faecalibacterium prausnitzii* was found at a lower abundance, including in patients with postoperative recurrence[84].

Abnormalities in the intestinal microbiota have also been detected in UC, although to a lesser degree compared to CD patients[85]. Nevertheless, a less diverse microbiota was also demonstrated in samples from patients with UC and, in particular, the finding of increased *C. perfringens* in faeces suggested its role in disease exacerbation[86]. In another study, investigators identified a decrease in *Fusicatenibacter saccharivorans* in patients with active UC, in contrast to the increase observed in patients with quiescent disease[87].

Whether dysbiosis consists of a primary or secondary phenomenon in IBD is a question that remains unanswered. There is evidence showing that the intestinal microbiota can be shaped by the host's genotype[88,89] but also by diet, habits, history of infections, use of antibiotics or other medications, and the inflammatory process[14,90-92]. On the other hand, it is

important to call attention to the fact that dysbiosis alone may not be sufficient to induce IBD.

Several defects in the inflammatory response against microbial agents that have been reported in IBD[93,94] lend support to the idea of an inadequate clearance of microbial-associated molecular patterns as an important underlying mechanism of disease[95]. This may be particularly relevant in CD, due to the well-established association of the disease with genetic polymorphisms of *NOD2* and *ATG16L1*, for example, which result in defective autophagy and impaired microbial clearance[96-98]. Another important mechanistic association in intestinal inflammation is believed to occur in response to the accumulation of unfolded proteins in the lumen of the endoplasmic reticulum (ER stress), resulting in the activation of intracellular signal transduction pathways, known as the unfolded protein response (UPR). In addition to the relationship with autophagy, ER stress has been associated with intestinal inflammation and IBD based on studies revealing primary genetic alterations involving *XBP1*, *ARG2*, *ORMDL3*, and other components of the UPR[99,100]. Another example of an inadequate microbial recognition and control comes from the reduced expression of defensins, antimicrobial peptides produced by Paneth cells, in patients with *NOD2* mutations, with expected implications for CD[101]. Individual or combined defects involving various genes such as *NOD2*, *ATG16L1* and *IRGM* might result in inadequate recognition of microorganisms present in the intestinal lumen[102] and subsequently defective induction of autophagy, activation of alternate pathways, and modulation of adaptive immunity[103]. In addition to *ATG16L1*, polymorphisms of the immunity-related GTPase family M (IRGM) gene, shown to be involved in the process of microbial control, have also been associated with CD[104,105]. Furthermore, the interaction between single nucleotide polymorphisms of ATG16L1 and IRGM has also been demonstrated in CD[106], indicating the probable integration of defective autophagy with mitochondrial dysfunction and apoptosis. Together, the knowledge accumulated in the last few years in the field of IBD, in addition to shedding light on new mechanisms, has revealed the multiple redundant and overlapping pathways underlying the disease pathogenesis. In addition, the information accumulated matches, in great part, the recent epidemiological changes in IBD distribution and reinforce the participation of dysbiosis in disease pathogenesis[56].

DIET, MICROBIOME AND EPIGENETIC CHANGES IN IBD

Environmental factors have been recognized as fundamental elements in the perinatal maturation of the immune system. In this sense, the microbial colonization of mucosal surfaces becomes of critical importance in the development and maturation of the mucosal immune system[107,108]. At birth, the transition from the sterile foetal environment is marked by exposure to a large number of exogenous stimuli. Interestingly, after natural birth, a newborn's microbiota composition tends to resemble that of the maternal vaginal or gut microbiota, while after Caesarean section, the microbiota contains a considerable number of environmental agents[109]. The subsequent microbiota that establishes thereafter has an increasingly diverse composition, but individualities are preserved and are relatively stable over time[110]. Among the environmental factors, food components contribute to the development of the immune response both directly and indirectly. Early in life, breast-feeding provides several important elements in the defence against pathogens, such as IgA, cytokines, growth factors, and high concentrations of oligosaccharides that foster the accumulation of lactic acid-producing bacteria in the gut[111]. Moreover, in terms of IBD, the effect of breast-feeding may prove to be more important than previously thought, as the results of a meta-analysis have suggested that it might play a protective role against the development of paediatric disease[112].

Several other data exist to support the participation of dietary elements in the definition of the microbiota itself and the interaction with the immune system. For example, Western-like diets with their ubiquitous food additives were shown to affect the composition and function of the microbiota[113]. Retinoic acid, a derivative of vitamin A, is important in the development of the neonatal immune system, for cellular and subcellular membrane stability and in epithelial surfaces[114], and in adults, where it is required for the expression of gut-homing molecules on immune cells, the induction of Tregs and IgA class switching[115]. Iron, an essential element in haematopoiesis, may also trigger inflammatory processes associated with CD progression, as luminal iron may directly modify epithelial cell function or generate a pathological milieu due to alterations of the intestinal microbiota[116]. Vitamin D induces tolerogenic dendritic cells and is now regarded as an important regulator of mucosal immunity[117]. The availability and functionality of vitamin D depends on both ingestion and exposure to sunlight with natural ultraviolet (UV) radiation. In the case of IBD, it has been suggested that low sunlight exposure constitutes a risk factor, particularly for CD[118,119]. This is in agreement with the notion that the incidence of IBD is higher in the northern hemisphere, where UV exposure is significantly lower[120]. Analysing these data together, it is rational to suggest that not only do early postnatal events influence the priming of the mucosal immune system and the immune response in adult life, but also that there are clearly innumerable other dietary-environmental intervening factors that might impact normal homeostasis and the risk of developing IBD.

In the last few years, epigenetic mechanisms have been implicated in the regulation of gene expression and cellular functions. The epigenome has been regarded as an interface between the environment and the genome, which plays a pivotal role in the definition of phenotypes and their maintenance. In this context, methylation of cytosine in CpG motifs has constituted the most extensively studied epigenetic event[121]. In the nucleus, DNA CpG methylation regulates gene expression through its effects on chromatin states and accessibility of factor binding sites in regulatory regions in gene promoters. While hypermethylation close to promoter regions is associated with gene silencing, in contrast, hypomethylation results in an opposite effect[122]. Recent data have reinforced the thought that epigenetic interactions connecting host DNA with environmental factors might have a key influence in the phenotypical expression of complex diseases such as IBD. This hypothesis is further supported by epidemiologic observations revealing the increased risk of developing IBD among people migrating from low to high incidence areas of the world[123]. Another example highlighting the importance of non-genetic processes in IBD development comes from studies showing a relatively high discordance rate among monozygotic twins[124].

Currently, there are indications that epigenetic mechanisms other than DNA methylation are implicated in the development of IBD, including the differential expression of microRNAs[125] and histone modifications[126]. However, most epigenetic modifications that have been correlated with the pathogenesis of IBD rely on DNA methylation studies[127]. One of these studies, for example, investigated the methylation status in the colonic mucosa from foetuses, control children and children with IBD. The analysis comparing IBD with control samples identified 233 differentially methylated regions (DMR), with a substantial overlap between paediatric IBD and control samples. This study supports probable novel physiological roles for DNA methylation in the human intestinal epithelium and presents data connecting developmentally acquired alterations in the DNA methylation profile to changes seen in paediatric IBD[128].

Regarding the question of whether epigenetic changes during development could be associated with a later onset of IBD, another group studied the colonic mucosa epigenome in association with the microbiome in children and adolescents. The investigators observed a strong connection between age-dependent and IBD-specific DNA methylation variations, remarkably more consistent with UC than CD, and DMRs with decreased methylation during late-onset paediatric disease. Of note, the authors called attention to the finding that the genera with epigenetically plastic DMRs during childhood and adolescence were *Roseburia* and *Streptococcus.* In particular, *Roseburia,* butyrate-producing bacteria, possess the potential to drive epigenetic changes in epithelial stem cells, since butyrate has been shown to be a histone deacetylase inhibitor[129].

Complex interactions between genotype, epigenome and environmental factors, leading to continuous remodelling of the epigenome, determine the phenotype of an individual. Among the environmental factors, food constituents emerge as important stimuli, which have been associated with specific epigenetic signatures and patterns of gene expression[130]. The one-carbon metabolism is dependent on dietary food components (*e.g.,* choline, betaine, folate) that participate in biochemical pathways of DNA methylation and/or supply of methyl groups[131]. Processed food, typical of Western diets, in most cases are deficient in micronutrients, including selenium and folate, which are both implicated in the progression of many diseases, including increased risk of developing colorectal cancer[132-135].

DNA hypomethylation represents an important phenomenon in human health, as it acts as the initial epigenetic alteration associated with carcinogenesis[136]. Since DNA methylation depends on the one-carbon metabolism pathway, requiring the activity of enzymes that depend on micronutrients provided by the diet, it is conceivable that hypomethylation might occur due to the lack of methyl donors. In fact, folate present in the diet, not synthesized endogenously, acts as a donor of one-carbon moieties, critical elements for the synthesis and repair of DNA and methylation that control gene expression[137]. Folate deficiency, in turn, has been demonstrated to induce DNA hypomethylation, while its supplementation has been able to correct some mutations and DNA strand breaks[138]. However, contradictory effects of folate deficiency on DNA methylation also have been reported[139,140]. Nevertheless, the ablation of two receptor/carrier-mediated pathways for folate transport in transgenic mice was shown to increase the risk of developing colitis-associated colorectal cancer in a chemically induced IBD model[141]. On the other hand, controversial results based on human or animal studies add some uncertainty about the actual role of folate in preventing cancer[142-144].

The micronutrient selenium has also been implicated in colorectal cancer susceptibility and DNA methylation. Selenium-deficient diets were shown to result in significantly hypomethylated liver and colon DNA in an experimental model[145]. Moreover, selenium-deficient diets contributed to the formation of more carcinogen-induced aberrant colon crypts in rats[138,146]. In experimental IBD, using a model of chemically induced colitis, selenium supplementation prevented tissue damage through the protection of the mitochondria and interfering in the expression of key genes responsible for inflammation[147]. In another model of experimental IBD, selenium deficiency was shown to worsen inflammation and promote tumour development and progression in inflammatory
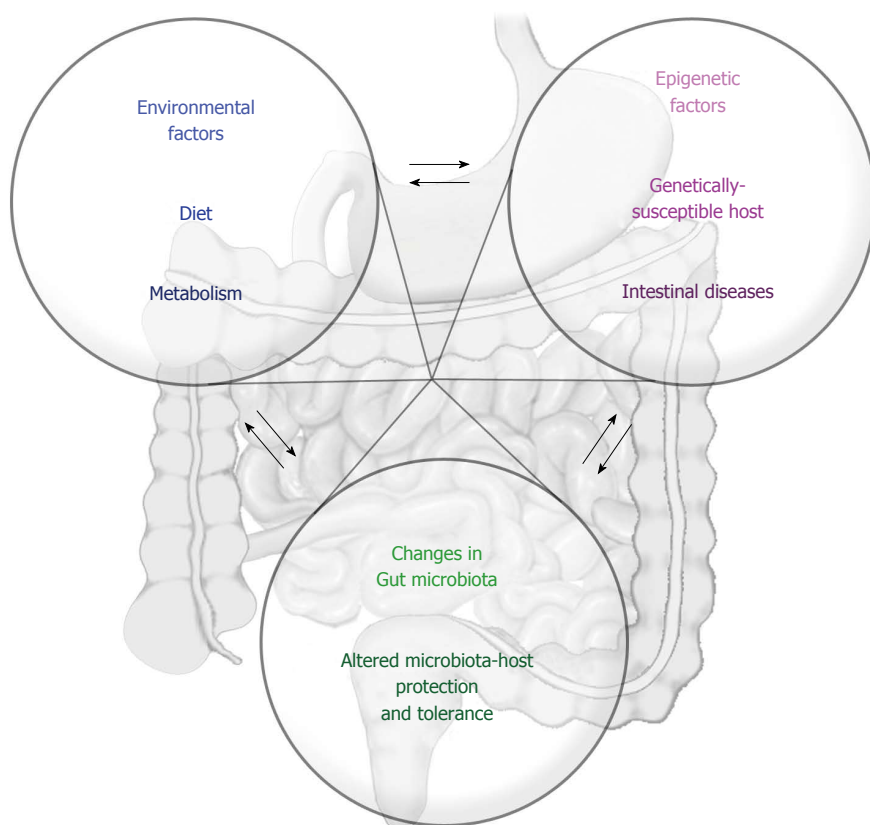
Figure 2 Interactive biological networks are affected by environmental factors. Environmental exposures, including dietary constituents and a dysbiotic microbiota, affect the host's genome and epigenome in a redundant and overlapping fashion, determining aberrant immunity and defective intestinal homeostasis, which lead to the development of inflammatory bowel disease.

carcinogenesis[148]. In human IBD, consistent studies regarding selenium and its potential impact in disease development are still limited. Recently, however, decreased serum selenium levels have been detected in patients with IBD[149].

Taken together, the current information available on dietary constituents and the potential effects on the epigenome is not sufficient to establish a clear relationship of cause and effect concerning IBD. Many questions remain unresolved, and it is urgent to address the interactions between the microbiome and epigenome, microbiome and diet, diet and epigenome, and the entire network of simultaneous, overlapping but also dynamic interactions that constitute the basis for intestinal homeostasis (Figure 2).

DIET AND INTESTINAL MICROBIOTA: THERAPEUTIC IMPLICATIONS IN IBD

Currently, consistent evidence to support specific dietary recommendations for patients with IBD is lacking. Nevertheless, it is fundamental to recognize particularities based on the heterogeneity of the patients and their complaints, with the frequent and spontaneous associations of symptoms with dietary habits and specific foods. Although interventional and well-controlled studies of dietary manipulation

are still required, it is agreed that the dietary intake should not be excessively restrictive in IBD[150]. However, considering the current knowledge on the direct effects of nutritional elements and the ability of food components to interact with microbial communities, it seems logical to continue pursuing dietary interventions in IBD, especially considering the modulatory potential of diet on the microbiota. On the other hand, a better comprehension of the complex mechanisms that underlie the interaction between the gut and its microbiota may clarify the defective relationships contributing to the development of diseases, such as IBD. Importantly, investigations of the gut-microbiota axis and the intervening modulating factors may unveil new mechanisms and, consequently, novel targets for therapeutic intervention[49]. The knowledge accumulated so far should allow exploration of the therapeutic potential of the intestinal microbiota in the treatment of several immune, metabolic and inflammatory disorders[151].

During the last decade, attempts to modulate the intestinal microbiota through the use of antibiotics, prebiotics, probiotics and synbiotics have represented a rational approach for the treatment of ubiquitous clinical disorders affecting the gastrointestinal tract[152,153]. The use of probiotics, including lactic acid bacteria, such as Lactobacilli and Bifidobacteria, for example, has been extensively studied in recent years.

Lactic acid bacteria are commonly present in yogurt and other fermented food products, but they are also commercialized in dietary supplements[154]. Data from the results of clinical trials suggest that probiotics consisting of lactic acid bacteria may be effective in treatment of pouchitis[155] and UC[156] and to a lesser extent in CD[157,158]. In UC, particularly, probiotics containing lactic acid bacteria have generated more promising results, although inconsistencies between studies may render the data difficult to interpret[159]. On the other hand, in CD, only relatively weak evidence exists to support a role for probiotics as effective therapeutic tools[160]. However, a lower rate of recurrence after surgery among CD patients who received early VSL#3 suggests its potential usefulness but also the need for additional studies on this probiotic in CD[161]. Another line of investigation in the field of IBD therapy analyses the potential use of prebiotics, oligosaccharides that are metabolized into SCFAs by commensal bacteria of the intestinal microbiota[162]. Interestingly, a synergistic effect between prebiotics and probiotics for the treatment of CD was proposed in an open-label study, where more effective results were observed when a mix of different lactic acid bacteria was used in combination with the prebiotic psyllium[163]. However, a consequent challenge that arises is how to maintain those lactic acid bacteria probiotics in the gut of patients with IBD, as clinical relapses tend to occur once the probiotic has been discontinued[164].

Recently, in a more audacious approach, another probiotic therapy based on faecal transplantation has been under investigation. Faecal microbiota transplantation (FMT) therapy is a process in which an abnormal, pathological microbiota is replaced by a supposedly normal one[165]. Although this type of intervention may sound like a rather extreme form of therapy, favourable outcomes have already been achieved in patients with recurrent *Clostridium difficile* infection, for example[166]. In IBD, the results of studies investigating FMT as a potential new alternative therapy are still difficult to interpret, because of distinct study designs and the relatively small number of controlled trials. However, some preliminary information suggests that FMT may be useful in the treatment of IBD, as most patients have exhibited symptomatic relief or even remission in several studies[167]. In a systematic literature search and meta-analysis investigating clinical outcomes, FMT was evaluated as safe, although with variable efficacy in IBD[168]. In a pilot study, high rates of clinical improvement and remission were observed after a single FMT was administered to patients with refractory CD[169]. Using a similar approach, the same group also investigated the efficacy and safety of a designed step-up FMT strategy for steroid-dependent UC. Almost sixty percent of the patients achieved clinical improvement, and the microbiota analysis showed that FMT altered its composition, which became highly similar to that of the donor, particularly in the patients with successful treatment[170]. In a recent randomized controlled trial, FMT was shown to induce remission in a significantly greater percentage of patients with active UC compared to a placebo, with no difference regarding adverse events[171]. Together, these data support the idea that FMT might develop into a promising new alternative for the treatment of IBD.

It is increasingly accepted that dietary constituents can affect the immune response and inflammatory status, in great part mediated through the modulation of the microbiota, as previously discussed in this article. Here, it is worth highlighting the fact that environmental compounds, including nutrients, can modify the genome activity in a manner that, although not changing the DNA sequence, can produce relevant, stable and, possibly, transgenerational alterations in the phenotype[172]. In this sense, alterations to the epigenome interface, which can determine long lasting phenotypic or even tissue structure and function modifications, are believed to be secondary to the nature and potency of the environmental stimuli, including dietary factors, in a dynamic process[173]. Support for the hypothesis of epigenetic programming constituting a permanent and even a transgenerational phenomenon is derived primarily from animal models, including studies involving dietary methyl donors and cofactors such as folic acid, choline and vitamin B12, for example[174,175]. The mechanisms by which environmental stimuli can induce long-term effects and be transmitted across generations are still unclear, and a better understanding of these processes has been regarded as essential for possible future interventions in dramatically increasing diseases such as obesity and diabetes[176], in an approach that hopefully can also be translated to IBD therapy.

In the interim, patients should be advised to pursue a healthier life, including a healthy diet, and avoiding sedentary behaviour, exposure to tobacco, pollutants and drugs in general. In terms of food, specifically, current knowledge suggests that the best approach relies on consuming a well-balanced diet containing predominantly fruits and vegetables and avoiding, as much as possible, processed foods and foods identified by the patient as prejudicial, capable of worsening symptoms or even triggering flares[43]. In this regard, for example, a high intake of red meat and processed meat, protein, alcoholic beverages, sulfur, and sulfate has been associated with an increased risk of flares in UC[177,178]. On the other hand, a high intake of saturated fat, monounsaturated fatty acids, and a higher ratio of omega-6:omega-3 polyunsaturated fatty acids have been associated with CD relapses[179,180].

The increase in and worldwide distribution of autoimmune and complex chronic inflammatory diseases such as IBD, especially in the last half-century, strongly suggest the crucial participation of environmental changes. Among the environmental factors, food and alimentary habits, progressively altered in modern societies, appear to be critical

modulators of the microbiota, contributing to or co-participating in dysbiosis, an important component of IBD pathogenesis. In addition, food components have also been shown to modulate epigenetic mechanisms, which can result in increased risk for the development and progression of IBD. Therefore, it seems reasonable to suppose that a better understanding of the role of the different food components in intestinal homeostasis and the resident microbiota will be essential for unravelling the complex molecular basis of the epigenetic, genetic and environment interactions underlying IBD pathogenesis as well as for offering dietary interventions with minimal expected side effects.

REFERENCES

1   **Hooper LV**, Macpherson AJ. Immune adaptations that maintain homeostasis with the intestinal microbiota. *Nat Rev Immunol* 2010; **10**: 159-169 [PMID: 20182457 DOI: 10.1038/nri2710]

2   **Råberg L**, Sim D, Read AF. Disentangling genetic variation for resistance and tolerance to infectious diseases in animals. *Science* 2007; **318**: 812-814 [PMID: 17975068 DOI: 10.1126/science.1148526]

3   **Medzhitov R**, Schneider DS, Soares MP. Disease tolerance as a defense strategy. *Science* 2012; **335**: 936-941 [PMID: 22363001 DOI: 10.1126/science.1214935]

4   **Hill DA**, Artis D. Intestinal bacteria and the regulation of immune cell homeostasis. *Annu Rev Immunol* 2010; **28**: 623-667 [PMID: 20192812 DOI: 10.1146/annurev-immunol-030409-101330]

5   **Hart AL**, Hendy P. The microbiome in inflammatory bowel disease and its modulation as a therapeutic manoeuvre. *Proc Nutr Soc* 2014; **73**: 452-456 [PMID: 25221893 DOI: 10.1017/S0029665114001153]

6   **Knights D**, Lassen KG, Xavier RJ. Advances in inflammatory bowel disease pathogenesis: linking host genetics and the microbiome. *Gut* 2013; **62**: 1505-1510 [PMID: 24037875 DOI: 10.1136/gutjnl-2012-303954]

7   **Costello EK**, Lauber CL, Hamady M, Fierer N, Gordon JI, Knight R. Bacterial community variation in human body habitats across space and time. *Science* 2009; **326**: 1694-1697 [PMID: 19892944 DOI: 10.1126/science.1177486]

8   **Dominguez-Bello MG**, Blaser MJ, Ley RE, Knight R. Development of the human gastrointestinal microbiota and insights from high-throughput sequencing. *Gastroenterology* 2011; **140**: 1713-1719 [PMID: 21530737 DOI: 10.1053/j.gastro.2011.02.011]

9   **Martin R**, Nauta AJ, Ben Amor K, Knippels LM, Knol J, Garssen J. Early life: gut microbiota and immune development in infancy. *Benef Microbes* 2010; **1**: 367-382 [PMID: 21831776 DOI: 10.3920/BM2010.0027]

10   **Zhu B**, Wang X, Li L. Human gut microbiome: the second genome of human body. *Protein Cell* 2010; **1**: 718-725 [PMID: 21203913 DOI: 10.1007/s13238-010-0093-z]

11   **Arumugam M**, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende DR, Fernandes GR, Tap J, Bruls T, Batto JM, Bertalan M, Borruel N, Casellas F, Fernandez L, Gautier L, Hansen T, Hattori M, Hayashi T, Kleerebezem M, Kurokawa K, Leclerc M, Levenez F, Manichanh C, Nielsen HB, Nielsen T, Pons N, Poulain J, Qin J, Sicheritz-Ponten T, Tims S, Torrents D, Ugarte E, Zoetendal EG, Wang J, Guarner F, Pedersen O, de Vos WM, Brunak S, Doré J, Antolín M, Artiguenave F, Blottiere HM, Almeida M, Brechot C, Cara C, Chervaux C, Cultrone A, Delorme C, Denariaz G, Dervyn R, Foerstner KU, Friss C, van de Guchte M, Guedon E, Haimet F, Huber W, van Hylckama-Vlieg J, Jamet A, Juste C, Kaci G, Knol J, Lakhdari O, Layec S, Le Roux K, Maguin E, Mérieux A, Melo Minardi R, M'rini C, Muller J, Oozeer R, Parkhill J, Renault P, Rescigno M, Sanchez N, Sunagawa S, Torrejon A, Turner

K, Vandemeulebrouck G, Varela E, Winogradsky Y, Zeller G, Weissenbach J, Ehrlich SD, Bork P. Enterotypes of the human gut microbiome. *Nature* 2011; **473**: 174-180 [PMID: 21508958 DOI: 10.1038/nature09944]

12   **Nava GM**, Stappenbeck TS. Diversity of the autochthonous colonic microbiota. *Gut Microbes* 2011; **2**: 99-104 [PMID: 21694499 DOI: 10.4161/gmic.2.2.15416]

13   **Lathrop SK**, Bloom SM, Rao SM, Nutsch K, Lio CW, Santacruz N, Peterson DA, Stappenbeck TS, Hsieh CS. Peripheral education of the immune system by colonic commensal microbiota. *Nature* 2011; **478**: 250-254 [PMID: 21937990 DOI: 10.1038/nature10434]

14   **Round JL**, Mazmanian SK. The gut microbiota shapes intestinal immune responses during health and disease. *Nat Rev Immunol* 2009; **9**: 313-323 [PMID: 19343057 DOI: 10.1038/nri2515]

15   **Medzhitov R**. Recognition of microorganisms and activation of the immune response. *Nature* 2007; **449**: 819-826 [PMID: 17943118 DOI: 10.1038/nature06246]

16   **Hooper LV**, Littman DR, Macpherson AJ. Interactions between the microbiota and the immune system. *Science* 2012; **336**: 1268-1273 [PMID: 22674334 DOI: 10.1126/science.1223490]

17   **Ivanov II**, Atarashi K, Manel N, Brodie EL, Shima T, Karaoz U, Wei D, Goldfarb KC, Santee CA, Lynch SV, Tanoue T, Imaoka A, Itoh K, Takeda K, Umesaki Y, Honda K, Littman DR. Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell* 2009; **139**: 485-498 [PMID: 19836068 DOI: 10.1016/j.cell.2009.09.033]

18   **Gaboriau-Routhiau V**, Rakotobe S, Lécuyer E, Mulder I, Lan A, Bridonneau C, Rochet V, Pisi A, De Paepe M, Brandi G, Eberl G, Snel J, Kelly D, Cerf-Bensussan N. The key role of segmented filamentous bacteria in the coordinated maturation of gut helper T cell responses. *Immunity* 2009; **31**: 677-689 [PMID: 19833089 DOI: 10.1016/j.immuni.2009.08.020]

19   **Wu HJ**, Ivanov II, Darce J, Hattori K, Shima T, Umesaki Y, Littman DR, Benoist C, Mathis D. Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells. *Immunity* 2010; **32**: 815-827 [PMID: 20620945 DOI: 10.1016/j.immuni.2010.06.001]

20   **Lee YK**, Menezes JS, Umesaki Y, Mazmanian SK. Proinflammatory T-cell responses to gut microbiota promote experimental autoimmune encephalomyelitis. *Proc Natl Acad Sci USA* 2011; **108** Suppl 1: 4615-4622 [PMID: 20660719 DOI: 10.1073/pnas.1000082107]

21   **Geuking MB**, Cahenzli J, Lawson MA, Ng DC, Slack E, Hapfelmeier S, McCoy KD, Macpherson AJ. Intestinal bacterial colonization induces mutualistic regulatory T cell responses. *Immunity* 2011; **34**: 794-806 [PMID: 21596591 DOI: 10.1016/j.immuni.2011.03.021]

22   **Smith PM**, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly-Y M, Glickman JN, Garrett WS. The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. *Science* 2013; **341**: 569-573 [PMID: 23828891 DOI: 10.1126/science.1241165]

23   **Hamer HM**, Jonkers D, Venema K, Vanhoutvin S, Troost FJ, Brummer RJ. Review article: the role of butyrate on colonic function. *Aliment Pharmacol Ther* 2008; **27**: 104-119 [PMID: 17973645 DOI: 10.1111/j.1365-2036.2007.03562.x]

24   **Pacheco RG**, Esposito CC, Müller LC, Castelo-Branco MT, Quintella LP, Chagas VL, de Souza HS, Schanaider A. Use of butyrate or glutamine in enema solution reduces inflammation and fibrosis in experimental diversion colitis. *World J Gastroenterol* 2012; **18**: 4278-4287 [PMID: 22969190 DOI: 10.3748/wjg.v18.i32.4278]

25   **Arpaia N**, Campbell C, Fan X, Dikiy S, van der Veeken J, deRoos P, Liu H, Cross JR, Pfeffer K, Coffer PJ, Rudensky AY. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature* 2013; **504**: 451-455 [PMID: 24226773 DOI: 10.1038/nature12726]

26   **Chang PV**, Hao L, Offermanns S, Medzhitov R. The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition. *Proc Natl Acad Sci USA* 2014; **111**: 2247-2252 [PMID: 24390544 DOI: 10.1073/pnas.1322269111]

27   **Furusawa Y**, Obata Y, Fukuda S, Endo TA, Nakato G, Takahashi D, Nakanishi Y, Uetake C, Kato K, Kato T, Takahashi M, Fukuda NN, Murakami S, Miyauchi E, Hino S, Atarashi K, Onawa S, Fujimura Y, Lockett T, Clarke JM, Topping DL, Tomita M, Hori S, Ohara O, Morita T, Koseki H, Kikuchi J, Honda K, Hase K, Ohno H. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. *Nature* 2013; **504**: 446-450 [PMID: 24226770 DOI: 10.1038/nature12721]

28   **Maslowski KM**, Mackay CR. Diet, gut microbiota and immune responses. *Nat Immunol* 2011; **12**: 5-9 [PMID: 21169997 DOI: 10.1038/ni0111-5]

29   **Duffy LC**, Raiten DJ, Hubbard VS, Starke-Reed P. Progress and challenges in developing metabolic footprints from diet in human gut microbial cometabolism. *J Nutr* 2015; **145**: 1123S-1130S [PMID: 25833886 DOI: 10.3945/jn.114.194936]

30   **Laitinen K**, Collado MC, Isolauri E. Early nutritional environment: focus on health effects of microbiota and probiotics. *Benef Microbes* 2010; **1**: 383-390 [PMID: 21831777 DOI: 10.3920/BM2010.0045]

31   **Kau AL**, Ahern PP, Griffin NW, Goodman AL, Gordon JI. Human nutrition, the gut microbiome and the immune system. *Nature* 2011; **474**: 327-336 [PMID: 21677749 DOI: 10.1038/nature10213]

32   **Clemente JC**, Ursell LK, Parfrey LW, Knight R. The impact of the gut microbiota on human health: an integrative view. *Cell* 2012; **148**: 1258-1270 [PMID: 22424233 DOI: 10.1016/j.cell.2012.01.035]

33   **Ley RE**, Hamady M, Lozupone C, Turnbaugh PJ, Ramey RR, Bircher JS, Schlegel ML, Tucker TA, Schrenzel MD, Knight R, Gordon JI. Evolution of mammals and their gut microbes. *Science* 2008; **320**: 1647-1651 [PMID: 18497261 DOI: 10.1126/science.1155725]

34   **De Filippo C**, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB, Massart S, Collini S, Pieraccini G, Lionetti P. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proc Natl Acad Sci USA* 2010; **107**: 14691-14696 [PMID: 20679230 DOI: 10.1073/pnas.1005963107]

35   **Wu GD**, Chen J, Hoffmann C, Bittinger K, Chen YY, Keilbaugh SA, Bewtra M, Knights D, Walters WA, Knight R, Sinha R, Gilroy E, Gupta K, Baldassano R, Nessel L, Li H, Bushman FD, Lewis JD. Linking long-term dietary patterns with gut microbial enterotypes. *Science* 2011; **334**: 105-108 [PMID: 21885731 DOI: 10.1126/science.1208344]

36   **David LA**, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, Ling AV, Devlin AS, Varma Y, Fischbach MA, Biddinger SB, Dutton RJ, Turnbaugh PJ. Diet rapidly and reproducibly alters the human gut microbiome. *Nature* 2014; **505**: 559-563 [PMID: 24336217 DOI: 10.1038/nature12820]

37   **Macfarlane S**, Macfarlane GT. Regulation of short-chain fatty acid production. *Proc Nutr Soc* 2003; **62**: 67-72 [PMID: 12740060 DOI: 10.1079/PNS2002207]

38   **Barcenilla A**, Pryde SE, Martin JC, Duncan SH, Stewart CS, Henderson C, Flint HJ. Phylogenetic relationships of butyrate-producing bacteria from the human gut. *Appl Environ Microbiol* 2000; **66**: 1654-1661 [PMID: 10742256]

39   **Hu Y**, Le Leu RK, Christophersen CT, Somashekar R, Conlon MA, Meng XQ, Winter JM, Woodman RJ, McKinnon R, Young GP. Manipulation of the gut microbiota using resistant starch is associated with protection against colitis-associated colorectal cancer in rats. *Carcinogenesis* 2016; **37**: 366-375 [PMID: 26905582 DOI: 10.1093/carcin/bgw019]

40   **Wang H**, Shi P, Zuo L, Dong J, Zhao J, Liu Q, Zhu W. Dietary Non-digestible Polysaccharides Ameliorate Intestinal Epithelial Barrier Dysfunction in IL-10 Knockout Mice. *J Crohns Colitis* 2016; **10**: 1076-1086 [PMID: 26944415 DOI: 10.1093/ecco-jcc/jjw065]

41   **Hung TV**, Suzuki T. Dietary Fermentable Fiber Reduces Intestinal Barrier Defects and Inflammation in Colitic Mice. *J Nutr* 2016; **146**: 1970-1979 [PMID: 27605405 DOI: 10.3945/jn.116.232538]

42   **Calder PC**. Polyunsaturated fatty acids and inflammation. *Biochem Soc Trans* 2005; **33**: 423-427 [PMID: 15787620 DOI: 10.1042/BST0330423]

43   **Lewis JD**, Abreu MT. Diet as a Trigger or Therapy for Inflammatory Bowel Diseases. *Gastroenterology* 2017; **152**: 398-414.e6 [PMID: 27793606 DOI: 10.1053/j.gastro.2016.10.019]

44   **Charlebois A**, Rosenfeld G, Bressler B. The Impact of Dietary Interventions on the Symptoms of Inflammatory Bowel Disease: A Systematic Review. *Crit Rev Food Sci Nutr* 2016; **56**: 1370-1378 [PMID: 25569442 DOI: 10.1080/10408398.2012.760515]

45   **Sigall-Boneh R**, Pfeffer-Gik T, Segal I, Zangen T, Boaz M, Levine A. Partial enteral nutrition with a Crohn's disease exclusion diet is effective for induction of remission in children and young adults with Crohn's disease. *Inflamm Bowel Dis* 2014; **20**: 1353-1360 [PMID: 24983973 DOI: 10.1097/MIB.0000000000000110]

46   **Olendzki BC**, Silverstein TD, Persuitte GM, Ma Y, Baldwin KR, Cave D. An anti-inflammatory diet as treatment for inflammatory bowel disease: a case series report. *Nutr J* 2014; **13**: 5 [PMID: 24428901 DOI: 10.1186/1475-2891-13-5]

47   **Mayneris-Perxachs J**, Bolick DT, Leng J, Medlock GL, Kolling GL, Papin JA, Swann JR, Guerrant RL. Protein- and zinc-deficient diets modulate the murine microbiome and metabolic phenotype. *Am J Clin Nutr* 2016; **104**: 1253-1262 [PMID: 27733402 DOI: 10.3945/ajcn.116.131797]

48   **Virtanen SM**. Dietary factors in the development of type 1 diabetes. *Pediatr Diabetes* 2016; **17** Suppl 22: 49-55 [PMID: 27411437 DOI: 10.1111/pedi.12341]

49   **Neish AS**. Microbes in gastrointestinal health and disease. *Gastroenterology* 2009; **136**: 65-80 [PMID: 19026645 DOI: 10.1053/j.gastro.2008.10.080]

50   **Kranich J**, Maslowski KM, Mackay CR. Commensal flora and the regulation of inflammatory and autoimmune responses. *Semin Immunol* 2011; **23**: 139-145 [PMID: 21292499 DOI: 10.1016/j.smim.2011.01.011]

51   **Bernstein CN**, Shanahan F. Disorders of a modern lifestyle: reconciling the epidemiology of inflammatory bowel diseases. *Gut* 2008; **57**: 1185-1191 [PMID: 18515412 DOI: 10.1136/gut.2007.122143]

52   **Molodecky NA**, Soon IS, Rabi DM, Ghali WA, Ferris M, Chernoff G, Benchimol EI, Panaccione R, Ghosh S, Barkema HW, Kaplan GG. Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. *Gastroenterology* 2012; **142**: 46-54.e42; quiz e30 [PMID: 22001864 DOI: 10.1053/j.gastro.2011.10.001]

53   **Albenberg LG**, Lewis JD, Wu GD. Food and the gut microbiota in inflammatory bowel diseases: a critical connection. *Curr Opin Gastroenterol* 2012; **28**: 314-320 [PMID: 22573192 DOI: 10.1097/MOG.0b013e328354586f]

54   **Rogler G**, Zeitz J, Biedermann L. The Search for Causative Environmental Factors in Inflammatory Bowel Disease. *Dig Dis* 2016; **34** Suppl 1: 48-55 [PMID: 27548430 DOI: 10.1159/000447283]

55   **Rook GA**. Hygiene hypothesis and autoimmune diseases. *Clin Rev Allergy Immunol* 2012; **42**: 5-15 [PMID: 22090147 DOI: 10.1007/s12016-011-8285-8]

56   **Saidel-Odes L**, Odes S. Hygiene hypothesis in inflammatory bowel disease. *Ann Gastroenterol* 2014; **27**: 189-190 [PMID: 24975779]

57   **García Rodríguez LA**, Ruigómez A, Panés J. Acute gastroenteritis is followed by an increased risk of inflammatory bowel disease. *Gastroenterology* 2006; **130**: 1588-1594 [PMID: 16697722 DOI: 10.1053/j.gastro.2006.02.004]

58   **Hviid A**, Svanström H, Frisch M. Antibiotic use and inflammatory bowel diseases in childhood. *Gut* 2011; **60**: 49-54 [PMID: 20966024 DOI: 10.1136/gut.2010.219683]

59   **Khor B**, Gardet A, Xavier RJ. Genetics and pathogenesis of inflammatory bowel disease. *Nature* 2011; **474**: 307-317 [PMID: 21677747 DOI: 10.1038/nature10209]

60   **Jostins L**, Ripke S, Weersma RK, Duerr RH, McGovern DP, Hui KY, Lee JC, Schumm LP, Sharma Y, Anderson CA, Essers J, Mitrovic M, Ning K, Cleynen I, Theatre E, Spain SL, Raychaudhuri S, Goyette P, Wei Z, Abraham C, Achkar JP, Ahmad

T, Amininejad L, Ananthakrishnan AN, Andersen V, Andrews JM, Baidoo L, Balschun T, Bampton PA, Bitton A, Boucher G, Brand S, Buning C, Cohain A, Cichon S, D'Amato M, De Jong D, Devaney KL, Dubinsky M, Edwards C, Ellinghaus D, Ferguson LR, Franchimont D, Fransen K, Gearry R, Georges M, Gieger C, Glas J, Haritunians T, Hart A, Hawkey C, Hedl M, Hu X, Karlsen TH, Kupcinskas L, Kugathasan S, Latiano A, Laukens D, Lawrance IC, Lees CW, Louis E, Mahy G, Mansfield J, Morgan AR, Mowat C, Newman W, Palmieri O, Ponsioen CY, Potocnik U, Prescott NJ, Regueiro M, Rotter JI, Russell RK, Sanderson JD, Sans M, Satsangi J, Schreiber S, Simms LA, Sventoraityte J, Targan SR, Taylor KD, Tremelling M, Verspaget HW, De Vos M, Wijmenga C, Wilson DC, Winkelmann J, Xavier RJ, Zeissig S, Zhang B, Zhang CK, Zhao H, International IBDGC, Silverberg MS, Annese V, Hakonarson H, Brant SR, Radford-Smith G, Mathew CG, Rioux JD, Schadt EE, Daly MJ, Franke A, Parkes M, Vermeire S, Barrett JC, Cho JH. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature* 2012; **491**: 119-124 [PMID: 23128233 DOI: 10.1038/nature11582]

61 **Shanahan F**. Irritable bowel syndrome: shifting the focus toward the gut microbiota. *Gastroenterology* 2007; **133**: 340-342 [PMID: 17631152 DOI: 10.1053/j.gastro.2007.05.030]

62 **Swidsinski A**, Loening-Baucke V, Verstraelen H, Osowska S, Doerffel Y. Biostructure of fecal microbiota in healthy subjects and patients with chronic idiopathic diarrhea. *Gastroenterology* 2008; **135**: 568-579 [PMID: 18570896 DOI: 10.1053/j.gastro.2008.04.017]

63 **Quigley EM**. Commensal bacteria: the link between IBS and IBD? *Curr Opin Clin Nutr Metab Care* 2011; **14**: 497-503 [PMID: 21673572 DOI: 10.1097/MCO.0b013e328348c033]

64 **Wen L**, Ley RE, Volchkov PY, Stranges PB, Avanesyan L, Stonebraker AC, Hu C, Wong FS, Szot GL, Bluestone JA, Gordon JI, Chervonsky AV. Innate immunity and intestinal microbiota in the development of Type 1 diabetes. *Nature* 2008; **455**: 1109-1113 [PMID: 18806780 DOI: 10.1038/nature07336]

65 **Wang Z**, Klipfell E, Bennett BJ, Koeth R, Levison BS, Dugar B, Feldstein AE, Britt EB, Fu X, Chung YM, Wu Y, Schauer P, Smith JD, Allayee H, Tang WH, DiDonato JA, Lusis AJ, Hazen SL. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. *Nature* 2011; **472**: 57-63 [PMID: 21475195 DOI: 10.1038/nature09922]

66 **Berer K**, Mues M, Koutrolos M, Rasbi ZA, Boziki M, Johner C, Wekerle H, Krishnamoorthy G. Commensal microbiota and myelin autoantigen cooperate to trigger autoimmune demyelination. *Nature* 2011; **479**: 538-541 [PMID: 22031325 DOI: 10.1038/nature10554]

67 **Sartor RB**. Microbial influences in inflammatory bowel diseases. *Gastroenterology* 2008; **134**: 577-594 [PMID: 18242222 DOI: 10.1053/j.gastro.2007.11.059]

68 **Quinton JF**, Sendid B, Reumaux D, Duthilleul P, Cortot A, Grandbastien B, Charrier G, Targan SR, Colombel JF, Poulain D. Anti-Saccharomyces cerevisiae mannan antibodies combined with antineutrophil cytoplasmic autoantibodies in inflammatory bowel disease: prevalence and diagnostic role. *Gut* 1998; **42**: 788-791 [PMID: 9691915]

69 **Lodes MJ**, Cong Y, Elson CO, Mohamath R, Landers CJ, Targan SR, Fort M, Hershberg RM. Bacterial flagellin is a dominant antigen in Crohn disease. *J Clin Invest* 2004; **113**: 1296-1306 [PMID: 15124021 DOI: 10.1172/JCI20295]

70 **Dotan I**, Fishman S, Dgani Y, Schwartz M, Karban A, Lerner A, Weishauss O, Spector L, Shtevi A, Altstock RT, Dotan N, Halpern Z. Antibodies against laminaribioside and chitobioside are novel serologic markers in Crohn's disease. *Gastroenterology* 2006; **131**: 366-378 [PMID: 16890590 DOI: 10.1053/j.gastro.2006.04.030]

71 **Xiong Y**, Wang GZ, Zhou JQ, Xia BQ, Wang XY, Jiang B. Serum antibodies to microbial antigens for Crohn's disease progression: a meta-analysis. *Eur J Gastroenterol Hepatol* 2014; **26**: 733-742 [PMID: 24901819 DOI: 10.1097/MEG.0000000000000102]

72 **D'Haens GR**, Geboes K, Peeters M, Baert F, Penninckx F, Rutgeerts P. Early lesions of recurrent Crohn's disease caused by infusion of intestinal contents in excluded ileum. *Gastroenterology* 1998; **114**: 262-267 [PMID: 9453485]

73 **Ananthakrishnan AN**, Khalili H, Konijeti GG, Higuchi LM, de Silva P, Korzenik JR, Fuchs CS, Willett WC, Richter JM, Chan AT. A prospective study of long-term intake of dietary fiber and risk of Crohn's disease and ulcerative colitis. *Gastroenterology* 2013; **145**: 970-977 [PMID: 23912083 DOI: 10.1053/j.gastro.2013.07.050]

74 **Racine A**, Carbonnel F, Chan SS, Hart AR, Bueno-de-Mesquita HB, Oldenburg B, van Schaik FD, Tjønneland A, Olsen A, Dahm CC, Key T, Luben R, Khaw KT, Riboli E, Grip O, Lindgren S, Hallmans G, Karling P, Clavel-Chapelon F, Bergman MM, Boeing H, Kaaks R, Katzke VA, Palli D, Masala G, Jantchou P, Boutron-Ruault MC. Dietary Patterns and Risk of Inflammatory Bowel Disease in Europe: Results from the EPIC Study. *Inflamm Bowel Dis* 2016; **22**: 345-354 [PMID: 26717318 DOI: 10.1097/MIB.0000000000000638]

75 **Tjonneland A**, Overvad K, Bergmann MM, Nagel G, Linseisen J, Hallmans G, Palmqvist R, Sjodin H, Hagglund G, Berglund G, Lindgren S, Grip O, Palli D, Day NE, Khaw KT, Bingham S, Riboli E, Kennedy H, Hart A. Linoleic acid, a dietary n-6 polyunsaturated fatty acid, and the aetiology of ulcerative colitis: a nested case-control study within a European prospective cohort study. *Gut* 2009; **58**: 1606-1611 [PMID: 19628674 DOI: 10.1136/gut.2008.169078]

76 **Ananthakrishnan AN**, Khalili H, Konijeti GG, Higuchi LM, de Silva P, Fuchs CS, Willett WC, Richter JM, Chan AT. Long-term intake of dietary fat and risk of ulcerative colitis and Crohn's disease. *Gut* 2014; **63**: 776-784 [PMID: 23828881 DOI: 10.1136/gutjnl-2013-305304]

77 **Devkota S**, Wang Y, Musch MW, Leone V, Fehlner-Peach H, Nadimpalli A, Antonopoulos DA, Jabri B, Chang EB. Dietary-fat-induced taurocholic acid promotes pathobiont expansion and colitis in Il10-/- mice. *Nature* 2012; **487**: 104-108 [PMID: 22722865 DOI: 10.1038/nature11225]

78 **Chassaing B**, Darfeuille-Michaud A. The commensal microbiota and enteropathogens in the pathogenesis of inflammatory bowel diseases. *Gastroenterology* 2011; **140**: 1720-1728 [PMID: 21530738 DOI: 10.1053/j.gastro.2011.01.054]

79 **Hansen R**, Russell RK, Reiff C, Louis P, McIntosh F, Berry SH, Mukhopadhya I, Bisset WM, Barclay AR, Bishop J, Flynn DM, McGrogan P, Loganathan S, Mahdi G, Flint HJ, El-Omar EM, Hold GL. Microbiota of de-novo pediatric IBD: increased Faecalibacterium prausnitzii and reduced bacterial diversity in Crohn's but not in ulcerative colitis. *Am J Gastroenterol* 2012; **107**: 1913-1922 [PMID: 23044767 DOI: 10.1038/ajg.2012.335]

80 **Man SM**, Kaakoush NO, Mitchell HM. The role of bacteria and pattern-recognition receptors in Crohn's disease. *Nat Rev Gastroenterol Hepatol* 2011; **8**: 152-168 [PMID: 21304476 DOI: 10.1038/nrgastro.2011.3]

81 **Sokol H**, Pigneur B, Watterlot L, Lakhdari O, Bermúdez-Humarán LG, Gratadoux JJ, Blugeon S, Bridonneau C, Furet JP, Corthier G, Grangette C, Vasquez N, Pochart P, Trugnan G, Thomas G, Blottière HM, Doré J, Marteau P, Seksik P, Langella P. Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. *Proc Natl Acad Sci USA* 2008; **105**: 16731-16736 [PMID: 18936492 DOI: 10.1073/pnas.0804812105]

82 **Takahashi K**, Nishida A, Fujimoto T, Fujii M, Shioya M, Imaeda H, Inatomi O, Bamba S, Sugimoto M, Andoh A. Reduced Abundance of Butyrate-Producing Bacteria Species in the Fecal Microbial Community in Crohn's Disease. *Digestion* 2016; **93**: 59-65 [PMID: 26789999 DOI: 10.1159/000441768]

83 **Gevers D**, Kugathasan S, Denson LA, Vázquez-Baeza Y, Van Treuren W, Ren B, Schwager E, Knights D, Song SJ, Yassour M, Morgan XC, Kostic AD, Luo C, González A, McDonald D, Haberman Y, Walters T, Baker S, Rosh J, Stephens M, Heyman M, Markowitz J, Baldassano R, Griffiths A, Sylvester F, Mack D, Kim S, Crandall W, Hyams J, Huttenhower C, Knight R, Xavier RJ. The treatment-naive microbiome in new-onset Crohn's disease. *Cell Host Microbe* 2014; **15**: 382-392 [PMID: 24629344 DOI: 10.1016/

j.chom.2014.02.005]

84   **Wright EK**, Kamm MA, Teo SM, Inouye M, Wagner J, Kirkwood CD. Recent advances in characterizing the gastrointestinal microbiome in Crohn's disease: a systematic review. *Inflamm Bowel Dis* 2015; **21**: 1219-1228 [PMID: 25844959 DOI: 10.1097/MIB.0000000000000382]

85   **Andoh A**, Imaeda H, Aomatsu T, Inatomi O, Bamba S, Sasaki M, Saito Y, Tsujikawa T, Fujiyama Y. Comparison of the fecal microbiota profiles between ulcerative colitis and Crohn's disease using terminal restriction fragment length polymorphism analysis. *J Gastroenterol* 2011; **46**: 479-486 [PMID: 21253779 DOI: 10.1007/s00535-010-0368-4]

86   **Li KY**, Wang JL, Wei JP, Gao SY, Zhang YY, Wang LT, Liu G. Fecal microbiota in pouchitis and ulcerative colitis. *World J Gastroenterol* 2016; **22**: 8929-8939 [PMID: 27833384 DOI: 10.3748/wjg.v22.i40.8929]

87   **Takeshita K**, Mizuno S, Mikami Y, Sujino T, Saigusa K, Matsuoka K, Naganuma M, Sato T, Takada T, Tsuji H, Kushiro A, Nomoto K, Kanai T. A Single Species of Clostridium Subcluster XIVa Decreased in Ulcerative Colitis Patients. *Inflamm Bowel Dis* 2016; **22**: 2802-2810 [PMID: 27824645 DOI: 10.1097/MIB.0000000000000972]

88   **Rausch P**, Rehman A, Künzel S, Häsler R, Ott SJ, Schreiber S, Rosenstiel P, Franke A, Baines JF. Colonic mucosa-associated microbiota is influenced by an interaction of Crohn disease and FUT2 (Secretor) genotype. *Proc Natl Acad Sci USA* 2011; **108**: 19030-19035 [PMID: 22068912 DOI: 10.1073/pnas.1106408108]

89   **Rehman A**, Sina C, Gavrilova O, Häsler R, Ott S, Baines JF, Schreiber S, Rosenstiel P. Nod2 is essential for temporal development of intestinal microbial communities. *Gut* 2011; **60**: 1354-1362 [PMID: 21421666 DOI: 10.1136/gut.2010.216259]

90   **Wang J**, Linnenbrink M, Künzel S, Fernandes R, Nadeau MJ, Rosenstiel P, Baines JF. Dietary history contributes to enterotype-like clustering and functional metagenomic content in the intestinal microbiome of wild mice. *Proc Natl Acad Sci USA* 2014; **111**: E2703-E2710 [PMID: 24912178 DOI: 10.1073/pnas.1402342111]

91   **Rehman A**, Rausch P, Wang J, Skieceviciene J, Kiudelis G, Bhagalia K, Amarapurkar D, Kupcinskas L, Schreiber S, Rosenstiel P, Baines JF, Ott S. Geographical patterns of the standing and active human gut microbiome in health and IBD. *Gut* 2016; **65**: 238-248 [PMID: 25567118 DOI: 10.1136/gutjnl-2014-308341]

92   **Frank DN**, St Amand AL, Feldman RA, Boedeker EC, Harpaz N, Pace NR. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proc Natl Acad Sci USA* 2007; **104**: 13780-13785 [PMID: 17699621 DOI: 10.1073/pnas.0706625104]

93   **Marks DJ**, Harbord MW, MacAllister R, Rahman FZ, Young J, Al-Lazikani B, Lees W, Novelli M, Bloom S, Segal AW. Defective acute inflammation in Crohn's disease: a clinical investigation. *Lancet* 2006; **367**: 668-678 [PMID: 16503465 DOI: 10.1016/S0140-6736(06)68265-2]

94   **Smith AM**, Rahman FZ, Hayee B, Graham SJ, Marks DJ, Sewell GW, Palmer CD, Wilde J, Foxwell BM, Gloger IS, Sweeting T, Marsh M, Walker AP, Bloom SL, Segal AW. Disordered macrophage cytokine secretion underlies impaired acute inflammation and bacterial clearance in Crohn's disease. *J Exp Med* 2009; **206**: 1883-1897 [PMID: 19652016 DOI: 10.1084/jem.20091233]

95   **Sewell GW**, Marks DJ, Segal AW. The immunopathogenesis of Crohn's disease: a three-stage model. *Curr Opin Immunol* 2009; **21**: 506-513 [PMID: 19665880 DOI: 10.1016/j.coi.2009.06.003]

96   **Cooney R**, Baker J, Brain O, Danis B, Pichulik T, Allan P, Ferguson DJ, Campbell BJ, Jewell D, Simmons A. NOD2 stimulation induces autophagy in dendritic cells influencing bacterial handling and antigen presentation. *Nat Med* 2010; **16**: 90-97 [PMID: 19966812 DOI: 10.1038/nm.2069]

97   **Fritz T**, Niederreiter L, Adolph T, Blumberg RS, Kaser A. Crohn's disease: NOD2, autophagy and ER stress converge. *Gut* 2011; **60**: 1580-1588 [PMID: 21252204 DOI: 10.1136/gut.2009.206466]

98   **Salem M**, Ammitzboell M, Nys K, Seidelin JB, Nielsen OH.

ATG16L1: A multifunctional susceptibility factor in Crohn disease. *Autophagy* 2015; **11**: 585-594 [PMID: 25906181 DOI: 10.1080/15548627.2015.1017187]

99   **Kaser A**, Martínez-Naves E, Blumberg RS. Endoplasmic reticulum stress: implications for inflammatory bowel disease pathogenesis. *Curr Opin Gastroenterol* 2010; **26**: 318-326 [PMID: 20495455 DOI: 10.1097/MOG.0b013e32833a9ff1]

100   **Hosomi S**, Kaser A, Blumberg RS. Role of endoplasmic reticulum stress and autophagy as interlinking pathways in the pathogenesis of inflammatory bowel disease. *Curr Opin Gastroenterol* 2015; **31**: 81-88 [PMID: 25426970 DOI: 10.1097/MOG.0000000000000144]

101   **Wehkamp J**, Salzman NH, Porter E, Nuding S, Weichenthal M, Petras RE, Shen B, Schaeffeler E, Schwab M, Linzmeier R, Feathers RW, Chu H, Lima H, Fellermann K, Ganz T, Stange EF, Bevins CL. Reduced Paneth cell alpha-defensins in ileal Crohn's disease. *Proc Natl Acad Sci USA* 2005; **102**: 18129-18134 [PMID: 16330776 DOI: 10.1073/pnas.0505256102]

102   **Billmann-Born S**, Lipinski S, Böck J, Till A, Rosenstiel P, Schreiber S. The complex interplay of NOD-like receptors and the autophagy machinery in the pathophysiology of Crohn disease. *Eur J Cell Biol* 2011; **90**: 593-602 [PMID: 21146253 DOI: 10.1016/j.ejcb.2010.10.015]

103   **Shaw MH**, Kamada N, Warner N, Kim YG, Nuñez G. The ever-expanding function of NOD2: autophagy, viral recognition, and T cell activation. *Trends Immunol* 2011; **32**: 73-79 [PMID: 21251876 DOI: 10.1016/j.it.2010.12.007]

104   **Hampe J**, Franke A, Rosenstiel P, Till A, Teuber M, Huse K, Albrecht M, Mayr G, De La Vega FM, Briggs J, Günther S, Prescott NJ, Onnie CM, Häsler R, Sipos B, Fölsch UR, Lengauer T, Platzer M, Mathew CG, Krawczak M, Schreiber S. A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. *Nat Genet* 2007; **39**: 207-211 [PMID: 17200669 DOI: 10.1038/ng1954]

105   **Parkes M**, Barrett JC, Prescott NJ, Tremelling M, Anderson CA, Fisher SA, Roberts RG, Nimmo ER, Cummings FR, Soars D, Drummond H, Lees CW, Khawaja SA, Bagnall R, Burke DA, Todhunter CE, Ahmad T, Onnie CM, McArdle W, Strachan D, Bethel G, Bryan C, Lewis CM, Deloukas P, Forbes A, Sanderson J, Jewell DP, Satsangi J, Mansfield JC, Cardon L, Mathew CG. Sequence variants in the autophagy gene IRGM and multiple other replicating loci contribute to Crohn's disease susceptibility. *Nat Genet* 2007; **39**: 830-832 [PMID: 17554261 DOI: 10.1038/ng2061]

106   **Hoefkens E**, Nys K, John JM, Van Steen K, Arijs I, Van der Goten J, Van Assche G, Agostinis P, Rutgeerts P, Vermeire S, Cleynen I. Genetic association and functional role of Crohn disease risk alleles involved in microbial sensing, autophagy, and endoplasmic reticulum (ER) stress. *Autophagy* 2013; **9**: 2046-2055 [PMID: 24247223 DOI: 10.4161/auto.26337]

107   **Elinav E**, Strowig T, Henao-Mejia J, Flavell RA. Regulation of the antimicrobial response by NLR proteins. *Immunity* 2011; **34**: 665-679 [PMID: 21616436 DOI: 10.1016/j.immuni.2011.05.007]

108   **Garrett WS**, Gallini CA, Yatsunenko T, Michaud M, DuBois A, Delaney ML, Punit S, Karlsson M, Bry L, Glickman JN, Gordon JI, Onderdonk AB, Glimcher LH. Enterobacteriaceae act in concert with the gut microbiota to induce spontaneous and maternally transmitted colitis. *Cell Host Microbe* 2010; **8**: 292-300 [PMID: 20833380 DOI: 10.1016/j.chom.2010.08.004]

109   **Dominguez-Bello MG**, Costello EK, Contreras M, Magris M, Hidalgo G, Fierer N, Knight R. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. *Proc Natl Acad Sci USA* 2010; **107**: 11971-11975 [PMID: 20566857 DOI: 10.1073/pnas.1002601107]

110   **Koenig JE**, Spor A, Scalfone N, Fricker AD, Stombaugh J, Knight R, Angenent LT, Ley RE. Succession of microbial consortia in the developing infant gut microbiome. *Proc Natl Acad Sci USA* 2011; **108** Suppl 1: 4578-4585 [PMID: 20668239 DOI: 10.1073/pnas.1000081107]

111   **Hanson LA**. Session 1: Feeding and infant development breast-feeding and immune function. *Proc Nutr Soc* 2007; **66**: 384-396 [PMID: 17637091 DOI: 10.1017/S0029665107005654]

112   **Barclay AR**, Russell RK, Wilson ML, Gilmour WH, Satsangi J, Wilson DC. Systematic review: the role of breastfeeding in the development of pediatric inflammatory bowel disease. *J Pediatr* 2009; **155**: 421-426 [PMID: 19464699 DOI: 10.1016/j.jpeds.2009.03.017]

113   **Nickerson KP**, McDonald C. Crohn's disease-associated adherent-invasive Escherichia coli adhesion is enhanced by exposure to the ubiquitous dietary polysaccharide maltodextrin. *PLoS One* 2012; **7**: e52132 [PMID: 23251695 DOI: 10.1371/journal.pone.0052132]

114   **Chabra S**, Arnold JD, Leslie GI, Bowen JR, Earl J, Wood F. Vitamin A status in preterm neonates with and without chronic lung disease. *J Paediatr Child Health* 1994; **30**: 432-435 [PMID: 7833081]

115   **Sawa S**, Cherrier M, Lochner M, Satoh-Takayama N, Fehling HJ, Langa F, Di Santo JP, Eberl G. Lineage relationship analysis of RORgammat+ innate lymphoid cells. *Science* 2010; **330**: 665-669 [PMID: 20929731 DOI: 10.1126/science.1194597]

116   **Werner T**, Wagner SJ, Martínez I, Walter J, Chang JS, Clavel T, Kisling S, Schuemann K, Haller D. Depletion of luminal iron alters the gut microbiota and prevents Crohn's disease-like ileitis. *Gut* 2011; **60**: 325-333 [PMID: 21076126 DOI: 10.1136/gut.2010.216929]

117   **Adorini L**, Penna G. Dendritic cell tolerogenicity: a key mechanism in immunomodulation by vitamin D receptor agonists. *Hum Immunol* 2009; **70**: 345-352 [PMID: 19405173]

118   **Nerich V**, Jantchou P, Boutron-Ruault MC, Monnet E, Weill A, Vanbockstael V, Auleley GR, Balaire C, Dubost P, Rican S, Allemand H, Carbonnel F. Low exposure to sunlight is a risk factor for Crohn's disease. *Aliment Pharmacol Ther* 2011; **33**: 940-945 [PMID: 21332762 DOI: 10.1111/j.1365-2036.2011.04601.x]

119   **Jørgensen SP**, Hvas CL, Agnholt J, Christensen LA, Heickendorff L, Dahlerup JF. Active Crohn's disease is associated with low vitamin D levels. *J Crohns Colitis* 2013; **7**: e407-e413 [PMID: 23403039 DOI: 10.1016/j.crohns.2013.01.012]

120   **Limketkai BN**, Bayless TM, Brant SR, Hutfless SM. Lower regional and temporal ultraviolet exposure is associated with increased rates and severity of inflammatory bowel disease hospitalisation. *Aliment Pharmacol Ther* 2014; **40**: 508-517 [PMID: 24943863 DOI: 10.1111/apt.12845]

121   **Jones PA**. Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nat Rev Genet* 2012; **13**: 484-492 [PMID: 22641018 DOI: 10.1038/nrg3230]

122   **Deaton AM**, Bird A. CpG islands and the regulation of transcription. *Genes Dev* 2011; **25**: 1010-1022 [PMID: 21576262 DOI: 10.1101/gad.2037511]

123   **Benchimol EI**, Mack DR, Guttmann A, Nguyen GC, To T, Mojaverian N, Quach P, Manuel DG. Inflammatory bowel disease in immigrants to Canada and their children: a population-based cohort study. *Am J Gastroenterol* 2015; **110**: 553-563 [PMID: 25756238 DOI: 10.1038/ajg.2015.52]

124   **Kellermayer R**. Epigenetics and the developmental origins of inflammatory bowel diseases. *Can J Gastroenterol* 2012; **26**: 909-915 [PMID: 23248794]

125   **Koukos G**, Polytarchou C, Kaplan JL, Oikonomopoulos A, Ziring D, Hommes DW, Wahed R, Kokkotou E, Pothoulakis C, Winter HS, Iliopoulos D. A microRNA signature in pediatric ulcerative colitis: deregulation of the miR-4284/CXCL5 pathway in the intestinal epithelium. *Inflamm Bowel Dis* 2015; **21**: 996-1005 [PMID: 25738378 DOI: 10.1097/MIB.0000000000000339]

126   **Scarpa M**, Stylianou E. Epigenetics: Concepts and relevance to IBD pathogenesis. *Inflamm Bowel Dis* 2012; **18**: 1982-1996 [PMID: 22407855 DOI: 10.1002/ibd.22934]

127   **Barnett M**, Bermingham E, McNabb W, Bassett S, Armstrong K, Rounce J, Roy N. Investigating micronutrients and epigenetic mechanisms in relation to inflammatory bowel disease. *Mutat Res* 2010; **690**: 71-80 [PMID: 20188748 DOI: 10.1016/j.mrfmmm.2010.02.006]

128   **Kraiczy J**, Nayak K, Ross A, Raine T, Mak TN, Gasparetto M, Cario E, Rakyan V, Heuschkel R, Zilbauer M. Assessing DNA methylation in the developing human intestinal epithelium:

129   potential link to inflammatory bowel disease. *Mucosal Immunol* 2016; **9**: 647-658 [PMID: 26376367 DOI: 10.1038/mi.2015.88]

129   **Harris RA**, Shah R, Hollister EB, Tronstad RR, Hovdenak N, Szigeti R, Versalovic J, Kellermayer R. Colonic Mucosal Epigenome and Microbiome Development in Children and Adolescents. *J Immunol Res* 2016; **2016**: 9170162 [PMID: 27006956 DOI: 10.1155/2016/9170162]

130   **Gallou-Kabani C**, Vigé A, Gross MS, Junien C. Nutri-epigenomics: lifelong remodelling of our epigenomes by nutritional and metabolic factors and beyond. *Clin Chem Lab Med* 2007; **45**: 321-327 [PMID: 17378726 DOI: 10.1515/CCLM.2007.081]

131   **Davis CD**, Uthus EO. DNA methylation, cancer susceptibility, and nutrient interactions. *Exp Biol Med* (Maywood) 2004; **229**: 988-995 [PMID: 15522834]

132   **Garfinkel MD**, Ruden DM. Chromatin effects in nutrition, cancer, and obesity. *Nutrition* 2004; **20**: 56-62 [PMID: 14698015]

133   **Al-Awadi FM**, Khan I, Dashti HM, Srikumar TS. Colitis-induced changes in the level of trace elements in rat colon and other tissues. *Ann Nutr Metab* 1998; **42**: 304-310 [PMID: 9812022]

134   **Wani NA**, Hamid A, Kaur J. Folate status in various pathophysiological conditions. *IUBMB Life* 2008; **60**: 834-842 [PMID: 18942083 DOI: 10.1002/iub.133]

135   **McKay JA**, Williams EA, Mathers JC. Gender-specific modulation of tumorigenesis by folic acid supply in the Apc mouse during early neonatal life. *Br J Nutr* 2008; **99**: 550-558 [PMID: 17868491 DOI: 10.1017/S0007114507819131]

136   **Ehrlich M**. DNA hypomethylation in cancer cells. *Epigenomics* 2009; **1**: 239-259 [PMID: 20495664 DOI: 10.2217/epi.09.33]

137   **Lu Q**, Qiu X, Hu N, Wen H, Su Y, Richardson BC. Epigenetics, disease, and therapeutic interventions. *Ageing Res Rev* 2006; **5**: 449-467 [PMID: 16965942 DOI: 10.1016/j.arr.2006.07.001]

138   **Davis CD**, Uthus EO. Dietary folate and selenium affect dimethylhydrazine-induced aberrant crypt formation, global DNA methylation and one-carbon metabolism in rats. *J Nutr* 2003; **133**: 2907-2914 [PMID: 12949386]

139   **Choi SW**, Friso S, Keyes MK, Mason JB. Folate supplementation increases genomic DNA methylation in the liver of elder rats. *Br J Nutr* 2005; **93**: 31-35 [PMID: 15705222]

140   **Arasaradnam RP**, Commane DM, Bradburn D, Mathers JC. A review of dietary factors and its influence on DNA methylation in colorectal carcinogenesis. *Epigenetics* 2008; **3**: 193-198 [PMID: 18682688]

141   **Chapkin RS**, Kamen BA, Callaway ES, Davidson LA, George NI, Wang N, Lupton JR, Finnell RH. Use of a novel genetic mouse model to investigate the role of folate in colitis-associated colon cancer. *J Nutr Biochem* 2009; **20**: 649-655 [PMID: 18926688 DOI: 10.1016/j.jnutbio.2008.07.001]

142   **Qin T**, Du M, Du H, Shu Y, Wang M, Zhu L. Folic acid supplements and colorectal cancer risk: meta-analysis of randomized controlled trials. *Sci Rep* 2015; **5**: 12044 [PMID: 26131763 DOI: 10.1038/srep12044]

143   **Tio M**, Andrici J, Cox MR, Eslick GD. Folate intake and the risk of upper gastrointestinal cancers: a systematic review and meta-analysis. *J Gastroenterol Hepatol* 2014; **29**: 250-258 [PMID: 24224911 DOI: 10.1111/jgh.12446]

144   **MacFarlane AJ**, Behan NA, Matias FM, Green J, Caldwell D, Brooks SP. Dietary folate does not significantly affect the intestinal microbiome, inflammation or tumorigenesis in azoxymethane-dextran sodium sulphate-treated mice. *Br J Nutr* 2013; **109**: 630-638 [PMID: 23021249 DOI: 10.1017/S0007114512001857]

145   **Davis CD**, Uthus EO, Finley JW. Dietary selenium and arsenic affect DNA methylation in vitro in Caco-2 cells and in vivo in rat liver and colon. *J Nutr* 2000; **130**: 2903-2909 [PMID: 11110844]

146   **Davis CD**, Uthus EO. Dietary selenite and azadeoxycytidine treatments affect dimethylhydrazine-induced aberrant crypt formation in rat colon and DNA methylation in HT-29 cells. *J Nutr* 2002; **132**: 292-297 [PMID: 11823593]

147   **Tirosh O**, Levy E, Reifen R. High selenium diet protects against TNBS-induced acute inflammation, mitochondrial dysfunction, and secondary necrosis in rat colon. *Nutrition* 2007; **23**: 878-886

[PMID: 17936198 DOI: 10.1016/j.nut.2007.08.019]

148   **Barrett CW**, Singh K, Motley AK, Lintel MK, Matafonova E, Bradley AM, Ning W, Poindexter SV, Parang B, Reddy VK, Chaturvedi R, Fingleton BM, Washington MK, Wilson KT, Davies SS, Hill KE, Burk RF, Williams CS. Dietary selenium deficiency exacerbates DSS-induced epithelial injury and AOM/DSS-induced tumorigenesis. *PLoS One* 2013; **8**: e67845 [PMID: 23861820 DOI: 10.1371/journal.pone.0067845]

149   **Castro Aguilar-Tablada T**, Navarro-Alarcón M, Quesada Granados J, Samaniego Sánchez C, Rufián-Henares JÁ, Nogueras-Lopez F. Ulcerative Colitis and Crohn's Disease Are Associated with Decreased Serum Selenium Concentrations and Increased Cardiovascular Risk. *Nutrients* 2016; **8**: [PMID: 27916926 DOI: 10.3390/nu8120780]

150   **Richman E**, Rhodes JM. Review article: evidence-based dietary advice for patients with inflammatory bowel disease. *Aliment Pharmacol Ther* 2013; **38**: 1156-1171 [PMID: 24102340 DOI: 10.1111/apt.12500]

151   **O'Hara AM**, Shanahan F. Gut microbiota: mining for therapeutic potential. *Clin Gastroenterol Hepatol* 2007; **5**: 274-284 [PMID: 17368226 DOI: 10.1016/j.cgh.2006.12.009]

152   **Sartor RB**, Muehlbauer M. Microbial host interactions in IBD: implications for pathogenesis and therapy. *Curr Gastroenterol Rep* 2007; **9**: 497-507 [PMID: 18377803]

153   **Macfarlane GT**, Blackett KL, Nakayama T, Steed H, Macfarlane S. The gut microbiota in inflammatory bowel disease. *Curr Pharm Des* 2009; **15**: 1528-1536 [PMID: 19442170]

154   **Mach T**. Clinical usefulness of probiotics in inflammatory bowel diseases. *J Physiol Pharmacol* 2006; **57** Suppl 9: 23-33 [PMID: 17242485]

155   **Mimura T**, Rizzello F, Helwig U, Poggioli G, Schreiber S, Talbot IC, Nicholls RJ, Gionchetti P, Campieri M, Kamm MA. Once daily high dose probiotic therapy (VSL#3) for maintaining remission in recurrent or refractory pouchitis. *Gut* 2004; **53**: 108-114 [PMID: 14684584]

156   **Sartor RB**. Therapeutic manipulation of the enteric microflora in inflammatory bowel diseases: antibiotics, probiotics, and prebiotics. *Gastroenterology* 2004; **126**: 1620-1633 [PMID: 15168372]

157   **Ewaschuk JB**, Dieleman LA. Probiotics and prebiotics in chronic inflammatory bowel diseases. *World J Gastroenterol* 2006; **12**: 5941-5950 [PMID: 17009391 DOI: 10.3748/wjg.v12.i37.5941]

158   **Mack DR**. Probiotics in inflammatory bowel diseases and associated conditions. *Nutrients* 2011; **3**: 245-264 [PMID: 22254095 DOI: 10.3390/nu3020245]

159   **Sokol H**. Probiotics and antibiotics in IBD. *Dig Dis* 2014; **32** Suppl 1: 10-17 [PMID: 25531348 DOI: 10.1159/000367820]

160   **Guslandi M**. Role of Probiotics in Crohn's Disease and in Pouchitis. *J Clin Gastroenterol* 2015; **49** Suppl 1: S46-S49 [PMID: 26447964 DOI: 10.1097/MCG.0000000000000351]

161   **Fedorak RN**, Feagan BG, Hotte N, Leddin D, Dieleman LA, Petrunia DM, Enns R, Bitton A, Chiba N, Paré P, Rostom A, Marshall J, Depew W, Bernstein CN, Panaccione R, Aumais G, Steinhart AH, Cockeram A, Bailey RJ, Gionchetti P, Wong C, Madsen K. The probiotic VSL#3 has anti-inflammatory effects and could reduce endoscopic recurrence after surgery for Crohn's disease. *Clin Gastroenterol Hepatol* 2015; **13**: 928-935.e2 [PMID: 25460016 DOI: 10.1016/j.cgh.2014.10.031]

162   **Scaldaferri F**, Gerardi V, Lopetuso LR, Del Zompo F, Mangiola F, Boškoski I, Bruno G, Petito V, Laterza L, Cammarota G, Gaetani E, Sgambato A, Gasbarrini A. Gut microbial flora, prebiotics, and probiotics in IBD: their current usage and utility. *Biomed Res Int* 2013; **2013**: 435268 [PMID: 23991417 DOI: 10.1155/2013/435268]

163   **Fujimori S**, Tatsuguchi A, Gudis K, Kishida T, Mitsui K, Ehara A, Kobayashi T, Sekita Y, Seo T, Sakamoto C. High dose probiotic and prebiotic cotherapy for remission induction of active Crohn's disease. *J Gastroenterol Hepatol* 2007; **22**: 1199-1204 [PMID: 17688660 DOI: 10.1111/j.1440-1746.2006.04535.x]

164   **Chandler M**, Wollins E, Toles A, Borum M, Doman DB. The

emerging therapeutic role of probiotics in inflammatory bowel disease. *Gastroenterol Hepatol* (N Y) 2008; **4**: 634-640 [PMID: 22798747]

165   **Khoruts A**, Sadowsky MJ. Therapeutic transplantation of the distal gut microbiota. *Mucosal Immunol* 2011; **4**: 4-7 [PMID: 21150894 DOI: 10.1038/mi.2010.79]

166   **Bakken JS**, Borody T, Brandt LJ, Brill JV, Demarco DC, Franzos MA, Kelly C, Khoruts A, Louie T, Martinelli LP, Moore TA, Russell G, Surawicz C. Treating Clostridium difficile infection with fecal microbiota transplantation. *Clin Gastroenterol Hepatol* 2011; **9**: 1044-1049 [PMID: 21871249 DOI: 10.1016/j.cgh.2011.08.014]

167   **Anderson JL**, Edney RJ, Whelan K. Systematic review: faecal microbiota transplantation in the management of inflammatory bowel disease. *Aliment Pharmacol Ther* 2012; **36**: 503-516 [PMID: 22827693 DOI: 10.1111/j.1365-2036.2012.05220.x]

168   **Colman RJ**, Rubin DT. Fecal microbiota transplantation as therapy for inflammatory bowel disease: a systematic review and meta-analysis. *J Crohns Colitis* 2014; **8**: 1569-1581 [PMID: 25223604 DOI: 10.1016/j.crohns.2014.08.006]

169   **Cui B**, Feng Q, Wang H, Wang M, Peng Z, Li P, Huang G, Liu Z, Wu P, Fan Z, Ji G, Wang X, Wu K, Fan D, Zhang F. Fecal microbiota transplantation through mid-gut for refractory Crohn's disease: safety, feasibility, and efficacy trial results. *J Gastroenterol Hepatol* 2015; **30**: 51-58 [PMID: 25168749 DOI: 10.1111/jgh.12727]

170   **Cui B**, Li P, Xu L, Zhao Y, Wang H, Peng Z, Xu H, Xiang J, He Z, Zhang T, Nie Y, Wu K, Fan D, Ji G, Zhang F. Step-up fecal microbiota transplantation strategy: a pilot study for steroid-dependent ulcerative colitis. *J Transl Med* 2015; **13**: 298 [PMID: 26363929 DOI: 10.1186/s12967-015-0646-2]

171   **Moayyedi P**, Surette MG, Kim PT, Libertucci J, Wolfe M, Onischi C, Armstrong D, Marshall JK, Kassam Z, Reinisch W, Lee CH. Fecal Microbiota Transplantation Induces Remission in Patients With Active Ulcerative Colitis in a Randomized Controlled Trial. *Gastroenterology* 2015; **149**: 102-109.e6 [PMID: 25857665 DOI: 10.1053/j.gastro.2015.04.001]

172   **Lee HS**. Impact of Maternal Diet on the Epigenome during In Utero Life and the Developmental Programming of Diseases in Childhood and Adulthood. *Nutrients* 2015; **7**: 9492-9507 [PMID: 26593940 DOI: 10.3390/nu7115467]

173   **Attig L**, Gabory A, Junien C. Nutritional developmental epigenomics: immediate and long-lasting effects. *Proc Nutr Soc* 2010; **69**: 221-231 [PMID: 20202279 DOI: 10.1017/S002966511000008X]

174   **MacLennan NK**, James SJ, Melnyk S, Piroozi A, Jernigan S, Hsu JL, Janke SM, Pham TD, Lane RH. Uteroplacental insufficiency alters DNA methylation, one-carbon metabolism, and histone acetylation in IUGR rats. *Physiol Genomics* 2004; **18**: 43-50 [PMID: 15084713 DOI: 10.1152/physiolgenomics.00042.2004]

175   **Vanhees K**, Vonhögen IG, van Schooten FJ, Godschalk RW. You are what you eat, and so are your children: the impact of micronutrients on the epigenetic programming of offspring. *Cell Mol Life Sci* 2014; **71**: 271-285 [PMID: 23892892 DOI: 10.1007/s00018-013-1427-9]

176   **Vickers MH**. Early life nutrition, epigenetics and programming of later life disease. *Nutrients* 2014; **6**: 2165-2178 [PMID: 24892374 DOI: 10.3390/nu6062165]

177   **Jowett SL**, Seal CJ, Pearce MS, Phillips E, Gregory W, Barton JR, Welfare MR. Influence of dietary factors on the clinical course of ulcerative colitis: a prospective cohort study. *Gut* 2004; **53**: 1479-1484 [PMID: 15361498 DOI: 10.1136/gut.2003.024828]

178   **Magee EA**, Edmond LM, Tasker SM, Kong SC, Curno R, Cummings JH. Associations between diet and disease activity in ulcerative colitis patients using a novel method of data analysis. *Nutr J* 2005; **4**: 7 [PMID: 15705205 DOI: 10.1186/1475-2891-4-7]

179   **Guerreiro CS**, Ferreira P, Tavares L, Santos PM, Neves M, Brito M, Cravo M. Fatty acids, IL6, and TNFalpha polymorphisms: an example of nutrigenetics in Crohn's disease. *Am J Gastroenterol* 2009; **104**: 2241-2249 [PMID: 19550417 DOI: 10.1038/ajg.2009.313]

180   **Ferreira P**, Cravo M, Guerreiro CS, Tavares L, Santos PM, Brito M. Fat intake interacts with polymorphisms of Caspase9, FasLigand and PPARgamma apoptotic genes in modulating Crohn's disease activity. *Clin Nutr* 2010; **29**: 819-823 [PMID: 20650551 DOI: 10.1016/j.clnu.2010.06.008]

181   **Takada T**, Kurakawa T, Tsuji H, Nomoto K. Fusicatenibacter saccharivorans gen. nov., sp. nov., isolated from human faeces. *Int J Syst Evol Microbiol* 2013; **63**: 3691-3696 [PMID: 23625266 DOI: 10.1099/ijs.0.045823-0]

182   **Miquel S**, Martín R, Rossi O, Bermúdez-Humarán LG, Chatel JM, Sokol H, Thomas M, Wells JM, Langella P. Faecalibacterium prausnitzii and human intestinal health. *Curr Opin Microbiol* 2013; **16**: 255-261 [PMID: 23831042 DOI: 10.1016/j.mib.2013.06.003]

183   **Park SK**, Kim MS, Bae JW. Blautia faecis sp. nov., isolated from human faeces. *Int J Syst Evol Microbiol* 2013; **63**: 599-603 [PMID: 22544782 DOI: 10.1099/ijs.0.036541-0]

184   **Swidsinski A**, Loening-Baucke V, Herber A. Mucosal flora in Crohn's disease and ulcerative colitis - an overview. *J Physiol Pharmacol* 2009; **60** Suppl 6: 61-71 [PMID: 20224153]

185   **Auchtung TA**, Holder ME, Gesell JR, Ajami NJ, Duarte RT, Itoh K, Caspi RR, Petrosino JF, Horai R, Zárate-Bladés CR. Complete Genome Sequence of Turicibacter sp. Strain H121, Isolated from the Feces of a Contaminated Germ-Free Mouse. *Genome Announc* 2016; **4**: [PMID: 27013036 DOI: 10.1128/genomeA.00114-16]

186   **Huys G**, Cnockaert M, Janda JM, Swings J. Escherichia albertii sp. nov., a diarrhoeagenic species isolated from stool specimens of Bangladeshi children. *Int J Syst Evol Microbiol* 2003; **53**: 807-810 [PMID: 12807204 DOI: 10.1099/ijs.0.02475-0]

187   **Ishikawa H**, Akedo I, Umesaki Y, Tanaka R, Imaoka A, Otani T. Randomized controlled trial of the effect of bifidobacteria-fermented milk on ulcerative colitis. *J Am Coll Nutr* 2003; **22**: 56-63 [PMID: 12569115]

ORIGINAL ARTICLE

Basic Study

Effect of a poloxamer 407-based thermosensitive gel on minimization of thermal injury to diaphragm during microwave ablation of the liver

Li-Li Zhang, Gui-Min Xia, Yu-Jiang Liu, Rui Dou, John Eisenbrey, Ji-Bin Liu, Xiao-Wei Wang, Lin-Xue Qian

Li-Li Zhang, Yu-Jiang Liu, Rui Dou, Lin-Xue Qian, Department of Ultrasound, Beijing Friendship Hospital, Capital Medical University, Beijing 100054, China

Gui-Min Xia, Xiao-Wei Wang, Department of Pharmaceutics, Institute of Medicinal Biotechnology, Chinese Academy of Medical Science and Peking Union Medical College, Beijing 100054, China

John Eisenbrey, Ji-Bin Liu, Department of Radiology, Thomas Jefferson University, Philadelphia, PA 19107, United States

Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Department of Ultrasound, Beijing Friendship Hospital, Capital Medical University (IACUC No. 15-4003).

Conflict-of-interest statement: The authors alone are responsible for the paper. The authors declare no conflicts of interest.

Data sharing statement: Technical appendix, statistical code and dataset available from the corresponding author at qianlinxue2002@163.com.

Abstract

AIM

To assess the insulating effect of a poloxamer 407 (P407)-based gel during microwave ablation of liver adjacent to the diaphragm.

METHODS

We prepared serial dilutions of P407, and 22.5% (w/w) concentration was identified as suitable for ablation procedures. Subsequently, microwave ablations were performed on the livers of 24 rabbits (gel, saline, control groups, n = 8 in each). The P407 solution and 0.9% normal saline were injected into the potential space between the diaphragm and liver in experimental groups. No barriers were applied to the controls. After microwave ablations, the frequency, size and degree of thermal injury were compared histologically among

the three groups. Subsequently, another 8 rabbits were injected with the P407 solution and microwave ablation was performed. The levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN) and creatinine (Cr) in serum were tested at 1 d before microwave ablation and 3 and 7 d after operation.

RESULTS
In vivo ablation thermal injury to the adjacent diaphragm was evaluated in the control, saline and 22.5% P407 gel groups (P = 0.001-0.040). However, there was no significant difference in the volume of ablation zone among the three groups ($P > 0.05$). Moreover, there were no statistical differences among the preoperative and postoperative gel groups according to the levels of ALT, AST, BUN and Cr in serum (all $P > 0.05$).

CONCLUSION
Twenty-two point five percent P407 gel could be a more effective choice during microwave ablation of hepatic tumors adjacent to the diaphragm. Further studies for clinical translation are warranted.

Key words: Microwave ablation; Injury; Hepatocellular carcinoma; Poloxamer; Hydrodissection

Core tip: Collateral thermal damage is the most common complication of microwave ablation. Conventional liquids can move away and be absorbed quickly, proving difficult to get a good separation effect. This study aimed to assess the insulating effect of a poloxamer 407 (P407)-based thermosensitive gel during microwave ablation of the liver adjacent to the diaphragm. We prepared serial dilutions of P407, and 22.5% (w/w) concentration was identified as suitable for ablation procedures. The 22.5% P407 effectively protected the diaphragm during microwave ablation of the liver, and was superior to 5% dextrose in water and 0.9% saline.

INTRODUCTION

Percutaneous thermal ablation has been widely used over the past 20 years as a minimally invasive procedure for treating liver tumors, especially hepatocellular carcinoma (HCC)[1]. Over the years, different types of ablation applicators have become widely accepted, such as microwave (MW), radiofrequency (RF) electrical current, laser and cryoablation[2,3]. Percutaneous thermal ablation has been credited with almost equivalent survival rates and a rapid return to normal status as compared to surgical resection[4,5]. Several studies have noted that MW ablation could create larger ablation zones compared to RF ablation[6,7]. A MW ablation at 60 ℃ has been found to immediately induce coagulative necrosis of the tumors[8].

In the treatment of HCC, a low energy can result in incomplete ablation and local progression. However, high-power MW ablations often result in thermal injury to non-target organs, including the gallbladder, diaphragm and so on[9]. This poses a challenge to the interventional doctors. As previous studies have reported, about 15% of liver tumors deemed as high risk are not suitable for thermal ablation[10,11].

To reduce such harmful effects, several methods have been suggested during the ablation of subcapsular hepatic lesions. Hydrodissection is the most commonly applied technique to insulate adjacent structures such as 5% dextrose in water (D5W) and 0.9% normal saline (NS)[12,13]. That has been found to be effective at decreasing unintended thermal injury, however D5W and NS tend to move away quickly from target sites, thereby reducing the insulating effect.

P407 is a nonionic surfactant composed of polyethylene oxide-polypropylene oxide-polyethylene oxide triblock copolymers[14]. It is currently used in clinical therapy as a drug carrier[15,16]. P407 has an attractive property that it can, from being in liquid state at low temperatures, transform into a semisolid gel state at elevated temperatures (gelation temperature), which depends on heat conduction of the surroundings[15,17]. This indicated that P407 gel may be useful in MW ablation of the liver. The aim of our study was to evaluate *in vivo* the insulating properties of a P407-based thermosensitive gel during MW ablation of the liver adjacent to the diaphragm.

MATERIALS AND METHODS

Study subjects
The subjects included in this study were 32 male and female healthy New Zealand white rabbits (weight range: 1.5-2.5 kg). The study protocol was approved by the Animal Care and Use Committee of our research institution. The treatment of animals was according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Concentration optimization
The sol-gel transformation of the injectable thermosensitive solution is expected to occur at slightly below room temperature. A series of dilutions ranging from 15%-30% (w/w) P407 (Batch No. WPWJ554C;
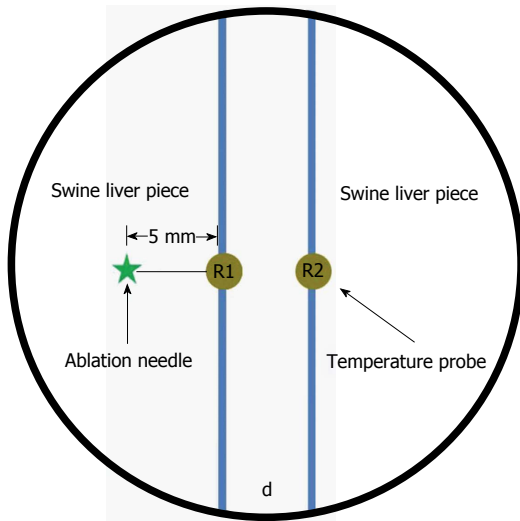
Figure 1 Schematic of the experimental set-up used for *ex vivo* microwave ablations in a 6-well plate. Two swine liver pieces were held at a specific distance, d, which were set to 5 mm or 10 mm. This separation provides a hydrodissection barrier. The ablation needle and temperature probes were positioned in parallel. The distance was always 5 mm between the ablation needle and the primary needle (R1).

BASF, Germany) in deionized water were prepared and their gelation temperatures were determined using a rotor equipped with a controlled heating system[18] (magnetic rotor size of 1.5 cm × 0.6 cm, stirring rate of 300 rotations/min). The samples were slowly heated at a rate of 0.5 ℃/min from the initial temperature of 15 ℃. The gelation temperatures were defined as when the magnetic rotor completely stopped rotating. Each concentration was tested in triplicate simultaneously. Eventually, an optimal concentration of the P407 solution was obtained. Then, the viscosity of 22.5% P407 gel was tested with a Brookfield R/S$^+$ rheometer (Stoughton, MA, United States) with a circulating water bath. The sample was heated from 15 ℃ to 30 ℃ at a constant shear rate of 5/s. The rheological behavior of 22.5% P407 was investigated.

MW ablation instrument
A water-cooled MW ablation system was used in this study (KY-2000; Kangyou Medical Instruments, Nanjing, China). The generator can produce 1-100 W of power at 2450 MHz. We used a Model T$_{11}$ (outer diameter of 15 G) MW ablation needle, with distance of 11 mm from the front end of the gap to the tip. An output setting of 40 W for 300 s was usually used for ablation sessions. However, since rabbit liver is small and fragile, such high MW power could easily penetrate the liver; therefore, ablation was performed for 180 s at 30 W in this study.

An iron/constantan thermocouple was used to monitor temperature in real time. The system had 21-gauge thermocouple needles, which were percutaneously placed at a designated location. For data acquisition, HP 34970A (Hewlett-Packard, Palo

Alto, CA, United States) with a 16-bit analog output function was used.

Ex vivo temperature measurement
Insulation effectiveness during MW ablation was evaluated *ex vivo* as described in Figure 1. Two swine liver pieces were placed in a six-well plate (diameter 2 cm and depth 2 cm) which was positioned in a water bath at 37 ℃. To obtain a 5 mm or 10 mm barrier between the liver pieces, 22.5% P407 gel was used as a hydrodissection. The ablation needles were placed vertically into the livers at a depth of 1.5 cm, 5 mm away from the barrier. The ablation needle and temperature probes were positioned in parallel, maintaining a distance of 5 mm between the ablation needle and the primary needle (R1). We compared the insulation effects of a 5 mm-thick barrier against a 10 mm-thick barrier. MWs were applied three times at 30 W for 3 min. The temperature differences between probes R1 and R2 were recorded every 30 s and mapped. Moreover, whenever R1 reached 60 ℃, the temperature at R2 was measured.

Preparation of experimental animals
Of the 32 rabbits used in this study, 8 were employed to study the safety of the P407 gel, as described later. The other 24 rabbits were randomly assigned to three experimental groups. Two experimental groups were injected with 5 mL P407 and 5 mL 0.9% NS, respectively, between the diaphragm and the liver. Such volume enabled the presence of a 5 mm barrier by ultrasonic examination. For control animals, no protective technique was used. Before each ablation procedure, the rabbits were anesthetized with 30 mg/kg intravenous pentobarbital sodium (Sigma, St Louis, MO, United States). The abdomen was shaved, disinfected routinely, and the animals were placed in a supine position for MW ablation.

MW ablation
All of the procedures described in this study were performed by two interventional clinicians. The animals were ultrasonically scanned to choose the best puncture sites (avoiding important blood vessels and ribs). A 2 mm incision was made at the edge of the skin with a sharp knife. The MW antennas were placed 5 mm away from the liver surface. The ablation applicator was used for 3 min at an output power of 30 W. During MW ablations, the thickness of the hydrodissection barrier was observed for each experimental group. All interventional procedures were monitored and guided by ultrasound examination.

Animal sacrifice and data analysis
The 24 rabbits were sacrificed and dissected immediately after MW ablation. The liver ablation zones and adjacent diaphragms were photographed and the ablation effects compared. Subsequently,
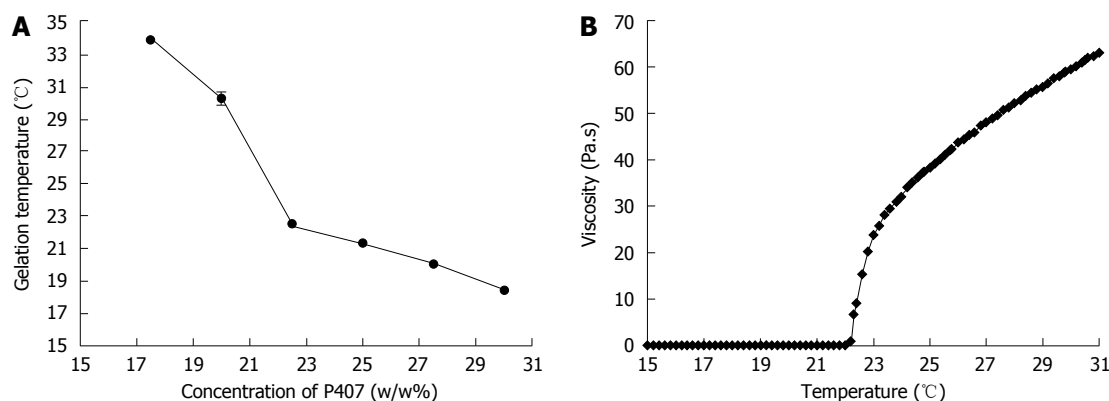
Figure 2 Optimal formulation. A: Several concentrations of P407 were prepared. A stirring magnetic bar was used to determine the gelation temperature. When the magnetic bar stopped moving, the solution was considered gelled. The reliable data were defined three times in parallel (mean ± SD, n = 3). A negative correlation was observed between gelation temperatures and concentrations of P407. A 22.5% (w/w) P407 solution was found to gel at 22.3 ℃; B: A Brookfield R/S$^+$ rheometer with a spindle attached was used to study the viscosity of 22.5% (w/w) P407 solution. It was programmed to increase the temperature from 15 ℃ to 30 ℃ at a shear rate of 5/s. The viscosity was relatively low at temperatures below 18 ℃ and characterized as a fluidic state. Then, a sharp increase in viscosity was observed as an inflexion point was reached at sol-gel transition temperature (22.3 ℃). By this time, it turned into a semi-solid.

the diaphragm and liver samples were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin. An experienced pathologist evaluated thermal injury to the diaphragm histologically. The volumes of ablation zones were calculated and compared using the following formula.

Volume (V) = $\pi/6 \times a\, b\, c$

where a is dimension 1, b is dimension 2, and c is dimension 3. (a is the largest diameter, and b and c are the other mutually perpendicular diameters).

Thermal injury to the diaphragm was expressed as a diameter of injured lesions. In addition, we graded the degree of thermal injury to the diaphragm according to a 4-point scoring system (none, 0; mild, 1; moderate, 2; severe, 3) based on a consensus of two of the contributing authors. If a diaphragm was seen discolored and having a thickened pale area that extended toward the pleural margin, it was considered seriously injured. The suspected injured diaphragms were sectioned and graded on a scale of 0-3 (0, no injury; 1, mild injury up to one-third thickness; 2, moderate injury to two-thirds thickness; 3, severe injury)[19].

In vivo safety experiment

Eight rabbits were injected with the P407 solution at a dose of 5 mL into the potential space between the diaphragm and liver under ultrasonic guidance and MW ablation was performed at 30 W for 3 min. Using 2 mL of ear vein blood, the levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), and creatinine (Cr) in serum were tested 1 d before and 3 and 7 d after the procedures, so as to check liver and renal functions.

Statistical analysis

The experimental data were analyzed using SPSS software, version 16.0. Quantitative data were described as mean ± SD and were evaluated using one-way analysis of variance. The levels of thermal injury were compared using the Mann-Whitney test (Kruskal-Wallis test). $P < 0.05$ was considered statistically significant.

RESULTS

Optimal formulation

The sol-gel transformation temperature decreased as P407 concentration increased (Figure 2A). Finally, gelation temperature of 22.5% P407 (BASF) solution was 22 ℃. For clinical purposes, the thermosensitive gel should have a relatively lower gelation temperature in order to gelate rapidly in target site and facilitate our operation smoothly. Our results show that 22.5% P407 solution gelled at about 1.5 min in a water bath at 37 ℃ but took 16 min at room temperature. Such short interval is beneficial to operate the surgery rapidly and smoothly. So, we propose that 22.5% P407 gel could be an ideal choice for ablation procedures.

The rheological behavior of P407 is also presented as a flow curve (Figure 2B). The sample exhibited low viscosity and characterized fluidic behavior below 18 ℃. It is feasible to be injected. When the gelation temperature was reached, the viscosity sharply increased. By this time, the sample had transformed into a semi-solid state.

Ex vivo MW ablation and temperature testing

After MW ablation for 120 s, the maximum temperature difference of 26.4 ± 0.5 ℃ was observed between R1 and R2 with a P407 gel thickness of 5 mm (Figure 3A). When the mean temperature of R1 reached 60 ℃ at 180 s, the temperature of R2 was 41.9 ± 1.1 ℃ (Figure 3B) and the temperature difference was 18.1 ± 1.5 ℃ (Figure 3A). However,
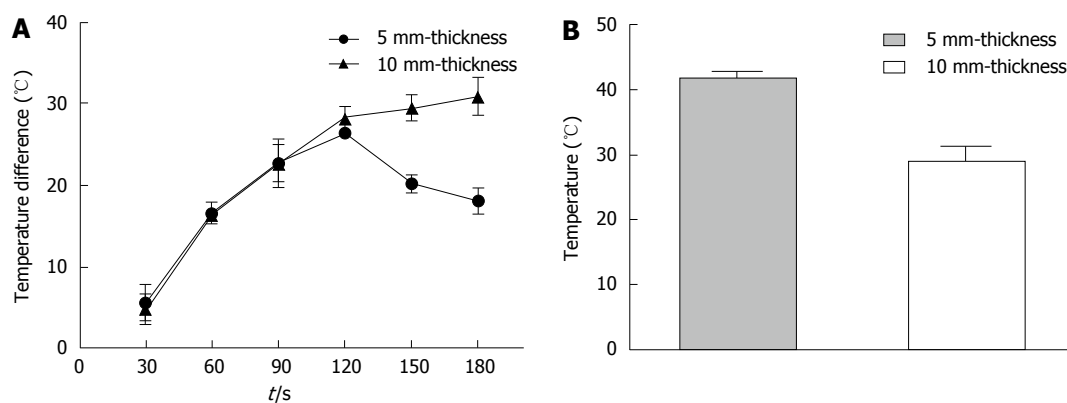
Figure 3 *Ex vivo* microwave ablation and temperature testing. A: Temperature differences between R1 and R2 when 5 mm-thick and 10 mm-thick gels were maintained; B: Temperatures of R2 when the temperature at R1 was 60 ℃. The mean temperature at R2 was 41.9 ± 1.1 ℃ with a 5 mm-thick gel. When the gel was prepared for 10 mm-thick separation, the mean temperature at R2 was 29.1 ± 2.4 ℃.
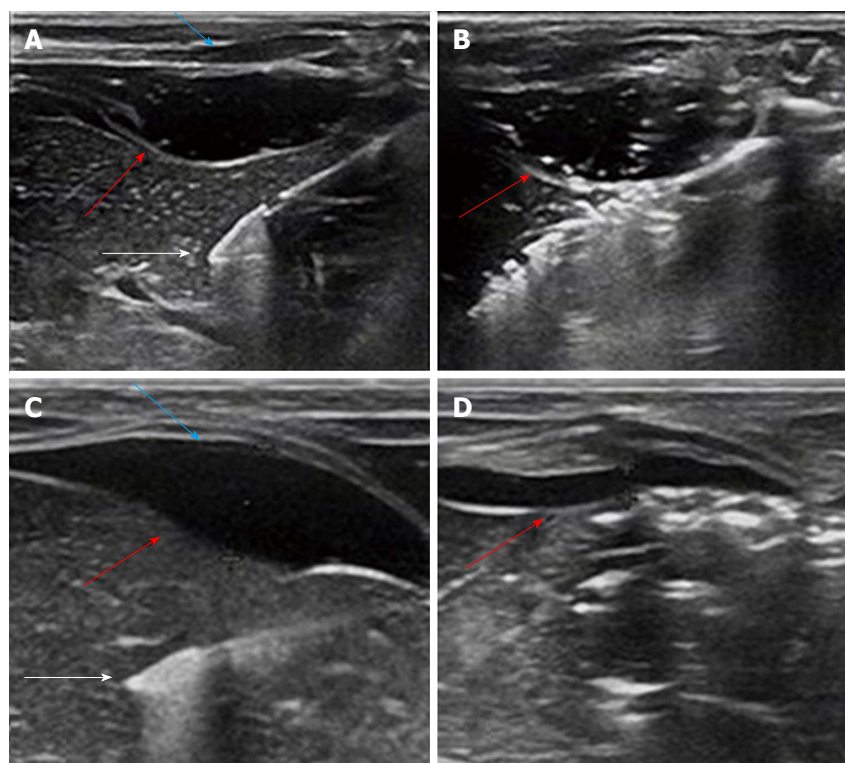
Figure 4 Ultrasonographic view. A: Ultrasonographic view of the placement of the ablation needle (white arrow) with the poloxamer 407 (P407) gel (red arrow) positioned between the diaphragm (blue arrow) and liver; B: Image captured to assess the change in the size of P407 barrier at 3 min during microwave ablation. No apparent thinning was observed (red arrow); C: Ultrasound image showing a saline barrier (red arrow) of 5 mm thickness between the diaphragm (blue arrow) and liver and the placement of ablation needle (white arrow); D: Ultrasound image showing a hydrodissection barrier of about 1.3 mm thickness (red arrow) at the end of the ablation procedure.

the maximum temperature difference of 30.9 ± 2.2 ℃ (Figure 3A) was observed after MW ablation for 3 min with a P407 gel thickness of 10 mm; the mean temperature of R1 was 60 ℃ and that of R2 was 29.1 ± 2.4 ℃ (Figure 3B). Our results demonstrate that a 5 mm P407 gel is adequate to insulate the surrounding tissue from thermal damage.

Gross pathology

When monitored ultrasonically, no changes in gel thickness were observed during MW ablation (Figure 4A and B). After MW ablation, laparotomy was

performed on the experimental animals immediately and the *in situ* gel and liver ablation zones were observed (Figure 5). Similarly, for the NS group, the initial barrier thickness was 5 mm (Figure 4C). The distance between ablation needle tip and the edge of the liver was approximately 5 mm. However, the thickness had become reduced to 1.3 mm at the end of the ablation procedure (Figure 4D). After several hours, the 22.5% P407 gel was undetectable by ultrasound.

The effects of ablations extended into the surrounding diaphragm in all of the control animals (*n* = 8),
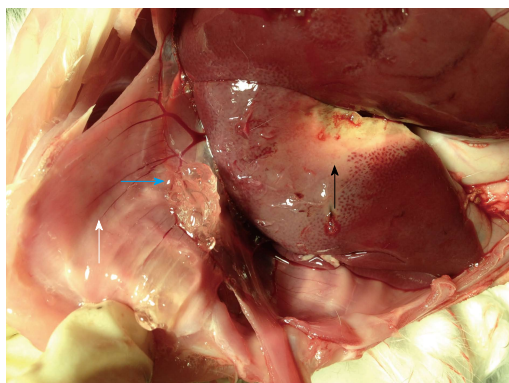
Figure 5 Upon performing a laparotomy, the gel (blue arrow) is seen between the diaphragm (white arrow) and liver lobe (black arrow). Photograph showing a microwave ablation zone at the liver lobe (black arrow), yet no thermal injury to the diaphragm can be observed.

in 5 of the NS-protected animals and none of the gel-protected animals. Table 1 shows that thermal damage to the diaphragm differed significantly in size and severity among the three groups (*P* < 0.05). However, no difference in the volume of ablation zone was detected among the three experimental groups (*P* = 0.353; Table 2). Representative photographs of gross specimens of ablated liver and injured diaphragm are shown in Figure 6.

Safety assessment
The levels of ALT, AST, BUN and Cr in serum were assayed before and after MW ablation (Table 3) as indicators of liver and renal functions. Our statistical analysis showed that there was no significant difference among the groups pre- and postoperatively (*P* > 0.05; Table 3).

DISCUSSION

MW ablation is considered an effective treatment for small HCCs[20,21]. However, several complications may occur, including hemorrhage, pleural effusion and thermal injury[1]. Among these, thermal injury to non-target tissue is the most common side effect, in particular when the tumor is close to vital organs, thereby resulting in poor prognosis. Therefore, ablation is not recommended for large tumors located close to the diaphragm or the gastrointestinal tract.

Many investigators have attempted to reduce collateral thermal damage by means of hydrodissection[13,22]. Although several conventional thermoprotective fluids are known, low viscosities as a result of their high mobility pose a challenge. In some cases, a continuous infusion of the fluid needs to be maintained during the entire ablation procedure, which can lead to fluid overload and patient discomfort[23,24]. Thus, we optimized the fluids to replace conventional hydrodissection applied for MW ablation of the liver.

In the present study, 22.5% P407 solution exhibited potential as a thermoprotective barrier during MW

Table 1 Comparison of thermal injury to the diaphragm among the three groups (*n* = 8)

Diaphragmatic injury	Gel group, *n* = 8	Saline group, *n* = 8	Control group, *n* = 8	*P* value
Injury rate	0	5%	8%	
Size in cm	0	0.9 ± 0.7[a]	1.7 ± 0.3[b,c]	0.001[1]
Grade, score	0	0.6 ± 1.1[d]	1.8 ± 0.7[e,f]	0.001[1]

[1]Statistically significant difference, Mann-Whitney test. The maximum diameter of thermal injury to the diaphragmatic surface is reported. [a]*P* = 0.011 *vs* gel group; [b]*P* = 0.001 *vs* gel group; [c]*P* = 0.005 *vs* saline group; [d]*P* = 0.010 *vs* gel group; [e]*P* = 0.001 *vs* gel group; [f]*P* = 0.040 *vs* saline group.

Table 2 Comparison of the size of microwave ablation zones among the three groups (*n* = 8)

	Gel group	Saline group	Control group	*P* value
Dimension 1 in cm	2.06 ± 0.38	2.14 ± 0.15	2.19 ± 0.14	
Dimension 2 in cm	1.28 ± 0.18	1.26 ± 0.22	1.38 ± 0.14	
Dimension 3 in cm	1.24 ± 0.18	1.23 ± 0.22	1.34 ± 0.16	
Volume in cm^3	1.76 ± 0.66	1.75 ± 0.54	2.11 ± 0.43	0.353

Table 3 Comparison of hepatic and renal functions before and after microwave ablation (*n* = 8)

Indicator	Baseline	Postablation Day 3	Day 7	*P* value
ALT in U/L	45.38 ± 5.24	41.57 ± 3.96	41.50 ± 4.14	0.166
AST in U/L	50.79 ± 3.95	47.25 ± 3.28	45.63 ± 5.10	0.062
BUN in mmol/L	7.50 ± 0.90	7.14 ± 1.10	7.12 ± 1.05	0.708
Cr in μmol/L	66.24 ± 4.14	62.77 ± 7.16	62.04 ± 3.39	0.244

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BUN: Blood urea nitrogen; Cr: Creatinine.

ablations. It exhibited low viscosity at below 18 ℃ as D5W and 0.9% NS, which allows for injectability without resistance through small needles. However, 22.5% P407 transformed into a semi-solid state rapidly at 37 ℃, providing a stable gel barrier at the injection site. It was not detected by ultrasound after several hours. Therefore, performance of more critical ablations, such as for high-risk liver cancers, is possible when using P407 as a thermoprotective agent, although MW ablation is generally not the preferred method for treating such cases. It is well known that conventional hydrodissection fluids flow away from target sites due to heat convection, thereby dissipating heat from the ablation site. Nevertheless, this appears to play little role in the mechanism of P407 gel. Instead, it appears to work mainly through heat conduction. Further studies are needed to establish the mechanisms of thermoprotection by P407.

According to *ex vivo* temperature studies, 22.5% P407 gel of 5 mm thickness can result in a temperature difference of about 18 ℃ between both sides of the gel. During ablation, the temperature was 29.1 ± 2.4 ℃ on the other side of the gel (corresponding to the one side of tissue necrosis temperature, 60 ℃). This
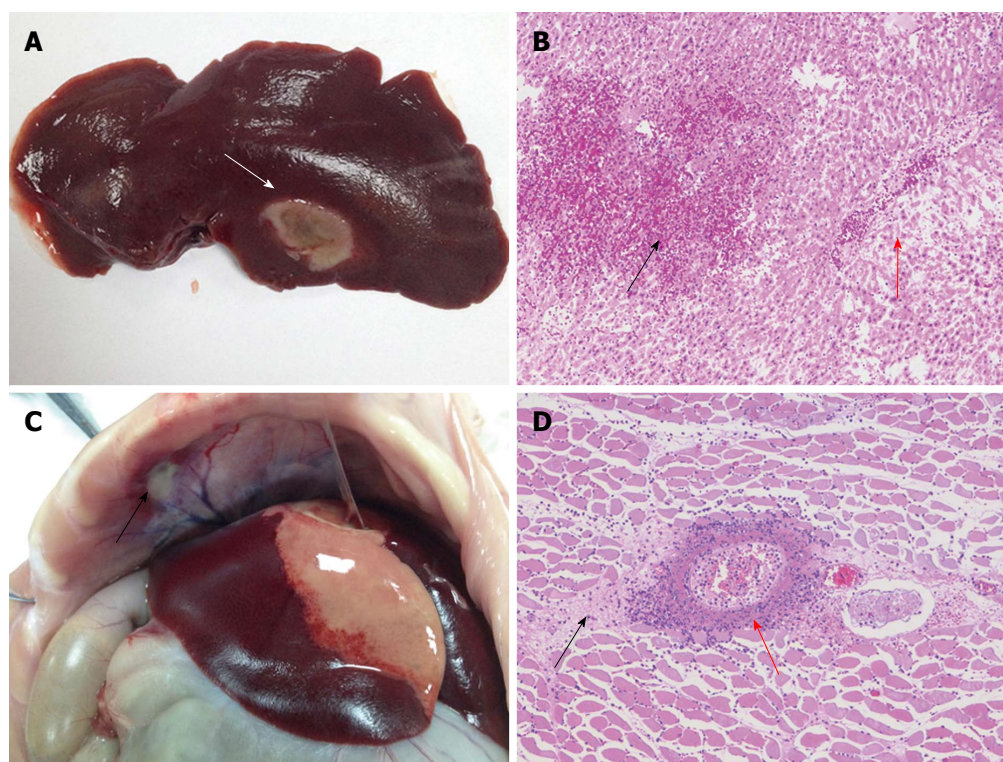
Figure 6 Histopathologic images of thermal lesions of the liver and diaphragm. A: Photograph showing a gray microwave ablation lesion (white arrow) in the liver lobe; B: Image depicting liver tissue congestion (black arrow), local hepatic sinus expansion, and hepatic cord disappearance due to atrophy and necrosis (red arrow); C: Photograph highlighting a gray-white lesion in the diaphragm (black arrow); D: Image showing a large number of inflammatory cells (red arrow) around the diaphragm and local necrocytosis of the muscular tissue (black arrow).

insulation effect is enough to protect the surrounding tissues adjacent to the ablation zones, as well as to reduce postoperative complications. Besides, since the volume of the fluid required to be injected into the body is significant low, it will be much more easily accepted by patients in a clinical setting.

Most important of all, none of the animals in the gel group experienced diaphragmatic injury, even when MW ablation was performed at the subcapsular region of the liver. This is partly due to the fact that the gel had been placed into a preset position and remained stable during the MW ablation. In contrast, thermal damage in the NS group was serious, due in part to the tendency of saline to flow away from the injection site, thereby providing partial protection to the diaphragm during liver ablations. In many cases, continuous infusion is unavoidable with saline, which is considered not suitable for use in clinical practice especially for patients susceptible to volume overload.

In addition, 22.5% P407 was found to be safe on our experimental animals, as demonstrated through *in vivo* safety studies involving liver and renal function tests. Yet, 3 and 7 d postoperatively are still in acute the timeframe and further studies over a longer period of time are necessary to establish the safety of P407 gel.

In spite of the accomplishments of the present study, there are a few limitations. Firstly, the sample size was relatively small (n = 24 ablations), yet the insulation effect showed statistical significance. Further

comprehensive studies are required to prove the safety and effectiveness of 22.5% P407 gel during MW ablation for small HCC. Secondly, healthy rabbits were included rather than tumor models; however, this may not affect our study findings, because the study aim was to assess thermoprotection properties rather than treatment effectiveness.

In conclusion, 22.5% P407 gel could be a more effective choice during MW ablation of subcapsular hepatic tumors adjacent to the diaphragm. Further studies for clinical translation are warranted.

COMMENTS

Background
Percutaneous thermal ablation has been a widely used method for treating liver tumors. However, about 15% of liver tumors deemed as high risk are not suitable for thermal ablation due to collateral thermal damage. Several methods, especially hydrodissection, have been suggested for ablation of subcapsular hepatic lesions.

Research frontiers
Hydrodissection, such as 5% dextrose in water (D5W) and 0.9% normal saline (NS), that has been found to be effective at decreasing unintended thermal injury; however, D5W and NS tend to move away quickly from target sites, thereby reducing the insulating effect.

Innovations and breakthroughs
In the study, the authors utilized the thermosensitivity of poloxamer 407 (P407) as novel hydrodissection to protect the surrounding tissues during microwave (MW) ablations.

Applications

In medical practice, percutaneous MW ablation has been credited with almost equivalent survival rates as surgical resection. As the results of this study suggest, critical ablations, such as for high-risk liver cancers, are possible to be performed when using P407 as a thermoprotective agent, although MW ablation is generally not recommended for treating such cases. In addition, since the volume of the fluid required to be injected into the body is significantly low, it would be more easily accepted by patients in a clinical setting. It should be noted that this material would be much-needed in most clinical situations, such as high-risk liver tumors.

Terminology

The study material is thermosensitive in nature. This means that it behaves like a liquid at low temperature and transforms into a get state at an increased temperature.

Peer-review

This is an interesting manuscript about the effect of a P407-based thermosensitive gel on minimization of thermal injury to diaphragm during MW ablation of the liver.

REFERENCES

1   **Liang P**, Wang Y, Yu X, Dong B. Malignant liver tumors: treatment with percutaneous microwave ablation--complications among cohort of 1136 patients. *Radiology* 2009; **251**: 933-940 [PMID: 19304921 DOI: 10.1148/radiol.2513081740]
2   **Mertyna P**, Goldberg W, Yang W, Goldberg SN. Thermal ablation a comparison of thermal dose required for radiofrequency-, microwave-, and laser-induced coagulation in an ex vivo bovine liver model. *Acad Radiol* 2009; **16**: 1539-1548 [PMID: 19836267 DOI: 10.1016/j.acra.2009.06.016]
3   **Callstrom MR**, Kurup AN. Percutaneous ablation for bone and soft tissue metastases--why cryoablation? *Skeletal Radiol* 2009; **38**: 835-839 [PMID: 19590871 DOI: 10.1007/s00256-009-0736-4]
4   **Lubner MG**, Brace CL, Hinshaw JL, Lee FT. Microwave tumor ablation: mechanism of action, clinical results, and devices. *J Vasc Interv Radiol* 2010; **21**: S192-S203 [PMID: 20656229 DOI: 10.1016/j.jvir.2010.04.007]
5   **Livraghi T**, Meloni F, Di Stasi M, Rolle E, Solbiati L, Tinelli C, Rossi S. Sustained complete response and complications rates after radiofrequency ablation of very early hepatocellular carcinoma in cirrhosis: Is resection still the treatment of choice? *Hepatology* 2008; **47**: 82-89 [PMID: 18008357 DOI: 10.1002/hep.21933]
6   **Laeseke PF**, Lee FT, Sampson LA, van der Weide DW, Brace CL. Microwave ablation versus radiofrequency ablation in the kidney: high-power triaxial antennas create larger ablation zones than similarly sized internally cooled electrodes. *J Vasc Interv Radiol* 2009; **20**: 1224-1229 [PMID: 19616970 DOI: 10.1016/j.jvir.2009.05.029]
7   **Laeseke PF**, Lee FT, van der Weide DW, Brace CL. Multiple-Antenna Microwave Ablation: Spatially Distributing Power Improves Thermal Profiles and Reduces Invasiveness. *J Interv Oncol* 2009; **2**: 65-72 [PMID: 21857888]
8   **Zhou P**, Liang P, Yu X, Wang Y, Dong B. Percutaneous microwave ablation of liver cancer adjacent to the gastrointestinal tract. *J Gastrointest Surg* 2009; **13**: 318-324 [PMID: 18825464 DOI: 10.1007/s11605-008-0710-9]
9   **Rhim H**, Yoon KH, Lee JM, Cho Y, Cho JS, Kim SH, Lee WJ, Lim HK, Nam GJ, Han SS, Kim YH, Park CM, Kim PN, Byun JY. Major complications after radio-frequency thermal ablation of hepatic tumors: spectrum of imaging findings. *Radiographics* 2003; **23**: 123-134; discussion 134-136 [PMID: 12533647 DOI: 10.1148/rg.231025054]
10 **Teratani T**, Yoshida H, Shiina S, Obi S, Sato S, Tateishi R, Mine N, Kondo Y, Kawabe T, Omata M. Radiofrequency ablation for hepatocellular carcinoma in so-called high-risk locations. *Hepatology* 2006; **43**: 1101-1108 [PMID: 16628706 DOI: 10.1002/hep.21164]
11 **Wong SN**, Lin CJ, Lin CC, Chen WT, Cua IH, Lin SM. Combined percutaneous radiofrequency ablation and ethanol injection for hepatocellular carcinoma in high-risk locations. *AJR Am J Roentgenol* 2008; **190**: W187-W195 [PMID: 18287411 DOI: 10.2214/AJR.07.2537]
12 **Hinshaw JL**, Laeseke PF, Winter TC, Kliewer MA, Fine JP, Lee FT. Radiofrequency ablation of peripheral liver tumors: intraperitoneal 5% dextrose in water decreases postprocedural pain. *AJR Am J Roentgenol* 2006; **186**: S306-S310 [PMID: 16632692 DOI: 10.2214/AJR.05.0140]
13 **Zhang M**, Liang P, Cheng ZG, Yu XL, Han ZY, Yu J. Efficacy and safety of artificial ascites in assisting percutaneous microwave ablation of hepatic tumours adjacent to the gastrointestinal tract. *Int J Hyperthermia* 2014; **30**: 134-141 [PMID: 24571176 DOI: 10.3109/02656736.2014.891765]
14 **Dumortier G**, Grossiord JL, Agnely F, Chaumeil JC. A review of poloxamer 407 pharmaceutical and pharmacological characteristics. *Pharm Res* 2006; **23**: 2709-2728 [PMID: 17096184 DOI: 10.1007/s11095-006-9104-4]
15 **Miyazaki S**, Takeuchi S, Yokouchi C, Takada M. Pluronic F-127 gels as a vehicle for topical administration of anticancer agents. *Chem Pharm Bull* (Tokyo) 1984; **32**: 4205-4208 [PMID: 6529816]
16 **Ricci EJ**, Lunardi LO, Nanclares DM, Marchetti JM. Sustained release of lidocaine from Poloxamer 407 gels. *Int J Pharm* 2005; **288**: 235-244 [PMID: 15620863 DOI: 10.1016/j.ijpharm.2004.09.028]
17 **Johnson A**, Sprangers A, Cassidy P, Heyrman S, Hinshaw JL, Lubner M, Puccinelli J, Brace C. Design and validation of a thermoreversible material for percutaneous tissue hydrodissection. *J Biomed Mater Res B Appl Biomater* 2013; **101**: 1400-1409 [PMID: 24591222 DOI: 10.1002/jbm.b.32959]
18 **Yong CS**, Choi JS, Quan QZ, Rhee JD, Kim CK, Lim SJ, Kim KM, Oh PS, Choi HG. Effect of sodium chloride on the gelation temperature, gel strength and bioadhesive force of poloxamer gels containing diclofenac sodium. *Int J Pharm* 2001; **226**: 195-205 [PMID: 11532582]
19 **Kim YS**, Rhim H, Paik SS. Radiofrequency ablation of the liver in a rabbit model: creation of artificial ascites to minimize collateral thermal injury to the diaphragm and stomach. *J Vasc Interv Radiol* 2006; **17**: 541-547 [PMID: 16567679 DOI: 10.1097/01.rvi.0000208305.65202.84]
20 **Martin RC**, Scoggins CR, McMasters KM. Microwave hepatic ablation: initial experience of safety and efficacy. *J Surg Oncol* 2007; **96**: 481-486 [PMID: 17654527 DOI: 10.1002/jso.20750]
21 **Thandassery RB**, Goenka U, Goenka MK. Role of local ablative therapy for hepatocellular carcinoma. *J Clin Exp Hepatol* 2014; **4**: S104-S111 [PMID: 25755601 DOI: 10.1016/j.jceh.2014.03.046]
22 **Chen EA**, Neeman Z, Lee FT, Kam A, Wood B. Thermal protection with 5% dextrose solution blanket during radiofrequency ablation. *Cardiovasc Intervent Radiol* 2006; **29**: 1093-1096 [PMID: 16802079 DOI: 10.1007/s00270-004-6216-2]
23 **Raman SS**, Lu DS, Vodopich DJ, Sayre J, Lassman C. Minimizing diaphragmatic injury during radio-frequency ablation: efficacy of subphrenic peritoneal saline injection in a porcine model. *Radiology* 2002; **222**: 819-823 [PMID: 11867807 DOI: 10.1148/radiol.2223001805]
24 **Bodily KD**, Atwell TD, Mandrekar JN, Farrell MA, Callstrom MR, Schmit GD, Charboneau JW. Hydrodisplacement in the percutaneous cryoablation of 50 renal tumors. *AJR Am J Roentgenol* 2010; **194**: 779-783 [PMID: 20173159 DOI: 10.2214/AJR.08.1570]

ORIGINAL ARTICLE

Basic Study

Protective effect of *Bifidobacterium infantis* CGMCC313-2 on ovalbumin-induced airway asthma and β-lactoglobulin-induced intestinal food allergy mouse models

Meng-Yun Liu, Zhen-Yu Yang, Wen-Kui Dai, Jian-Qiong Huang, Yin-Hu Li, Juan Zhang, Chuang-Zhao Qiu, Chun Wei, Qian Zhou, Xin Sun, Xin Feng, Dong-Fang Li, He-Ping Wang, Yue-Jie Zheng

Meng-Yun Liu, Jian-Qiong Huang, He-Ping Wang, Yue-Jie Zheng, Department of Respiratory, Shenzhen Children's Hospital, Shenzhen 518026, China

Zhen-Yu Yang, Wen-Kui Dai, Yin-Hu Li, Chuang-Zhao Qiu, Qian Zhou, Xin Feng, Dong-Fang Li, WeHealthGene Co., Shenzhen 518129, China

Juan Zhang, Chun Wei, Xin Sun, Xijing Hospital, The Fourth Military Medical University, Xi'an 7100032, Shann'xi Province, China

Abstract

AIM

To determine whether oral administration of *Bifidobacterium infantis* CGMCC313-2 (*B. infantis* CGMCC313-2) inhibits allergen-induced airway inflammation and food allergies in a mouse model.

METHODS

Ovalbumin (OVA)-induced allergic asthma and β-lactoglobulin-induced food allergy mouse models were used in this study. Following oral administration of *B. infantis* CGMCC313-2 during or after allergen sensitization, histopathologic changes in the lung and intestine were

evaluated by hematoxylin and eosin (HE) staining. In the allergic asthma mouse model, we evaluated the proportion of lung-infiltrating inflammatory cells. OVA-specific IgE and IgG1 levels in serum and cytokine levels in bronchoalveolar lavage fluid (BALF) were also assessed. In the food allergy mouse model, the levels of total IgE and cytokines in serum were measured.

RESULTS
Oral administration of *B. infantis* CGMCC313-2 during or after allergen sensitization suppressed allergic inflammation in lung and intestinal tissues, while the proportion of infiltrating inflammatory cells was significantly decreased in the BALF of allergic asthma mice. Moreover, *B. infantis* CGMCC313-2 decreased the serum levels of total IgE in food allergy mice, and reductions in IgE and IgG1 were also observed in OVA-induced allergic asthma mice. The expression of interleukin-4 (IL-4) and IL-13 in both serum and BALF was suppressed following the administration of *B. infantis* CGMCC313-2, while an effect on serum IL-10 levels was not observed.

CONCLUSION
B. infantis CGMCC313-2 inhibits the secretion of allergen-induced IgE, IL-4 and IL-13, and attenuates allergic inflammation.

Core tip: *Bifidobacterium infantis* CGMCC313-2 significantly decreased the serum concentration of IgE and IgG1 in asthma and food allergy mouse models. The number of infiltrating cells in bronchoalveolar lavage fluid was reduced, and eosinophil infiltration in lungs was relieved by *B. infantis* CGMCC313-2 in allergic asthma mice. Body weight was regained in food allergy mice, and intestinal inflammation was attenuated by *B. infantis* CGMCC313-2. Following administration of *B. infantis* CGMCC313-2, the concentrations of interleukin-4 (IL-4) and IL-13 decreased in both allergic asthma and food allergy mice.

INTRODUCTION
The prevalence of asthma, food allergies, eczema, and allergic rhinitis in developed countries has increased over the last three decades. In China, childhood allergic diseases are generally lower than those in Western countries; however, the prevalence of asthma, allergic rhinitis, and eczema in children has increased markedly during the past two decades[1-4]. A number of environmental factors including air pollution, cigarette smoking, and allergen exposure have been proposed to explain the changes in the prevalence of allergic diseases; however, no major risk factors have been identified. A common explanation for the increased incidence rates of childhood allergy and asthma observed in industrialized countries during the past few decades is the "hygiene hypothesis," which states that a lack of early childhood exposure to infectious agents, symbiotic microorganisms, and parasites increase susceptibility to allergic diseases by suppressing the natural development of the immune system[5,6]. Recent epidemiological and experimental studies have both renewed the "hygiene hypothesis" and extended it to a more specific theorem, the "microflora hypothesis"[6-8].

Probiotics are live microorganisms that confer a health benefit to the host when administered in adequate numbers[9]. In other words, ingested probiotics can modify microbial flora, which benefit the host[10,11]. Previous studies have shown that probiotics can reduce allergic diseases by modifying the immune system of the host. Some probiotic genera including *Lactobacilli* and *Bifidobacteria* are intensively investigated as novel alternative options for the management of allergic diseases including asthma and food allergy[12,13].

Experimental studies have shown that probiotics have strain-specific effects. In the present study, mice received nebulized ovalbumin and were used as an asthma model, while mice fed with β-lactoglobulin were used as a food allergy model (details in Materials and Methods). The effects of *Bifidobacterium infantis* CGMCC313-2, which is extensively used as a probiotic drug in China, were investigated in these two mouse models during (prevention) or after allergen sensitization (pre-treatment).

MATERIALS AND METHODS
Mice
Male BALB/c mice aged 6-8 wk were obtained from the Laboratory Animal Center of the Fourth Military Medical University. All experimental procedures involving animals were approved by the Ethics Committee for Animal Studies of the Fourth Military Medical University and performed in accordance with their guidelines (approval ID: 20150902).

Probiotic bacterial preparations
Bifidobacterium infantis CGMCC313-02 powder (Kexing Biotech Company Limited, Shenzhen, China) was stored at -20 ℃. Solutions were prepared using normal saline only or normal saline plus *B. infantis*

A

B

C
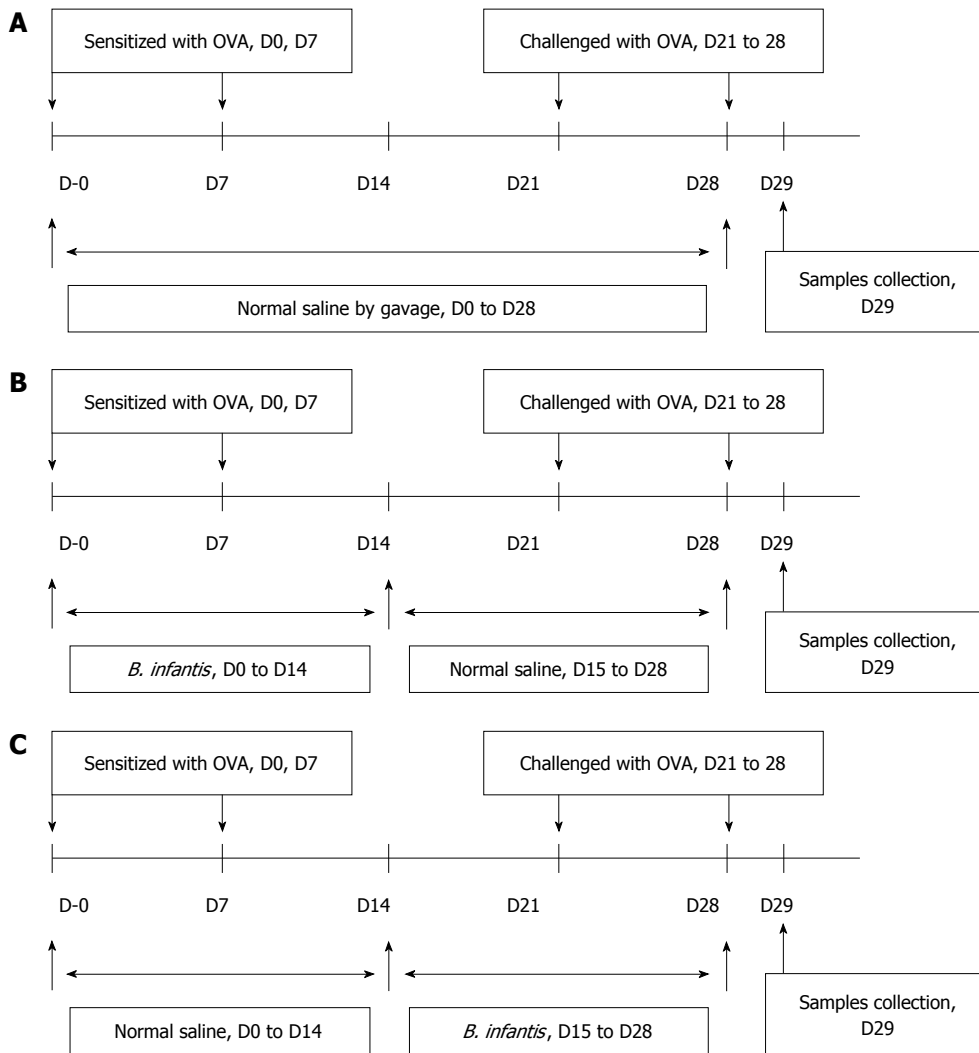
Figure 1 Protocols used for establishment of the mouse models. A: Allergic asthma; B: Prevention; C: Pre-treatment of OVA-induced airway allergy with *B. infantis* CGMCC313-2.

CGMCC313-2. *B. infantis* CGMCC313-2 preparations were adjusted at concentrations of 5×10^{10} colony-forming units (CFU)/mL.

Mouse model of OVA-induced allergic asthma

The mice were divided into four experimental groups, and each group consisted of 10 mice. Four groups of mice were treated as follows: (Group 1) the normal control group received normal saline plus 1.5 mg alum intraperitoneally. The mice were placed in an atomizing chamber (20 cm × 20 cm × 35 cm), and 8 mL saline was administered by nebulization. The mice were incubated for 30 min each time for 7 continuous days; (Group 2) the positive group (as shown in Figure 1A) received 100 μg ovalbumin (OVA) (Sigma, Buchs, Switzerland) plus 1.5 mg alum intraperitoneally from Day 0 to Day 7, and subsequently challenged with 1% OVA inhaled by nebulizer from Day 21 to Day 28; and (Group 3) the prevention and (Group 4) pre-treatment groups received 100 μg OVA plus alum intraperitoneally and 1% OVA inhaled, and were fed 0.2

mL/d (5×10^{10} CFU/mL) of *B. infantis* CGMCC313-2 from Day 0 to Day 14 (prevention group, as shown in Figure 1B), or from Day 15 to Day 28 (pre-treatment group, as shown in Figure 1C). Serum and BALF samples were collected from mice at sacrifice on Day 29.

Mouse model of β-lactoglobulin-induced food allergy

The mice were divided into four experimental groups, and each group consisted of 10 mice. Four groups of mice were treated as follows: (Group 1) the normal control group was fed normal saline (2 mL each time for 7 continuous days); (Group 2) the positive group (as shown in Figure 2A) received the mixture of 20 mg β-lactoglobulin (BLG) (Sigma, Buchs, Switzerland) and 10 μg CTX (Cholera toxin, List Biological Laboratories, Campbell, CA, United States) on days 0, 7, and 14 by intragastric gavage (2 mL of the mixture was used each time). Subsequently, the mice were challenged with 100 mg BLG (3 mL) on day 21 by intragastric gavage; and (Group 3) the prevention and (Group 4)
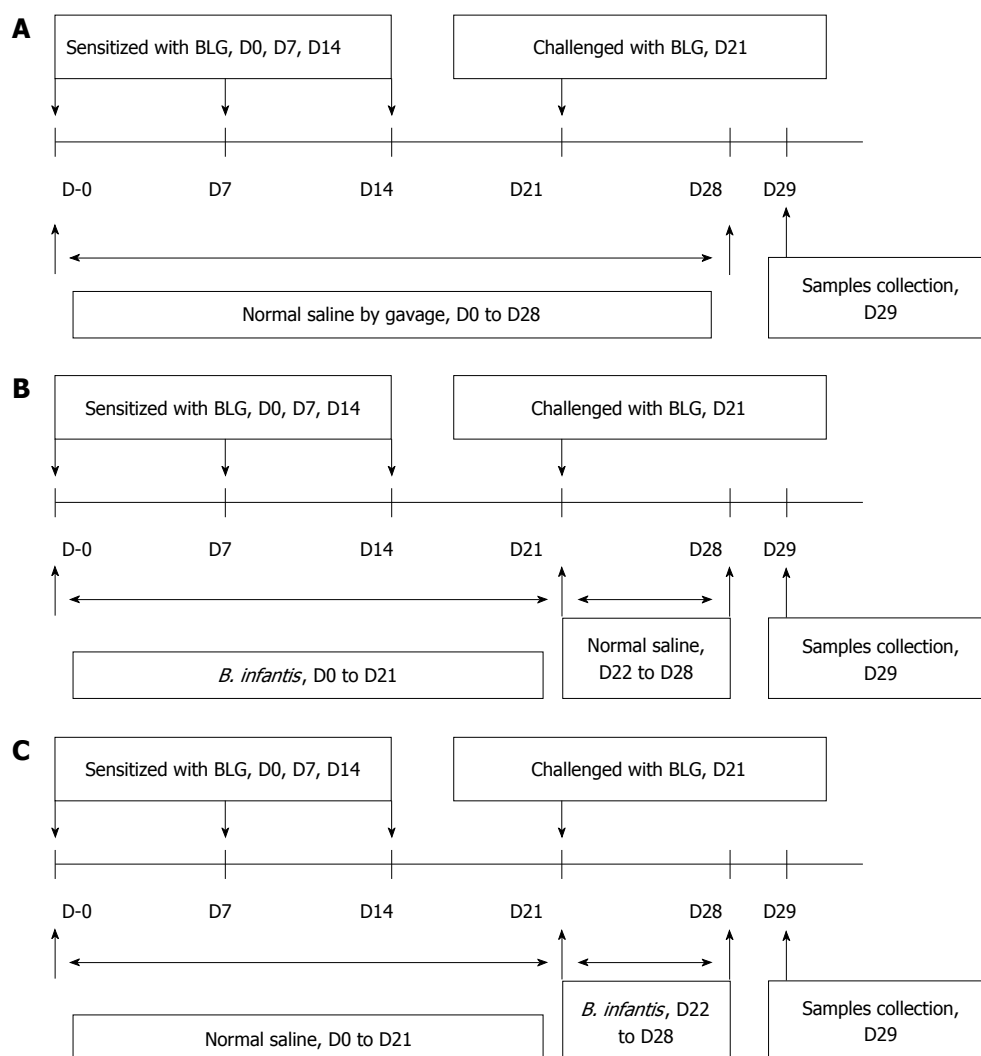
Figure 2 Protocols used for establishment of the mouse models. A: Food allergy; B: Prevention; C: Pre-treatment of β-lactoglobulin-induced food allergies with *B. infantis* CGMCC313-2.

pre-treatment groups received 20 mg BLG plus 10 μg CTX and challenged with 100 mg BLG by intragastric gavage, and were fed 0.2 mL/d (5×10^{10} CFU/mL) of *B. infantis* CGMCC313-2 from Day 0 to Day 21 (prevention group, as shown in Figure 2B), or from Day 22 to Day 28 (pre-treatment group, as shown in Figure 2C). Body weight was measured on Day 29, and then serum samples were collected after the mice were sacrificed.

Measurement of serum immunoglobulins

Serum samples from the mouse model of OVA-induced allergic asthma were assayed for OVA-specific IgE and IgG1 levels using ELISA kits (Chondrex Inc., United States) following the manufacturer's protocol. The serum level of total IgE was assayed in BLG-induced food allergy mice using ELISA kits (Chondrex, Inc., United States).

Measurement of cytokines

IL-4, IL-10, IL-13, and IFN-γ levels in serum (from the BLG-induced food allergy mouse model) or in BALF (from the OVA-induced allergic asthma mouse model) were assayed using ELISA Kits (R&D Systems, Boston, MA, United States) according to the manufacturer's protocol.

Cell counts of BALF

BALF was isolated in 1 mL of phosphate buffered saline (PBS) from the mouse model of OVA-induced allergic asthma. The BALF cellularity was determined using a hemocytometer. A 10 μL aliquot of centrifuged cells (4000 rpm, 5 min) was transferred onto slides, and all leukocytes were fixed for staining using Giemsa. The observer counted 200-300 cells per slide, and standard morphological criteria were adopted to identify the individual leukocyte populations. The number of leukocytes was counted twice, and the average value was calculated.

Histological analysis

To assess the pathological changes, samples from
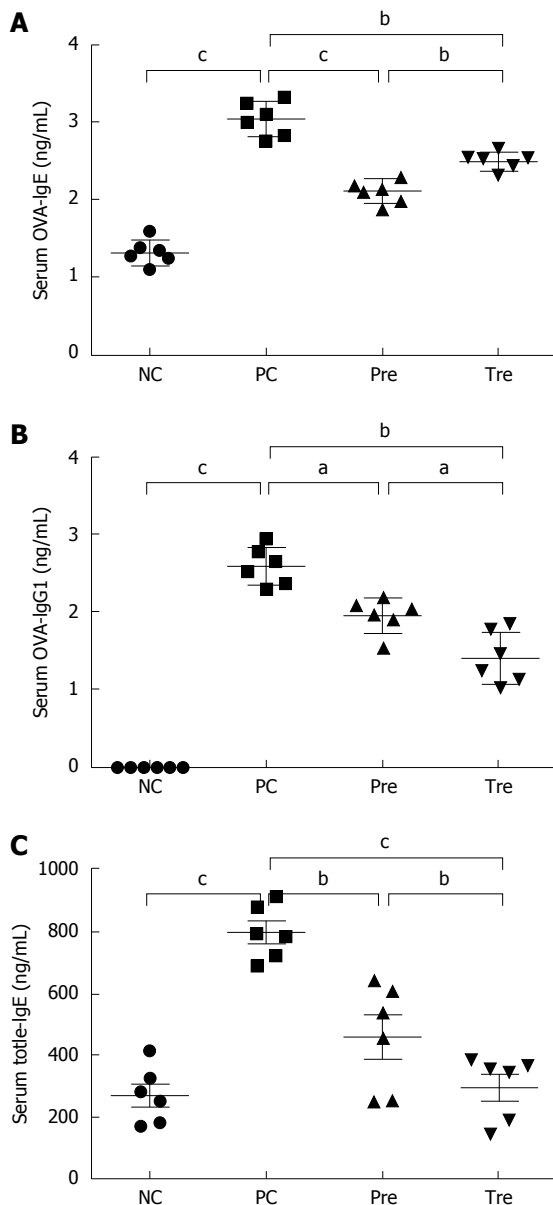
Figure 3 Effect of *B. infantis* CGMCC313-2 on the reversal of IgE and IgG1 in ovalbumin-induced asthma and β-lactoglobulin-induced food allergy mouse models. A and B: There were significant increases in OVA-specific IgE and IgG1 expression in the positive control (PC; Group 2) group compared with the normal control (NC; Group 1) group in the allergic asthma mouse model. The prevention (pre; Group 3) and pre-treatment (tre; Group 4) groups following *B. infantis* CGMCC313-2 administration showed decreased expression; C: A significant increase in total IgE expression was seen in the positive control (PC; Group 2) group compared with the normal control (NC; Group 1) group in the BLG-induced food allergy mouse model. The prevention (pre; Group 3) and pre-treatment (tre; Group 4) groups following *B. infantis* CGMCC313-2 administration showed decreased expression. The statistical differences are represented as follows: [a]$P < 0.05$; [b]$P < 0.01$, and [c]$P < 0.001$.

either lungs (OVA-induced allergic asthma) or intestine (BLG-induced food allergy) were collected. The samples were fixed in neutrally buffered 10% formaldehyde and embedded in paraffin. Sections 4 μm thick were stained with HE to detect inflammatory cell infiltration in intestinal tissue (BLG-induced food allergy), or to assess the extent of inflammation in the lungs (OVA-induced asthma) at 200 × magnification.

Statistical analysis

All data points represent the mean ± SEM in each mouse group. Analysis was performed using SPSS 19.0 software for Windows. Variance analysis of single factor and multi factor was conducted to determine the statistical significance. A *P* value lower than 0.05 was considered statistically significant.

RESULTS

B. infantis decreased the levels of IgE and IgG1 in OVA-induced asthma and BLG-induced food allergy mouse models

We determined whether oral *B. infantis* CGMCC313-2 affected serum levels of allergen-induced specific IgE and IgG1, and ELISA was used for data analysis in the OVA-induced allergic asthma mouse model. The serum levels of OVA-specific IgE and IgG1 were significantly elevated in the OVA sensitization/challenge (Group 2) compared with the normal control group (Group 1). In groups which received *B. infantis* CGMCC313-2 for prevention (Group 3) and pre-treatment (Group 4) during the OVA sensitization/challenge, the serum levels of IgE and IgG1 were significantly decreased (*P* < 0.05; Figure 3A and B). Moreover, the levels of serum IgE in the prevention group were also significantly decreased compared with the pre-treatment group (*P* < 0.05; Figure 3A).

Due to the unavailability of reagents for BLG-specific IgE and IgG1 detection, the serum levels of total IgE were evaluated in the BLG-induced food allergy mouse model. The serum levels of total IgE were significantly increased after the BLG sensitization/challenge (Group 2) compared with the normal control group (Group 1). In the groups challenged with *B. infantis* CGMCC313-2 for prevention (Group 3) and pre-treatment (Group 4), the levels of total IgE were significantly decreased. Moreover, the total IgE serum levels in the pre-treatment group were also significantly decreased compared with the prevention group (*P* < 0.05; Figure 3C).

B. infantis administration increases body weight in BLG-induced food allergy mice

Compared with the normal control group, mice in the BLG-sensitization/challenge group showed weight loss. However, the prevention and pre-treatment groups showed weight gain following *B. infantis* CGMCC313-2 (Figure 4), and the pre-treatment group gained more weight than the prevention group.

B. infantis alters the proportion of lung-infiltrating cells in OVA-induced allergic asthma mice

In order to evaluate the degree of inflammatory cell infiltration in the lungs of OVA-induced allergic asthma mice, leukocyte counts were conducted in BALF tissue. Inflammatory cell number was significantly increased in the OVA-sensitized/challenged mice compared to the
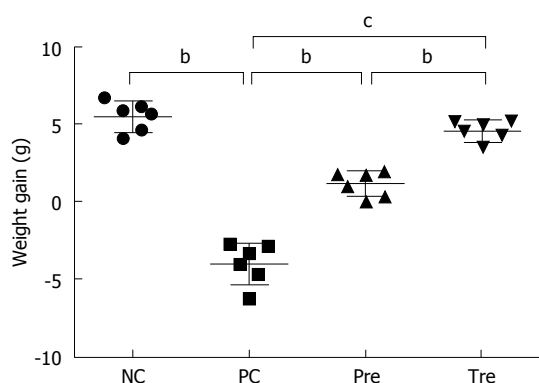
Figure 4 Effect of *B. infantis* CGMCC313-2 on body weight in BLG-induced food allergy mice. Average body weight decreased significantly in the positive control (PC; Group 2) group compared with the normal control (NC; Group 1) group. The prevention (pre; Group 3) and pre-treatment (tre; Group 4) groups following *B. infantis* CGMCC313-2 administration showed an increase in body weight. The statistical differences are represented as follows: [a]$P < 0.05$; [b]$P < 0.01$, and [c]$P < 0.001$.
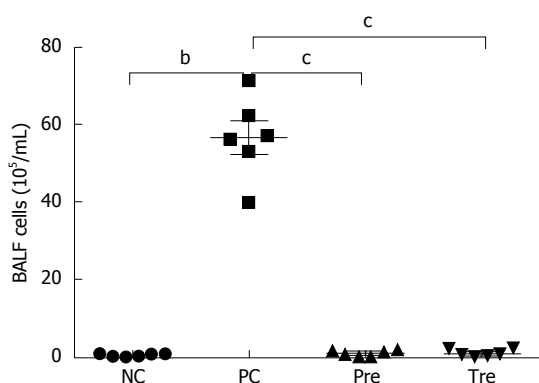
Figure 5 Effects of *B. infantis* CGMCC313-2 on infiltrating cells in the lungs of ovalbumin-induced allergic asthma mice. Total cell number in BALF increased significantly in the positive control (PC; Group 2) group compared with the normal control (NC; Group 1) group. The prevention (pre; Group 3), and pre-treatment (tre; Group 4) groups following *B. infantis* CGMCC313-2 administration showed a decrease. The statistical differences are represented as: [a]$P < 0.05$; [b]$P < 0.01$, and [c]$P < 0.001$.

control group. However, the proportion of infiltrating cells in the lung was significantly decreased in the groups treated with *B. infantis* CGMCC313-2 (Figure 5). Differential cell counts using Giemsa staining failed to identify cell types in our study.

Impact of B. infantis on allergic inflammation in OVA-induced asthma and BLG-induced food allergy mouse models

The effect of *B. infantis* CGMCC313-2 in the OVA or BLG-sensitized/challenged mice was evaluated from the perspective of overall lung or intestinal inflammation using histological HE staining (Figures 6 and 7, respectively). Compared with normal control mice (Figure 6A and Figure 7A), allergen sensitized/challenged mice (Figure 6B and Figure 7B) had severe inflammation; while the prevention (Figure 6C and Figure 7C) and pre-treatment (Figure 6D and Figure

7D) mice showed significantly diminished signs of inflammation following *B. infantis* CGMCC313-2 treatment.

Effect of B. infantis on cytokines in serum and BALF

To further elucidate possible mechanisms responsible for the effects of *B. infantis* CGMCC313-2 on systemic sensitization and allergic inflammation, ELISA was used to determine the expression of IL-4, IL-10, IL-13, and IFN-γ in serum and BALF was collected from BLG-induced food allergy mice and OVA-induced allergic asthma mice, respectively. IL-4 and IL-13 in serum (Figure 8A and B) or BALF (Figure 8D and E) increased significantly in the positive control (PC; Group 2) group compared with the normal control (NC; Group 1) group. In the groups that received *B. infantis* CGMCC313-2, the serum levels of IL-4 and IL-13 in the prevention group were significantly decreased. In addition, a reduction in IL-4 and IL-13 in the prevention and treatment groups was also observed in OVA-induced allergic asthma mice. Serum IL-10 (Figure 8C) decreased significantly in the positive control (PC; Group 2) group, prevention (Pre; Group 3) group, and pre-treatment (Tre; Group 4) group compared with the normal control (NC; Group 1) group. IL-10 was not detected in BALF from OVA-induced allergic asthma mice, and IFN-γ was not detected in either serum or BALF from any of the mice.

DISCUSSION

There is increasing evidence to show that intestinal microbiota and ingested probiotics may induce important metabolic and physiological reactions in the host, and drive maturation of the immune system in early life. Of these diverse probiotics, *Lactobacilli* and *Bifidobacteria*, which are part of the gut flora in infants, are the most promising candidates that naturally affect immune system development[14,15]. For the same reason, *Lactobacilli* and *Bifidobacteria* are the most frequently used probiotics for clinical intervention studies[16-20]. However, the most important characteristic of probiotics is their strain-specificity effect[21]. In this study, we investigated the role of *Bifidobacterium infantis* CGMCC313-2 in allergic disease prevention and treatment in two mouse models, as *B. infantis* CGMCC313-2 has been extensively used in the treatment and prevention of diarrhea including antibiotic-associated diarrhea in China. In OVA-sensitized/challenged mice, severe lung inflammation and infiltrating cells in the lungs were observed, and the administration of *B. infantis* CGMCC313-2 significantly diminished inflammation. Similarly, in β-lactoglobulin-induced food allergy mice, *B. infantis* CGMCC313-2 decreased intestinal inflammation, and ameliorated weight loss in BLG-sensitized/challenged mice. These results demonstrate that oral administration of *B. infantis* CGMCC313-2
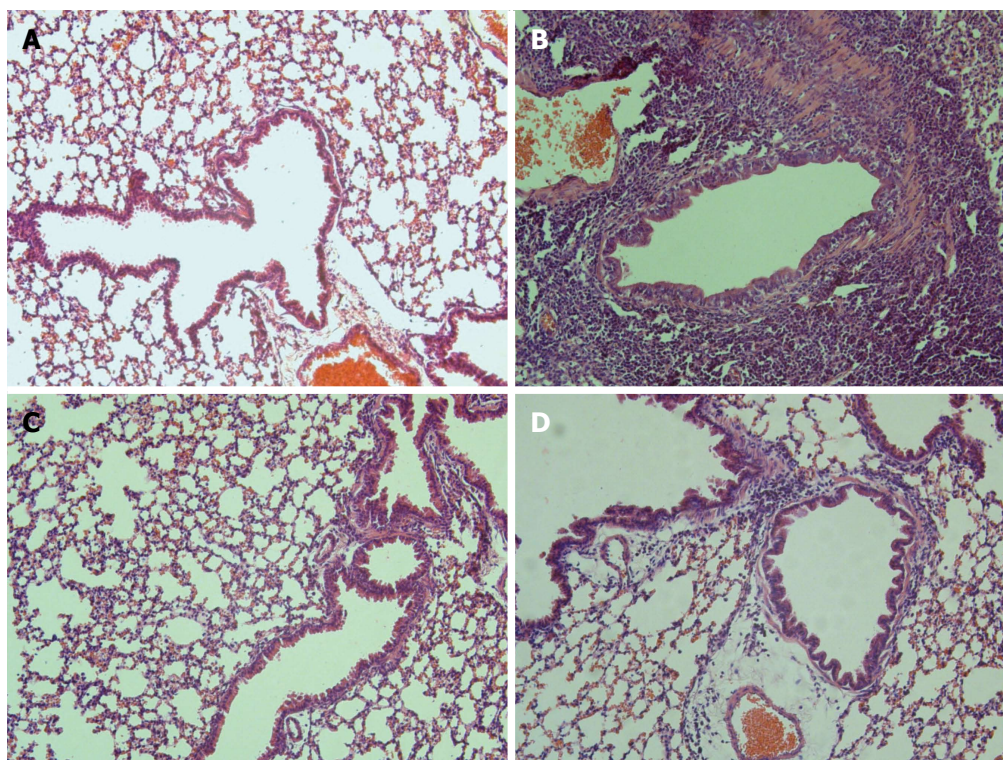
Figure 6 Effects of *B. infantis* CGMCC313-2 on OVA-induced airway inflammation. Lung tissues were obtained from the (C) prevention group and (D) pre-treatment group treated with *B. infantis* CGMCC313-2, and from (A) the normal control group and (B) the ovalbumin sensitized/challenged group on Day 29. The tissues were stained and observed under × 200 magnification. The positive control group showed severe airway inflammation, while the groups treated with *B. infantis* CGMCC313-2 showed attenuation of airway inflammation.
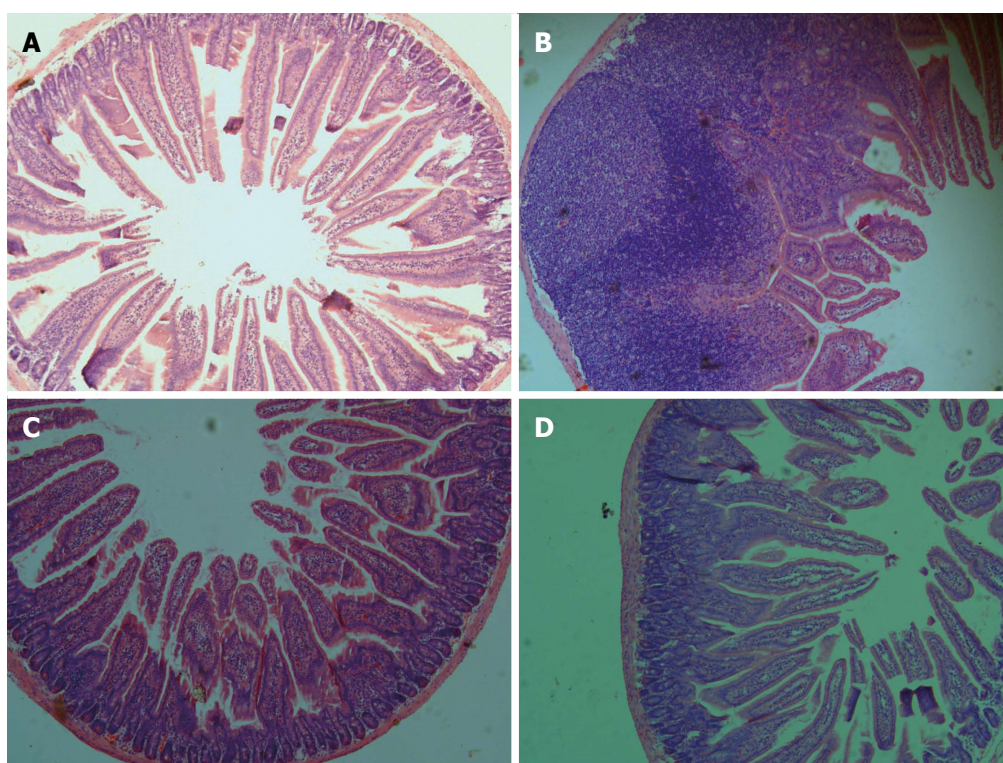
Figure 7 Effects of *B. infantis* CGMCC313-2 on BLG-induced intestinal inflammation. Intestinal tissues were obtained from (A) the normal control group and (B) the BLG-sensitized/challenged group on Day 29, and from the (C) prevention group and (D) pre-treatment group which were treated with *B. infantis* CGMCC313-2. The tissues were stained and observed under 200 × magnification. The positive control group showed severe intestinal inflammation, while the groups treated with *B. infantis* CGMCC313-2 showed attenuated intestinal inflammation.
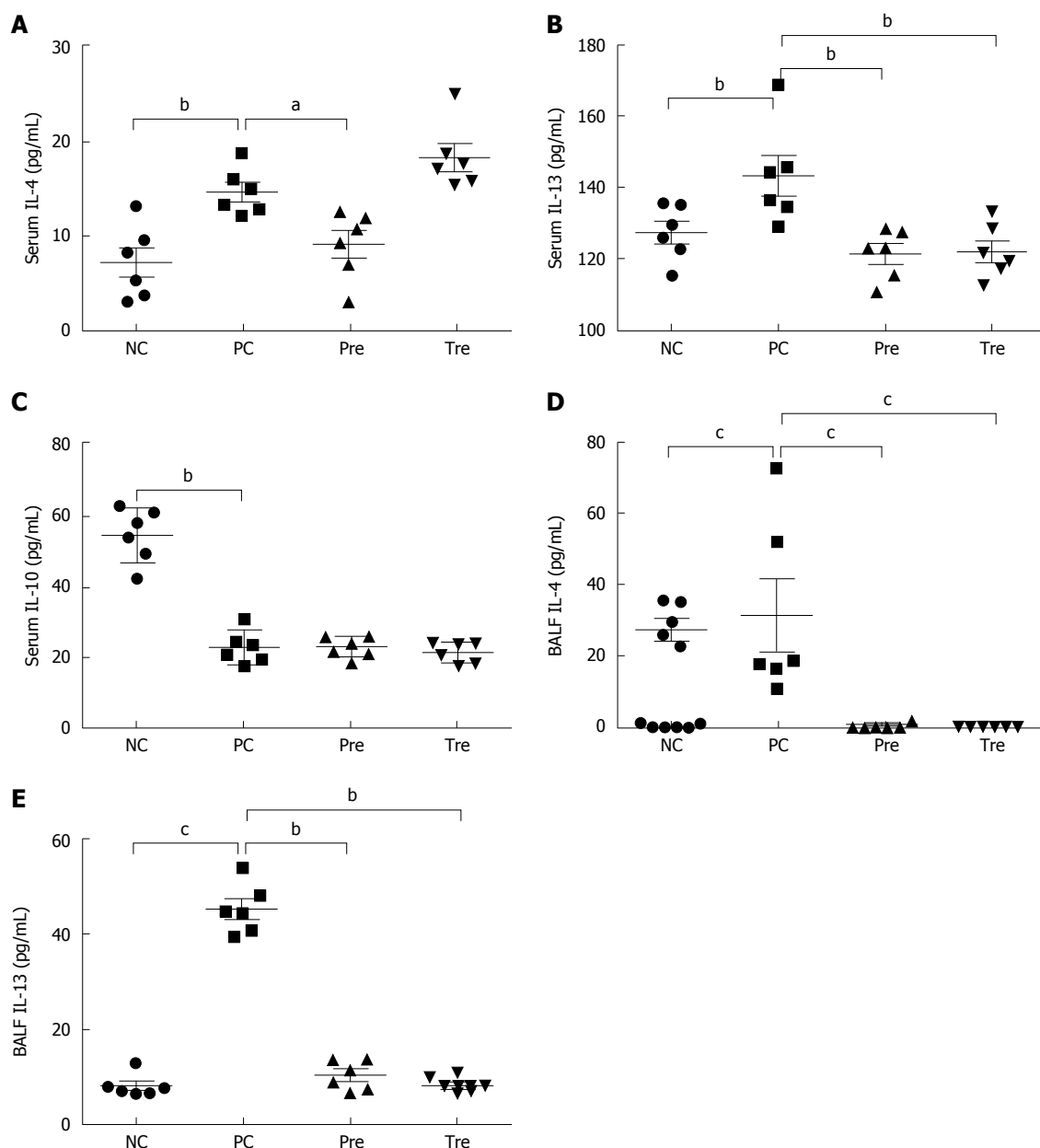
Figure 8 Effects of *B. infantis* CGMCC313-2 on cytokines in serum and bronchoalveolar lavage fluid. IL-4, IL-10, and IL-13 in serum and BALF were determined in BLG-induced food allergy mice and OVA-induced allergic asthma mice, respectively. A: Serum IL-4 in the prevention (pre; Group 3) group; B: serum IL-13 in the prevention (pre; Group 3) and pre-treatment (tre; Group 4) groups were significantly decreased compared with the positive control (PC; Group 2) group; C: There was no significant difference in IL-10 between the positive control group (PC; Group 2), prevention group (pre; Group 3), and pre-treatment (tre; Group 4) groups, which was significantly decreased when compared with the normal control (NC; Group 1) group; D: The concentrations of BALF IL-4 and (E) BALF IL-13 were significantly decreased in the prevention (pre; Group 3) group and the pre-treatment (tre; Group 4) group treated with *B. infantis* CGMCC313-2. The statistical differences are represented as follows: [a]$P < 0.05$; [b]$P < 0.01$, and [c]$P < 0.001$.

during or after allergen sensitization may relieve allergic inflammation in the airway and intestine.

In the allergen sensitized/challenged mice, IL-4, IL-13, total IgE, and allergen-induced serum specific IgE and IgG1 levels were highly expressed. Based on the immunological basis of allergy, the overexpression of IL-4 and IL-13, which is modulated by type 2 T helper cells, could promote IgE production and eosinophil infiltration in target organs. In the present study, following the oral administration of *B. infantis* CGMCC313-2, the levels of IL-13 and total IgE were significantly decreased, which was accompanied by the attenuation of inflammatory symptoms. We deduced that the metabolites of *B. infantis* CGMCC313-2, including butyrate and short-chain fatty acids, can suppress the inflammatory responses triggered by Th2 cytokines[22-28]. However, the level of IL-4 was higher in the treatment group, which was opposite to the results of IL-13 and IgE. Due to the complexity of the immune system and response, the role of IL-4 as an allergic disorder marker requires further investigation in our future study. In addition, the oral administration of this

probiotic helped in the prevention and treatment of airway and intestine allergy.

On the other hand, there was a decrease in IL-10 serum levels in mice sensitized/challenged with BLG. There is strong evidence to indicate that the production of IL-10, which is affected by antigens exposure, is associated with T cell tolerance and Treg secretion, which in turn plays important roles in controlling allergic diseases. However, the administration of *B. infantis* CGMCC313-2 did not promote the secretion of IL-10. This phenomenon was inconsistent with previous preclinical studies in which probiotic strains promoted Treg responses[29,30]. We deduced that the different probiotic strains adopted in different studies may have strain-specific effects, or the immunomodulatory effect of *B. infantis* CGMCC313-2 suppressed Th2 responses. In our study, the levels of IFN-γ in both serum and BALF were too low to be detected in all mice, and this may have been due to the poor sensitivity of the measurement technique. This is a limitation of our study.

In the present study, which included allergic asthma and food allergy mouse models, we found that *B. infantis* CGMCC313-2 inhibited the secretion of allergen-induced IgE and Th2 cytokines, and further attenuated allergic inflammation. Our study also suggested that the modulatory activity of *B. infantis* CGMCC313-2 was not only confined to intestinal allergic diseases, but also to allergic airway disease. Therefore, *B. infantis* CGMCC313-2 may be regarded as a candidate probiotic strain in the prevention and treatment of allergic diseases. However, further clinical and experimental studies are required to delineate the potential preventive and treatment effects of *B. infantis* CGMCC313-2.

ACKNOWLEDGMENTS

COMMENTS

Background
Probiotics exhibit beneficial effects on allergies based on the "microflora hypothesis", and experimental studies have also shown that probiotics have strain-specific effects. In this study, the specific effects of *B. infantis* CGMCC313-2, which is widely used as a probiotic drug in China, on allergic asthma and food allergy mouse models were determined.

Research frontiers
Previous studies have indicated that ingested *Lactobacilli* and *Bifidobacteria* can modify microbial flora, and can reduce the symptoms of allergic diseases by modifying the host immune system. Some probiotic drugs have been intensively investigated as novel alternative options for the management of allergic diseases including asthma and food allergy.

Innovations and breakthroughs
In this study, *B. infantis* CGMCC313-2 was administered to allergic asthma and food allergy mouse models. Following *B. infantis* CGMCC313-2 treatment, the serum concentrations of IgE and IgG1 significantly decreased, and the concentrations of interleukin-4 (IL-4) and IL-13 also reduced in mice with allergic asthma and food allergy. Inflammatory cell infiltration and inflammation were also attenuated.

Applications
B. infantis CGMCC313-2 can inhibit the secretion of allergen-induced IgE and Th2 cytokines, and can attenuate allergic inflammation. *B. infantis* CGMCC313-2 provides an important reference for the prevention and treatment of intestinal and airway allergic diseases.

Terminology
IgE and IgG, which are closely related with anaphylaxis, are higher in patients with allergies. IL-4 and IL-13, which are secreted by Th2 cells, are involved in the humoral immune response and indicate the degree of allergic disease.

Peer-review
The authors performed clear experiments on asthma mouse model and food allergy mouse model to detect the effects of *B. infantis* on allergy diseases. They found that *B. infantis* could inhibit the secretion of allergens induced IgG, IgE, IL-4 and IL-13, and allergic inflammation were also attenuated. The study shed the light on the prevention and treatment of intestinal and airway allergic diseases. However, further clinical studies are still required.

REFERENCES

1   **Zhao J**, Bai J, Shen K, Xiang L, Huang S, Chen A, Huang Y, Wang J, Ye R. Self-reported prevalence of childhood allergic diseases in three cities of China: a multicenter study. *BMC Public Health* 2010; **10**: 551 [PMID: 20836838 DOI: 10.1186/1471-2458-10-551]

2   **Yangzong Y**, Shi Z, Nafstad P, Håheim LL, Luobu O, Bjertness E. The prevalence of childhood asthma in China: a systematic review. *BMC Public Health* 2012; **12**: 860 [PMID: 23050953 DOI: 10.1186/1471-2458-12-860]

3   **Zhang Y**, Zhang L. Prevalence of allergic rhinitis in china. *Allergy Asthma Immunol Res* 2014; **6**: 105-113 [PMID: 24587945 DOI: 10.4168/aair.2014.6.2.105]

4   **Li F**, Zhou Y, Li S, Jiang F, Jin X, Yan C, Tian Y, Zhang Y, Tong S, Shen X. Prevalence and risk factors of childhood allergic diseases in eight metropolitan cities in China: a multicenter study. *BMC Public Health* 2011; **11**: 437 [PMID: 21645361 DOI: 10.1186/1471-2458-11-437]

5   **Strachan DP**. Family size, infection and atopy: the first decade of the "hygiene hypothesis". *Thorax* 2000; **55** Suppl 1: S2-10 [PMID: 10943631]

6   **Strannegård O**, Strannegård IL. The causes of the increasing prevalence of allergy: is atopy a microbial deprivation disorder? *Allergy* 2001; **56**: 91-102 [PMID: 11167368]

7   **Kalliomäki M**, Isolauri E. Role of intestinal flora in the development of allergy. *Curr Opin Allergy Clin Immunol* 2003; **3**: 15-20 [PMID: 12582309 DOI: 10.1097/01.all.0000053262.39029.a1]

8   **Björkstén B**. Effects of intestinal microflora and the environment on the development of asthma and allergy. *Springer Semin Immunopathol* 2004; **25**: 257-270 [PMID: 15007630 DOI: 10.1007/s00281-003-0142-2]

9   Food and Agriculture Organization of the United Nations and World Health Organization (FAO-WHO) (2002). Guideline for the evaluation of probiotics in food. FAO of the United Nations and WHO working group report; Online: Available from: URL: http://who.int/foodsafety/publications.fs-management/probiotics/en/

10 **Zuccotti G**, Meneghin F, Aceti A, Barone G, Callegari ML, Di Mauro A, Fantini MP, Gori D, Indrio F, Maggio L, Morelli L, Corvaglia L. Probiotics for prevention of atopic diseases in infants:

systematic review and meta-analysis. *Allergy* 2015; **70**: 1356-1371 [PMID: 26198702 DOI: 10.1111/all.12700]

11   **Floch MH**, Walker WA, Sanders ME, Nieuwdorp M, Kim AS, Brenner DA, Qamar AA, Miloh TA, Guarino A, Guslandi M, Dieleman LA, Ringel Y, Quigley EM, Brandt LJ. Recommendations for Probiotic Use--2015 Update: Proceedings and Consensus Opinion. *J Clin Gastroenterol* 2015; **49** Suppl 1: S69-S73 [PMID: 26447969 DOI: 10.1097/MCG.0000000000000420]

12   **Cuello-Garcia CA**, Brożek JL, Fiocchi A, Pawankar R, Yepes-Nuñez JJ, Terracciano L, Gandhi S, Agarwal A, Zhang Y, Schünemann HJ. Probiotics for the prevention of allergy: A systematic review and meta-analysis of randomized controlled trials. *J Allergy Clin Immunol* 2015; **136**: 952-961 [PMID: 26044853 DOI: 10.1016/j.jaci.2015.04.031]

13   **Szajewska H**, Shamir R, Turck D, van Goudoever JB, Mihatsch WA, Fewtrell M. Recommendations on probiotics in allergy prevention should not be based on pooling data from different strains. *J Allergy Clin Immunol* 2015; **136**: 1422 [PMID: 26329511 DOI: 10.1016/j.jaci.2015.07.022]

14   **Saavedra JM**. Use of probiotics in pediatrics: rationale, mechanisms of action, and practical aspects. *Nutr Clin Pract* 2007; **22**: 351-365 [PMID: 17507735]

15   **Ringel-Kulka T**, Cheng J, Ringel Y, Salojärvi J, Carroll I, Palva A, de Vos WM, Satokari R. Intestinal microbiota in healthy U.S. young children and adults--a high throughput microarray analysis. *PLoS One* 2013; **8**: e64315 [PMID: 23717595 DOI: 10.1371/journal.pone.0064315]

16   **Weston S**, Halbert A, Richmond P, Prescott SL. Effects of probiotics on atopic dermatitis: a randomised controlled trial. *Arch Dis Child* 2005; **90**: 892-897 [PMID: 15863468 DOI: 10.1136/adc.2004.060673]

17   **Viljanen M**, Savilahti E, Haahtela T, Juntunen-Backman K, Korpela R, Poussa T, Tuure T, Kuitunen M. Probiotics in the treatment of atopic eczema/dermatitis syndrome in infants: a double-blind placebo-controlled trial. *Allergy* 2005; **60**: 494-500 [PMID: 15727582 DOI: 10.1111/j.1398-9995.2004.00514.x]

18   **Kalliomäki M**, Salminen S, Arvilommi H, Kero P, Koskinen P, Isolauri E. Probiotics in primary prevention of atopic disease: a randomised placebo-controlled trial. *Lancet* 2001; **357**: 1076-1079 [PMID: 11297958 DOI: 10.1016/S0140-6736(00)04259-8]

19   **Kalliomäki M**, Salminen S, Poussa T, Arvilommi H, Isolauri E. Probiotics and prevention of atopic disease: 4-year follow-up of a randomised placebo-controlled trial. *Lancet* 2003; **361**: 1869-1871 [PMID: 12788576 DOI: 10.1016/S0140-6736(03)13490-3]

20   **Rautava S**, Kalliomäki M, Isolauri E. Probiotics during pregnancy and breast-feeding might confer immunomodulatory protection against atopic disease in the infant. *J Allergy Clin Immunol* 2002; **109**: 119-121 [PMID: 11799376]

21   **Hougee S**, Vriesema AJ, Wijering SC, Knippels LM, Folkerts G, Nijkamp FP, Knol J, Garssen J. Oral treatment with probiotics reduces allergic symptoms in ovalbumin-sensitized mice: a bacterial strain comparative study. *Int Arch Allergy Immunol* 2010; **151**: 107-117 [PMID: 19752564 DOI: 10.1159/000236000]

22   **Schouten B**, van Esch BC, Hofman GA, van Doorn SA, Knol J, Nauta AJ, Garssen J, Willemsen LE, Knippels LM. Cow milk allergy symptoms are reduced in mice fed dietary synbiotics during oral sensitization with whey. *J Nutr* 2009; **139**: 1398-1403 [PMID: 19474160 DOI: 10.3945/jn.109.108514]

23   **Thang CL**, Baurhoo B, Boye JI, Simpson BK, Zhao X. Effects of Lactobacillus rhamnosus GG supplementation on cow's milk allergy in a mouse model. *Allergy Asthma Clin Immunol* 2011; **7**: 20 [PMID: 22145744 DOI: 10.1186/1710-1492-7-20]

24   **Frossard CP**, Steidler L, Eigenmann PA. Oral administration of an IL-10-secreting Lactococcus lactis strain prevents food-induced IgE sensitization. *J Allergy Clin Immunol* 2007; **119**: 952-959 [PMID: 17316776 DOI: 10.1016/j.jaci.2006.12.615]

25   **Forsythe P**, Inman MD, Bienenstock J. Oral treatment with live Lactobacillus reuteri inhibits the allergic airway response in mice. *Am J Respir Crit Care Med* 2007; **175**: 561-569 [PMID: 17204726 DOI: 10.1164/rccm.200606-821OC]

26   **Li CY**, Lin HC, Hsueh KC, Wu SF, Fang SH. Oral administration of Lactobacillus salivarius inhibits the allergic airway response in mice. *Can J Microbiol* 2010; **56**: 373-379 [PMID: 20555399 DOI: 10.1139/w10-024]

27   **Wu CT**, Chen PJ, Lee YT, Ko JL, Lue KH. Effects of immuno-modulatory supplementation with Lactobacillus rhamnosus on airway inflammation in a mouse asthma model. *J Microbiol Immunol Infect* 2016; **49**: 625-635 [PMID: 25440975 DOI: 10.1016/j.jmii.2014.08.001]

28   **Fujimura KE**, Demoor T, Rauch M, Faruqi AA, Jang S, Johnson CC, Boushey HA, Zoratti E, Ownby D, Lukacs NW, Lynch SV. House dust exposure mediates gut microbiome Lactobacillus enrichment and airway immune defense against allergens and virus infection. *Proc Natl Acad Sci USA* 2014; **111**: 805-810 [PMID: 24344318 DOI: 10.1073/pnas.1310750111]

29   **Berin MC**. Bugs versus bugs: probiotics, microbiome and allergy. *Int Arch Allergy Immunol* 2014; **163**: 165-167 [PMID: 24481028 DOI: 10.1159/000357946]

30   **Yoshida T**, Fujiwara W, Enomoto M, Nakayama S, Matsuda H, Sugiyama H, Shimojoh M, Okada S, Hattori M. An increased number of CD4+CD25+ cells induced by an oral administration of Lactobacillus plantarum NRIC0380 are involved in antiallergic activity. *Int Arch Allergy Immunol* 2013; **162**: 283-289 [PMID: 24135451 DOI: 10.1159/000354924]

ORIGINAL ARTICLE

Basic Study

Diagnostic value evaluation of trefoil factors family 3 for the early detection of colorectal cancer

Hui Xie, Jian-Hai Guo, Wei-Min An, Sheng-Tao Tian, Hai-Peng Yu, Xue-Ling Yang, Hua-Ming Wang, Zhi Guo

Hui Xie, Hai-Peng Yu, Xue-Ling Yang, Zhi Guo, Department of Interventional Therapy, Tianjin Medical University Cancer Institute and Hospital, National Clinical Cancer Research Center, Key Laboratory of Cancer Prevention and Therapy, Tianjin 300070, China

Hui Xie, Sheng-Tao Tian, Hua-Ming Wang, Department of interventional therapy, 302 Hospital of People's Liberation Army, Beijing 100039, China

Jian-Hai Guo, Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education/Beijing), Interventional Therapy Department, Peking University Cancer Hospital and Institute, Beijing 100142, China

Wei-Min An, Center of radiology, 302 Hospital of People's Liberation Army, Beijing 100039, China

Abstract

AIM

The purpose of this study was to evaluate the diagnostic value of trefoil factor family 3 (TFF3) for the

early detection of colorectal cancer (CC).

METHODS
Serum TFF3 and carcino-embryonic antigen (CEA) were detected in 527 individuals, including 115 healthy control (HC), 198 colorectal adenoma (CA), and 214 CC individuals in the training group.

RESULTS
Serum TFF3 showed no significant correlation with age, gender, or tumor location but showed significant correlation with the tumor stage. Serum TFF3 in the CC group was significantly higher than in the HC or CA group. The AUC values of TFF3 for discriminating between HC and CC and between CA and CC were 0.930 (0.903, 0.958) and 0.834 (0.796, 0.873). A multivariate model combining TFF3 and CEA was built. Compared to TFF3 or CEA alone, the multivariate model showed significant improvement ($P < 0.001$). For discriminating between HC and CC, HC and early stage CC, HC and advanced stage CC, CA and CC, CA and early stage CC, and CA and advanced stage CC in the training group, the sensitivities were 92.99%, 91.46%, 93.18%, 73.83%, 76.83%, and 81.82%, and the specificities were 91.30%, 91.30%, 93.91%, 88.38%, 77.27%, and 88.38%, respectively. After validation, the sensitivities were 89.39%, 85.71%, 90.79%, 72.73%, 71.43%, and 78.95%, and the specificities were 87.85%, 87.85%, 2.52%, 87.85%, 80.77%, and 87.50%, respectively.

CONCLUSION
The multivariate diagnostic model that included TFF3 and CEA showed significant improvement over the conventional biomarker CEA and might provide a potential method for the early detection of CC.

Key words: Trefoil factor family 3; Colorectal cancer; Colorectal adenoma; Multivariate model; Diagnostic value

Core tip: Serum level of trefoil factor family 3 (TFF3) was used for evaluation the diagnostic value of for the early detection of colorectal cancer (CC). A multivariate model combining TFF3 and carcino-embryonic antigen (CEA) was built. Compared to TFF3 or CEA alone, the multivariate model showed significant improvement. The multivariate diagnostic model that included TFF3 and CEA showed significant improvement over the conventional biomarker CEA and might provide a potential method for the early detection of CC.

INTRODUCTION
Colorectal cancer (CC) is one of the most common cancers worldwide. An estimated 131700 new colorectal cancer patients (69090 male and 62610 female) are estimated to have occurred in the United States in 2015[1], and in China, the incidence of colorectal cancer showed a clearly increased tendency. The prognosis of CC is strongly related to the tumor stage. The 5-year relative survival ratio ranges from greater than 90% in patients with stage I to slightly greater than 10% in patients with stage IV[2]. Although various detection methods are used in clinical practice, such as colonoscopy, fecal occult blood testing, stool DNA testing, and carcino-embryonic antigen (CEA), their diagnostic value is limited by disadvantages, and they cannot meet the needs of clinical detection[3]. A detection method with high sensitivity and specificity, easy availability and low cost is urgently needed for the early detection of CC in clinical practice.

The trefoil factor family proteins (TFFs), secreted by the mammalian gastrointestinal tract, are small and stable molecules. They include three thermo stable and protease-resistant proteins (TFF1, TFF2, and TFF3) and are widely distributed in the gastrointestinal tract[4]. Studies have demonstrated that they play important roles in the mucosal protection and repair of epithelial surfaces and are involved in the development and progression of various types of cancer. TFF levels in plasma were found to be heightened in advanced prostate cancer[5] but reduced in the oral mucosal tissues of oral squamous cell carcinoma patients[6]. The levels of TFF3 in the serum and lung tissues were also increased and indicated that TFF3 might serve as a promising biomarker of lung cancer[7]. TFF3 was also found to be expressed in hepatocellular carcinoma, and its expression correlates with tumor grade[8]. In addition to these kinds of cancers, current studies of TFF3 focus mainly on gastric cancer. The level of TFF3 in serum was found to be a better marker of gastric cancer than pepsinogen, and the combination of the levels of serum pepsinogen and TFF3 could improve screening for gastric cancer[9,10], possibly becoming applicable for the chemoprevention of gastrointestinal cancer associated with chronic persistent inflammation[11].

As described above, although many studies have been performed to evaluate the diagnostic value for different kinds of cancers, there are only a handful of studies evaluating the clinical value of TFF3 for CC, and they focused mainly on metastasis and therapy effect. They found that TFFs may be potential serum biomarkers in patients with metastatic colorectal cancer. Compared to CEA and CA19-9, TFF3 showed higher sensitivity and the same specificity, and it was strongly correlated with the extent of liver disease and seemed to have prognostic value[12]. It was also demonstrated to be a risk factor for early recurrence[13]. In addition, serum TFF3 was found to be an effective biomarker for

the detection of tumor stages and distant metastasis and as a predictor of responses to chemotherapy in colorectal cancer[14]. However, to date, there has been no study evaluating the clinical diagnostic value of TFF3 for the early detection of colorectal cancer.

In our study, we aimed to evaluate the diagnostic value of TFF3 for the detection of CC and to build a multivariate diagnostic model that might improve the diagnostic value compared to the indicator alone. It may serve as a potential assistant detection method.

MATERIALS AND METHODS

Patients
Written consent was obtained from all participants enrolled in this study. Our study was approved by the Ethics Committee of Tianjin Medical University Cancer Institute and Hospital. Serum samples of 527 individuals, including 115 healthy control (HC), 198 colorectal adenoma (CA), and 214 CC individuals, were collected for the training group. After the training group, an independent 343 individuals, including 107 HC, 104 CA, and 132 CC individuals, were collected to validate the diagnostic value of the training group. Serum samples were collected before surgery, chemotherapy, radiation therapy or immunotherapy. Age-matched healthy controls were enrolled based on their negative results on the blood biomarker test, computed tomography examination and fecal occult blood testing. The patients enrolled in our study were confirmed by histopathological analysis. The tumor stage was categorized according to the Dukes staging system. Duke stages A and B were categorized as early stage colorectal cancer. Dukes stages C and D were categorized as advanced stage CRC[15]. The clinical characteristics of the patients are shown in Table 1.

Serum collection
Ten milliliters of peripheral blood was collected in a tube that contained separating gel and clot activator, and then the tube was centrifuged at 3400 rpm for 7 min. The supernatant was transferred into another new tube. The sample serum was stored in aliquots at -80 ℃ until detection. No freeze-thawing was allowed prior to cytokine detection.

Detection of TFF3 and CEA
The levels of TFF3 (Item ID: E-EL-H1108c) in serum were detected by ELISA kits, which were provided by Elabscience Biotechnology Co., Ltd. (Wuhan, China). The detection protocol was performed according to the manufacturer's instructions. Briefly, 100 μL of standard, blank, or sample was added per well. Solutions were added to the bottom of the well and incubated for 90 min at 37 ℃. The liquid was removed and 100 μL of biotinylated detection Ab working solution added to each well, followed by incubation for

1 h at 37 ℃. The liquid was aspirated and the wells washed three times. Then, any remaining wash buffer was removed, and 100 μL of HRP conjugate working solution was added, followed by incubation for 30 min at 37 ℃. The wash process was repeated five times, and then 90 μL of substrate solution was added. Incubation was performed for 15 min at 37 ℃ followed by the addition of 50 μL of stop solution to each well. The optical density (OD value) of each well at 450 nm was measured by a Bio-Rad iMark Microplate Absorbance Reader (Bio-Rad Laboratories Inc.). The level of TFF3 was calculated according to the standard curve. The coefficient of variation of all kits was less than 10%. The levels of CEA in serum were detected by a Roche Modular Analytics E 170 instrument (Roche Diagnostics, Mannheim, Germany). The detection assays were provided by Roche Diagnostics, United States.

Statistical analysis
All the data were analyzed using MedCalc 12.7.0.0 (MedCalc Software, Mariakerke, Belgium) and SPSS 19.0 (SPSS, Brussels, Belgium). The levels of TFF3 and CEA between groups were compared by one-way analysis of variance with the Bonferroni correction. Binary logistic regression analysis was used to establish the multivariate diagnostic model. Receiver operating characteristic curves were used to evaluate the diagnostic value, and the areas under the curves (AUC) were compared by Z-scores[16]. The Youden index was used to choose the cutoff value that determined the sensitivity and specificity. A two-sided P value of less than 0.05 was considered statistically significant.

RESULTS

Correlation of TFF3 with clinical characteristics and comparison of level in groups
The serum level of TFF3 showed no significant correlation with age, gender, or tumor location but showed a significant correlation with tumor stage. As shown in Figure 1, compared to the healthy control group, the levels of TFF3 in the HC, CA, and CC groups were 14.10 (11.28, 23.19), 23.08 (18.72, 29.09), and 37.66 (29.87, 47.61) pg/mL, respectively. Compared to the HC group, the levels of TFF3 in both the CA group and the CC group showed significant increases ($P < 0.001$). Compared to the CA group, the level of TFF3 in the CC group showed a significant increase ($P < 0.001$).

Diagnostic evaluation of TFF3 and CEA for discriminating HC and CC groups
We first analyzed the diagnostic value of TFF3 or CEA alone for discriminating between the HC and CC groups, and then we analyzed the diagnostic value of the combination of TFF3 and CEA. The diagnostic values are given in Supplementary Table 1.

Table 1 Clinical characteristic of the individuals in our study *n* (%)

Clinical characteristics	Colorectal cancer group		Colorectal adenoma group		Healthy control group	
	Training (*n* = 214)	Validation (*n* = 132)	Training (*n* = 198)	Validation (*n* = 104)	Training (*n* = 115)	Validation (*n* = 107)
Age, yr						
Median	58	60	57	56	55	53
Range	43-72	41-76	41-70	36-73	38-68	39-62
Sex						
Male	128 (59.81)	84 (63.64)	107 (54.04)	62 (59.62)	61 (53.04)	56 (52.34)
Female	86 (40.19)	48 (36.36)	91 (45.96)	42 (40.38)	54 (46.96)	51 (47.66)
Location						
Colon	102 (47.66)	64 (48.48)	104 (52.53)	56 (53.85)	-	-
Rectum	112 (52.34)	68 (51.52)	94 (47.47)	48 (46.15)	-	-
Differentiation grade						
Well + moderately	126 (58.88)	68 (51.52)	-	-	-	-
Poorly	88 (41.12)	64 (48.48)	-	-	-	-
Stage						
A + B	82 (38.32)	56 (42.42)	-	-	-	-
C + D	132 (61.68)	76 (57.58)	-	-	-	-

CRC: Colorectal cancer; CA: Colorectal adenoma; HC: Healthy control; CEA: Carcino-embryonic antigen.
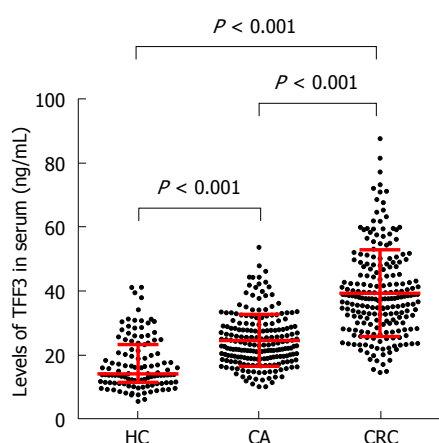
Figure 1 Comparisons of serum trefoil factor family 3 levels in colorectal cancer, colorectal adenoma, and healthy control groups. CRC: Colorectal cancer; CA: Colorectal adenoma; HC: Healthy control groups.

For discriminating between HC and CC, as shown in Figure 2A, the AUC of TFF3 was 0.930 (0.903, 0.958), and at the cutoff value of 21.30 pg/mL, the sensitivity and specificity were 94.86% and 73.91%, respectively. As shown in Figure 2D, the AUC of CEA was 0.850 (0.809, 0.890), and at the cutoff value of 3.09 U/mL, the sensitivity and specificity were 70.56% and 92.17%, respectively. Then, TFF3 and CEA were combined for analysis by binary logistic regression analysis to build the multivariate diagnostic model. The formula of the model was $Y = \text{logit}(P) = -6.498 + 0.189 X_{TFF3} + 0.651 X_{CEA}$. As shown in Figure 2G, the AUC of the multivariate model was 0.968 (0.951, 0.984), and at the cutoff value of 0.60, the sensitivity and specificity were 92.99% and 91.30%, respectively.

For discriminating between HC and early stage CC, as shown in Figure 2B, the AUC of TFF3 was 0.892 (0.849, 0.935), and at the cutoff value of 21.30 pg/ml, the sensitivity and specificity were 92.68% and

73.91%, respectively. As shown in Figure 2E, the AUC of CEA was 0.814 (0.745, 0.882), and at the cutoff value of 3.00 U/mL, the sensitivity and specificity were 69.51% and 90.43%, respectively. As shown in Figure 2H, the AUC of the multivariate model built to discriminate between HC and CC was 0.953 (0.926, 0.981), and at the cutoff value of 0.60, the sensitivity and specificity were 91.46% and 91.30%, respectively.

For discriminating between HC and advanced stage CC, as shown in Figure 2C, the AUC of TFF3 was 0.954 (0.931, 0.977), and at the cutoff value of 31.77 pg/mL, the sensitivity and specificity were 81.82% and 95.65%, respectively. As shown in Figure 2F, the AUC of CEA was 0.872 (0.828, 0.917), and at the cutoff value of 3.09 U/mL, the sensitivity and specificity were 72.73% and 92.17%, respectively. As shown in Figure 2I, the AUC of the multivariate model built to discriminate between HC and CC was 0.976 (0.961, 0.992), and at the cutoff value of 0.72, the sensitivity and specificity were 93.18% and 93.91%, respectively.

Diagnostic evaluation of TFF3 for discriminating between CA and CC groups in the training group
After discriminating between the HC and CC groups, we analyzed the diagnostic value of TFF3 and CEA alone or in combination for discriminating between the CA and CC groups. The diagnostic value is shown in Table 2, and the AUCs are shown in Supplementary Figure 1.

For discriminating between CA and CC, the AUC of TFF3 was 0.834 (0.796, 0.873), and at the cutoff value of 29.89 pg/mL, the sensitivity and specificity were 75.23% and 78.28%, respectively. The AUC of CEA was 0.683 (0.630, 0.737), and at the cutoff value of 4.96 U/mL, the sensitivity and specificity were 57.01% and 85.86%, respectively. Then, TFF3 and CEA were combined by binary logistic regression analysis to build the multivariate diagnostic model. The formula of the
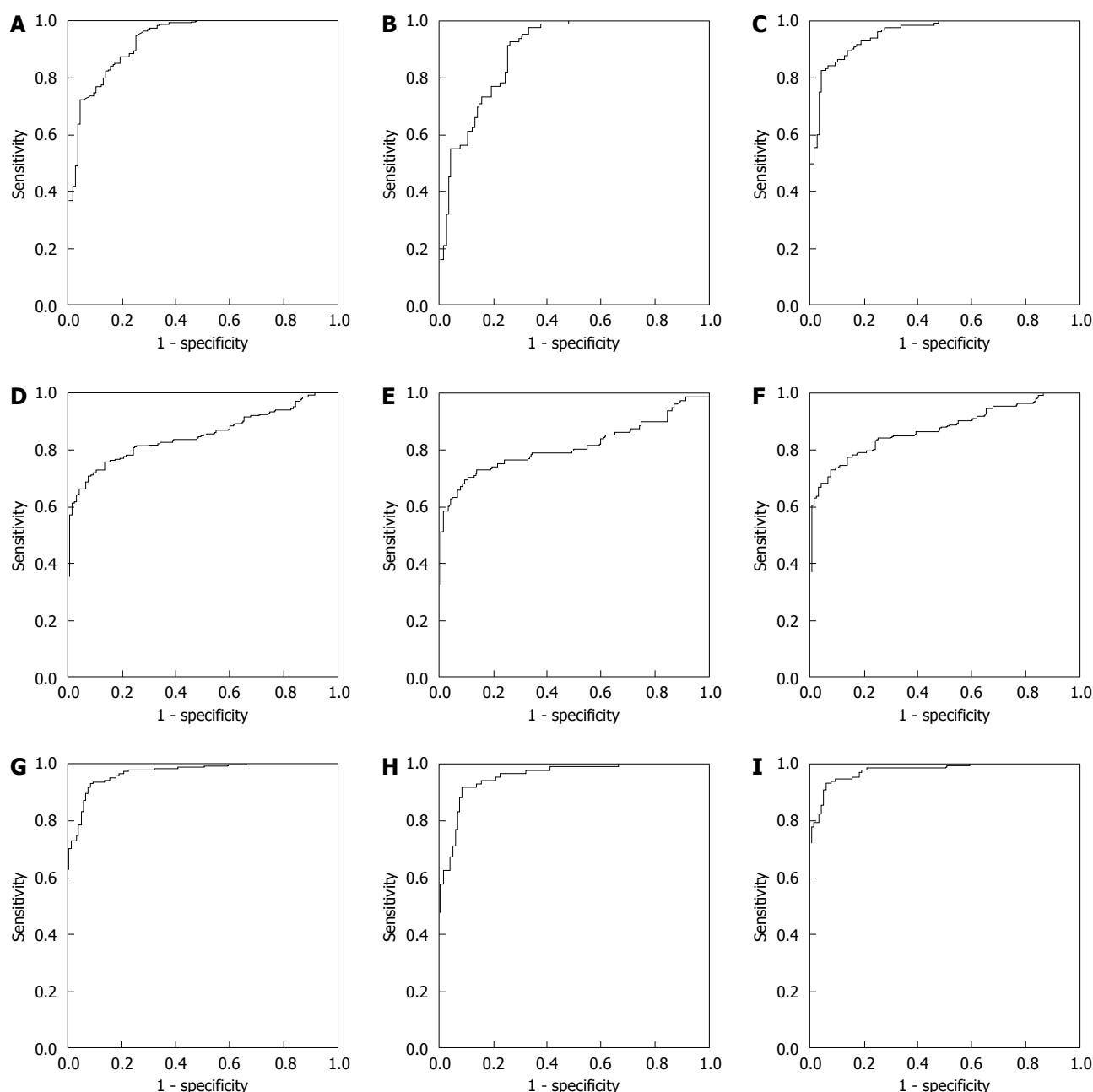
Figure 2 Analysis the Trefoil factors family and carcino-embryonic antigen diagnostic evaluation for discriminating the healthy control and colorectal cancer groups by receiver operating characteristic method in the training group. A: ROC of TFF3 for discriminating HC and CC; B: ROC of TFF3 for discriminating HC and early stage CC; C: ROC of TFF3 for discriminating HC and advanced stage CC; D: ROC of CEA for discriminating HC and CC; E: ROC of CEA for discriminating HC and early stage CC; F: ROC of CEA for discriminating HC and advanced stage CC; G: ROC of multivariate model for discriminating HC and CC; H: ROC of multivariate model for discriminating HC and early stage CC; I: ROC of multivariate model for discriminating HC and advanced stage CC. TFF3: Trefoil factors family; ROC: Receiver operating characteristic; HC: Healthy control; CC: Colorectal cancer.

model was $Y=logit(P)=-5.478+0.139X_{TFF3}+0.265X_{CEA}$. The AUC of the multivariate model was 0.883 (0.851, 0.915), and at the cutoff value of 0.57, the sensitivity and specificity were 73.83% and 88.38%, respectively. Compared to TFF3 or CEA alone, the AUC of the multivariate model showed significant improvement (P < 0.001 and P < 0.001).

For discriminating between CA and early stage CC, the AUC of TFF3 was 0.751 (0.691, 0.812), and at the cutoff value of 29.89 pg/mL, the sensitivity and specificity were 58.54% and 78.28%, respectively.

The AUC of CEA was 0.648 (0.563, 0.734), and at the cutoff value of 4.53 U/mL, the sensitivity and specificity were 56.10% and 81.31%, respectively. The AUC of the multivariate model built to discriminate between CA and CC was 0.823 (0.768, 0.878), and at the cutoff value of 0.41, the sensitivity and specificity were 76.83% and 77.27%, respectively.

For discriminating between CA and advanced stage CC, the AUC of TFF3 was 0.886 (0.849, 0.923), and at the cutoff value of 34.07 pg/mL, the sensitivity and specificity were 75.00% and 89.39%, respectively.

Table 2 Diagnostic evaluation of trefoil factors family, carcino-embryonic antigen alone or combination for discriminating colorectal adenoma and colorectal cancer in the training group

Indicator	Groups	AUC (95%CI)	Cutoff value	Sensitivity	Specificity
TFF3	CA *vs* CRC	0.834 (0.796, 0.873)	29.89	75.23%	78.28%
	CA *vs* early stage CRC	0.751 (0.691, 0.812)	29.89	58.54%	78.28%
	CA *vs* advanced CRC	0.886 (0.849, 0.923)	34.07	75.00%	89.39%
CEA	CA *vs* CRC	0.683 (0.630, 0.737)	4.96	57.01%	85.86%
	CA *vs* early CRC	0.648 (0.563, 0.734)	4.53	56.10%	81.31%
	CA *vs* advanced stage CRC	0.705 (0.641, 0.770)	4.96	60.61%	85.86%
TFF3+CEA	CA *vs* CRC	0.883 (0.851, 0.915)	0.57	73.83%	88.38%
	CA *vs* early stage CRC	0.823 (0.768, 0.878)	0.41	76.83%	77.27%
	CA *vs* advanced stage CRC	0.919 (0.888, 0.951)	0.57	81.82%	88.38%

TFF3: Trefoil factors family; CA: Colorectal adenoma; CRC: Colorectal cancer; AUC: Area under curve.

The AUC of CEA was 0.705 (0.641, 0.770), and at the cutoff value of 4.96 U/mL, the sensitivity and specificity were 60.61% and 86.86%, respectively. The AUC of the multivariate model built to discriminate between CA and CC was 0.919 (0.888, 0.951), and at the cutoff value of 0.57, the sensitivity and specificity were 81.82% and 88.38%, respectively.

Validation of the multivariate model for discriminating between HC and CC and between CA and CC in the validation group

After building the multivariate models to discriminate between HC and CC and between CA and CC, independent HC, CA and CC individuals were chosen to validate the diagnostic value of the multivariate models, as shown in Supplementary Table 2.

For discriminating between the HC and CC groups, as shown in Figure 3A, the AUC was 0.941 (0.912, 0.970), and at the cutoff value of 0.60, the sensitivity and specificity were 89.39% and 87.85%, respectively. For discriminating between the HC and early stage CC groups, as shown in Figure 3B, the AUC was 0.910 (0.856, 0.965), and at the cutoff value of 0.60, the sensitivity and specificity were 85.71% and 87.85%, respectively. For discriminating between the HC and advanced stage CC groups, as shown in Figure 3C, the AUC was 0.961 (0.938, 0.991), and at the cutoff value of 0.72, the sensitivity and specificity were 90.79% and 92.52%, respectively. Compared to TFF3 or CEA alone, the AUC of the multivariate model showed significant improvement ($P < 0.001$ and $P < 0.001$).

For discriminating between the CA and CC groups, as shown in Figure 3D, the AUC was 0.850 (0.799, 0.902), and at the cutoff value of 0.57, the sensitivity and specificity were 72.73% and 87.50%, respectively. For discriminating between the HC and early stage CC groups, as shown in Figure 3E, the AUC was 0.814 (0.741, 0.887), and at the cutoff value of 0.41, the sensitivity and specificity were 71.43% and 80.77%, respectively. For discriminating between the HC and advanced stage CC groups, as shown in Figure 3F, the AUC was 0.877 (0.824, 0.929), and at the cutoff value of 0.57, the sensitivity and specificity were 78.95%

and 87.50%, respectively.

DISCUSSION

TFF3, also called intestinal trefoil factor, consists of 59 amino acid peptides and occurs mainly in the gastrointestinal tract and in the serum. TFF3 expression is elevated during gastrointestinal adenoma progression and has been shown to promote mucosal wound healing. The induction of mucinous metaplasia was observed in mice with high TFF3 expression[17]. The TFFs can be used as biomarkers in various human cancers[18]. For gastric cancer, the serum TFF3 level may be a better biomarker of gastric cancer than the pepsinogen test. When combined with the serum pepsinogen test, TFF3 showed better diagnostic value for the screening of gastric cancer[9,10] and might be a potential non-endoscopic detection method for the screening of gastric cancer[19]. It also acted as an angiogenic factor and functions as a promoter to enhance tumor progression in mammary carcinoma[20]. In addition, the Cytosponge-TFF3 test is a safe and acceptable approach to identify patients with reflux symptoms who warrant endoscopy to diagnose Barrett's esophagus[21]. TFF3 plays an important role in the development of Barrett's metaplasia and may have diagnostic value for the early stages of Barrett's esophagus[22]. Although many studies have been performed to evaluate its diagnostic value for different cancers, few studies have evaluated the diagnostic value of TFF3 for the early detection of CC.

In our study, serum TFF3 showed significant correlation with tumor stage. This result was consistent with previous studies. The relationship between serum TFF3 and lymph node metastases of CC may make it a potentially useful marker for predicting the lymph node metastases[23], and it may also serve as a potential biomarker for the prediction of CC metastasis[24]. TFF3 up-regulation after neoadjuvant chemoradiotherapy for rectal cancer is associated with a higher risk of relapse[25]. Serum TFF3 can potentially be used as a biomarker to assess mucosal healing in ulcerative colitis patients[26]. In our study,
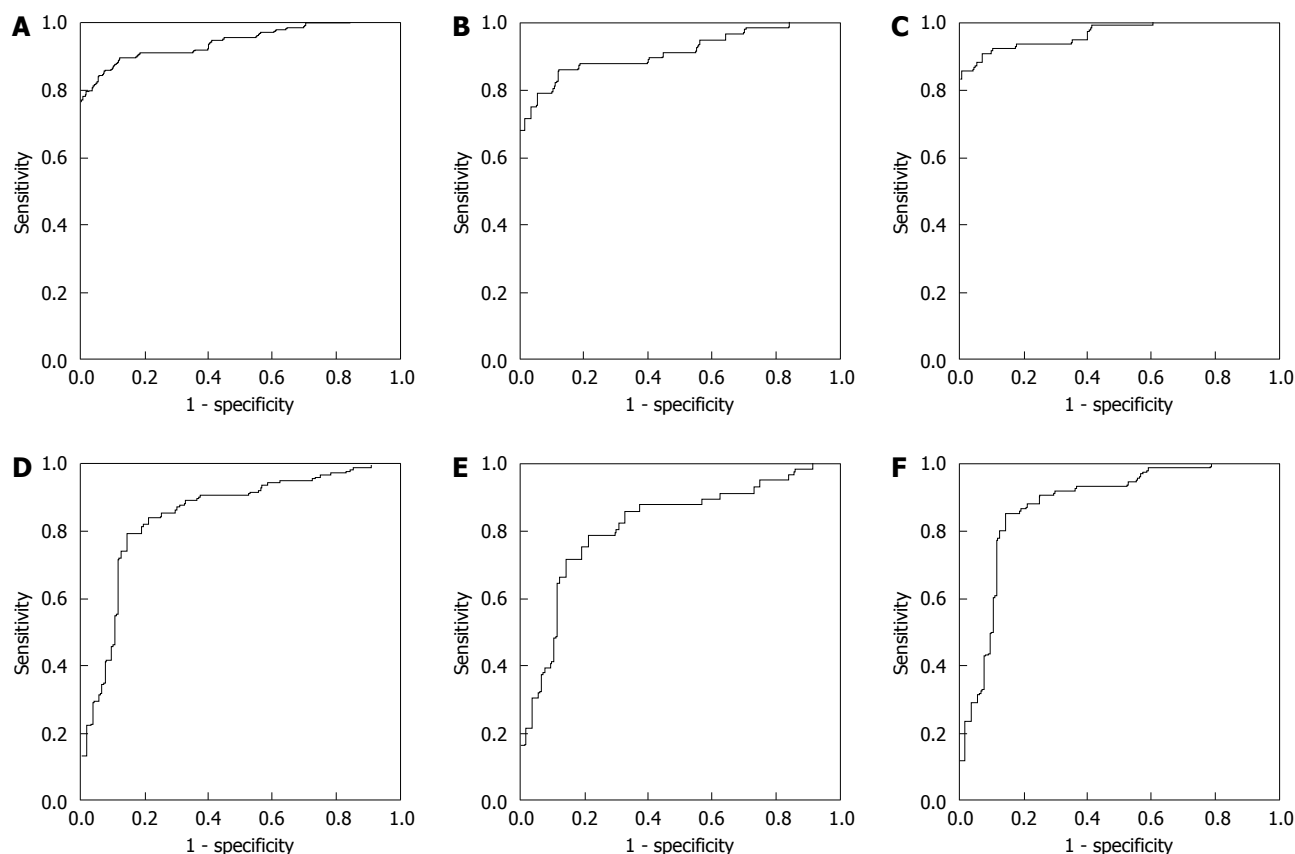
Figure 3 Analysis the multivariate model diagnostic evaluation by receiver operating characteristic method in the validation group. A: ROC of multivariate model for discriminating HC and CC; B: ROC of multivariate model for discriminating HC and early stage CC; C: ROC of multivariate model for discriminating HC and advanced stage CC; D: ROC of multivariate model for discriminating CA and CC; E: ROC of multivariate model for discriminating CA and early stage CC; F: ROC of multivariate model for discriminating CA and advanced stage CC. TFF3: Trefoil factors family; ROC: Receiver operating characteristic; CA: Colorectal adenoma; CC: Colorectal cancer.

compared to the HC and CA groups, serum TFF3 in the CC group showed a significant increase. It may contribute to the development of CC. In previous studies, TFF3 was demonstrated to contribute to the malignant behavior of colon cancer cells[27], and it was up-regulated in mucosal protection and repair. Its levels were increased in correlation with disease activity indices[28]. TFF3 level was also found to correlate with an aggressive phenotype in rat colon cancer cells. These findings provide evidence that TFF3 contributes to the malignant behavior of cancer cells[29]. There are some proposed mechanisms by which TFF3 participates in the development of CC. Signal transducer and activator of transcription (STAT) 3 has been demonstrated to be over expressed in most types of human cancers and classified as an oncogene. TFF3 may exert potent invasive activity through STAT3 signaling in human colorectal cancer cells[30]. In addition, TFF may also promote the proliferation and migration of gastric mucosal epithelial cells by activation of the PI3K/Akt signaling pathway, which has been demonstrated to be strongly related to the development of various cancers[31,32]. IL4-induced Stat6 signaling is active in various cell types, included immune cells and cancer cells. STAT6 activation

mediates a transcriptional enhancement of TFF3 by de novo induction, which plays an important role in host protective immunity against the infection synthesized protein in goblet cells[33]. TFF3 has been found to inhibit the TLR4/NF-kappaB signaling pathways, with potential treatment value for the inflammatory bowel disease[34]. Perturbation of the E-cadherin/catenin complex at intercellular junctions appears to be a functional pathway through which TFF2 and TFF3 promote cell migration[35]. In our study, for discriminating between HC and CC, the multivariate model showed significant improvement compared to CEA alone; however, because the prevalence of colorectal cancer was not taken into consideration, the diagnostic value of our study could be biased, and the disparity in the number of patients recruited in our study for the training group may also cause some bias in the diagnostic value. For discriminating between CA and CC, the multivariate model also showed significant improvement compared to CEA, as a method based on non-invasive discrimination. It was better than the conventional non-invasive method. In future research, the multivariate model should be compared with other discrimination methods, such as colonoscopy and fecal occult blood testing.

There are some limitations in our study. First, the number of individuals in the training group was relatively small, causing some bias in the results of our study. A larger sample size and multi-center sampling should be used to validate the diagnostic value of TFF3 and the multivariate diagnostic model. Second, although the diagnostic value of the multivariate model for discriminating between HC and CC was high, the diagnostic value for other kinds of cancers was not evaluated. The multivariate model built in our study currently can only be recognized as an assistant detection method that should be combined with the detection methods used in clinical practice, such as colonoscopy, fecal occult blood testing, and stool DNA testing. Third, in our study, we only evaluated the diagnostic value of TFF3 for the early detection of CC. The levels of the TFF3 after surgery, chemotherapy, radiotherapy, and other kinds of therapy methods were not evaluated. In future research, we will analyze TFF3 for evaluation of the effect of therapy or its correlation with prognosis.

In conclusion, we evaluated the diagnostic value of TFF3 for differentiating between the HC and CC and between the CA and CC groups, and we evaluated a multivariate diagnostic model that included TFF3 and CEA for differentiating between the HC and CC and between the CA and CC groups. Compared to the conventional biomarker CEA, the multivariate diagnostic model showed significant improvement. It could be used as an assistant detection method alongside the conventional screening methods for colorectal cancer, and it could also be used as a potentially effective diagnostic method for discriminating between CA and CC patients in clinical detection.

COMMENTS

Background
Colorectal cancer is one of the most common cancers worldwide. Although various detection methods are used in clinical practice, their diagnostic value is limited by disadvantages, and they cannot meet the needs of clinical detection. A detection method with high sensitivity and specificity, easy availability and low cost is urgently needed for the early detection in clinical practice.

Research frontiers
Although many studies have been performed to evaluate the diagnostic value of trefoil factor family 3 (TFF3) for different kinds of cancers, such as, however, to date, there has been no study evaluating the clinical diagnostic value of TFF3 for the early detection of colorectal cancer.

Innovations and breakthroughs
Serum level of TFF3 was used for evaluation the diagnostic value of for the early detection of colorectal cancer. A multivariate model combining TFF3 and carcino-embryonic antigen (CEA) was built. Compared to TFF3 or CEA alone, the multivariate model showed significant improvement.

Applications
The multivariate diagnostic model that included TFF3 and CEA showed significant improvement over the conventional biomarker CEA and might provide a potential method for the early detection of colorectal cancer.

Terminology
TFFs play important roles in the mucosal protection and repair of epithelial surfaces and are involved in the development and progression of various types of cancer.

Peer-review
This study is an interesting study about the diagnostic value evaluation of trefoil factors family 3 for the early detection of colorectal cancer. The multivariate diagnostic model which included TFF3 and CEA showed significant improvement when compared to the conventional biomarker CEA, and may provide a potential method for the early detection of colorectal cancer. Overall, this study is well designed and the manuscript is well written.

REFERENCES

1   **Siegel RL**, Miller KD, Jemal A. Cancer statistics, 2015. *CA Cancer J Clin* 2015; **65**: 5-29 [PMID: 25559415 DOI: 10.3322/caac.21254]
2   **Brenner H**, Kloor M, Pox CP. Colorectal cancer. *Lancet* 2014; **383**: 1490-1502 [PMID: 24225001 DOI: 10.1016/S0140-6736(13)61649-9]
3   **Gonzalez-Pons M**, Cruz-Correa M. Colorectal Cancer Biomarkers: Where Are We Now? *Biomed Res Int* 2015; **2015**: 149014 [PMID: 26106599 DOI: 10.1155/2015/149014]
4   **Lafontaine PO**, Arnal M, Buron N, Solary E, Lizard S, Bron A, Bara J, Gespach C, Creuzot-Garcher C. [Trefoil factor family gene and peptide expression in pterygium]. *J Fr Ophtalmol* 2003; **26**: 1007-1014 [PMID: 14691392]
5   **Vestergaard EM**, Borre M, Poulsen SS, Nexø E, Tørring N. Plasma levels of trefoil factors are increased in patients with advanced prostate cancer. *Clin Cancer Res* 2006; **12**: 807-812 [PMID: 16467092 DOI: 10.1158/1078-0432.CCR-05-1545]
6   **Chaiyarit P**, Utrawichian A, Leelayuwat C, Vatanasapt P, Chanchareonsook N, Samson MH, Giraud AS. Investigation of trefoil factor expression in saliva and oral mucosal tissues of patients with oral squamous cell carcinoma. *Clin Oral Investig* 2012; **16**: 1549-1556 [PMID: 22205269 DOI: 10.1007/s00784-011-0667-z]
7   **Qu Y**, Yang Y, Ma D, Xiao W. Increased trefoil factor 3 levels in the serum of patients with three major histological subtypes of lung cancer. *Oncol Rep* 2012; **27**: 1277-1283 [PMID: 22246423 DOI: 10.3892/or.2012.1627]
8   **Khoury T**, Chadha K, Javle M, Donohue K, Levea C, Iyer R, Okada H, Nagase H, Tan D. Expression of intestinal trefoil factor (TFF-3) in hepatocellular carcinoma. *Int J Gastrointest Cancer* 2005; **35**: 171-177 [PMID: 16110118 DOI: 10.1385/IJGC:35:3:171]
9   **Aikou S**, Ohmoto Y, Gunji T, Matsuhashi N, Ohtsu H, Miura H, Kubota K, Yamagata Y, Seto Y, Nakajima A, Goldenring JR, Kaminishi M, Nomura S. Tests for serum levels of trefoil factor family proteins can improve gastric cancer screening. *Gastroenterology* 2011; **141**: 837-845.e1-7 [PMID: 21699780 DOI: 10.1053/j.gastro.2011.05.040]
10 **Huang Z**, Zhang X, Lu H, Wu L, Wang D, Zhang Q, Ding H. Serum trefoil factor 3 is a promising non-invasive biomarker for gastric cancer screening: a monocentric cohort study in China. *BMC Gastroenterol* 2014; **14**: 74 [PMID: 24720760 DOI: 10.1186/1471-230X-14-74]
11 **Katoh M**. Trefoil factors and human gastric cancer (review). *Int J Mol Med* 2003; **12**: 3-9 [PMID: 12792801]
12 **Vocka M**, Langer D, Petrtyl J, Vockova P, Hanus T, Kalousova M, Zima T, Petruzelka L. Trefoil factor family (TFF) proteins as potential serum biomarkers in patients with metastatic colorectal cancer. *Neoplasma* 2015; **62**: 470-477 [PMID: 25866228 DOI: 10.4149/neo_2015_056]
13 **Morito K**, Nakamura J, Kitajima Y, Kai K, Tanaka T, Kubo H, Miyake S, Noshiro H. The value of trefoil factor 3 expression in predicting the long-term outcome and early recurrence of colorectal

cancer. *Int J Oncol* 2015; **46**: 563-568 [PMID: 25405728 DOI: 10.3892/ijo.2014.2755]

14   **Xiao L**, Liu YP, Xiao CX, Ren JL, Guleng B. Serum TFF3 may be a pharamcodynamic marker of responses to chemotherapy in gastrointestinal cancers. *BMC Clin Pathol* 2014; **14**: 26 [PMID: 25031551 DOI: 10.1186/1472-6890-14-26]

15   **Wong JM**, Yen MF, Lai MS, Duffy SW, Smith RA, Chen TH. Progression rates of colorectal cancer by Dukes' stage in a high-risk group: analysis of selective colorectal cancer screening. *Cancer J* 2004; **10**: 160-169 [PMID: 15285925]

16   **Pengjun Z**, Xinyu W, Feng G, Xinxin D, Yulan L, Juan L, Xingwang J, Zhennan D, Yaping T. Multiplexed cytokine profiling of serum for detection of colorectal cancer. *Future Oncol* 2013; **9**: 1017-1027 [PMID: 23837764 DOI: 10.2217/fon.13.71]

17   **Ge H**, Gardner J, Wu X, Rulifson I, Wang J, Xiong Y, Ye J, Belouski E, Cao P, Tang J, Lee KJ, Coberly S, Wu X, Gupte J, Miao L, Yang L, Nguyen N, Shan B, Yeh WC, Véniant MM, Li Y, Baribault H. Trefoil Factor 3 (TFF3) Is Regulated by Food Intake, Improves Glucose Tolerance and Induces Mucinous Metaplasia. *PLoS One* 2015; **10**: e0126924 [PMID: 26083576 DOI: 10.1371/journal.pone.0126924]

18   **May FE**. The potential of trefoil proteins as biomarkers in human cancer. *Biomark Med* 2012; **6**: 301-304 [PMID: 22731904 DOI: 10.2217/bmm.12.22]

19   **Kaise M**, Miwa J, Tashiro J, Ohmoto Y, Morimoto S, Kato M, Urashima M, Ikegami M, Tajiri H. The combination of serum trefoil factor 3 and pepsinogen testing is a valid non-endoscopic biomarker for predicting the presence of gastric cancer: a new marker for gastric cancer risk. *J Gastroenterol* 2011; **46**: 736-745 [PMID: 21455714 DOI: 10.1007/s00535-011-0396-8]

20   **Lau WH**, Pandey V, Kong X, Wang XN, Wu Z, Zhu T, Lobie PE. Trefoil Factor-3 (TFF3) Stimulates De Novo Angiogenesis in Mammary Carcinoma both Directly and Indirectly via IL-8/CXCR2. *PLoS One* 2015; **10**: e0141947 [PMID: 26559818 DOI: 10.1371/journal.pone.0141947]

21   **Ross-Innes CS**, Debiram-Beecham I, O'Donovan M, Walker E, Varghese S, Lao-Sirieix P, Lovat L, Griffin M, Ragunath K, Haidry R, Sami SS, Kaye P, Novelli M, Disep B, Ostler R, Aigret B, North BV, Bhandari P, Haycock A, Morris D, Attwood S, Dhar A, Rees C, Rutter MD, Sasieni PD, Fitzgerald RC. Evaluation of a minimally invasive cell sampling device coupled with assessment of trefoil factor 3 expression for diagnosing Barrett's esophagus: a multi-center case-control study. *PLoS Med* 2015; **12**: e1001780 [PMID: 25634542 DOI: 10.1371/journal.pmed.1001780]

22   **Dunn LJ**, Jankowski JA, Griffin SM. Trefoil Factor Expression in a Human Model of the Early Stages of Barrett's Esophagus. *Dig Dis Sci* 2015; **60**: 1187-1194 [PMID: 25424203 DOI: 10.1007/s10620-014-3440-8]

23   **Huang YG**, Li YF, Wang LP, Zhang Y. Aberrant expression of trefoil factor 3 is associated with colorectal carcinoma metastasis. *J Cancer Res Ther* 2015; **9**: 376-380 [PMID: 24125969 DOI: 10.4103/0973-1482.119308]

24   **Xue H**, Lü B, Zhang J, Wu M, Huang Q, Wu Q, Sheng H, Wu D, Hu J, Lai M. Identification of serum biomarkers for colorectal cancer metastasis using a differential secretome approach. *J Proteome Res* 2010; **9**: 545-555 [PMID: 19924834 DOI: 10.1021/pr9008817]

25   **Casado E**, Garcia VM, Sánchez JJ, Gómez Del Pulgar MT, Feliu J, Maurel J, Castelo B, Moreno Rubio J, López RA, García-Cabezas MÁ, Burgos E, de Castro J, Belda-Iniesta C, López-Gómez M, Gómez-Raposo C, Zambrana F, Sereno M, Fernández-Martos C, Vázquez P, Lacal JC, González-Barón M, Cejas P. Upregulation of trefoil factor 3 (TFF3) after rectal cancer chemoradiotherapy is an adverse prognostic factor and a potential therapeutic target. *Int J Radiat Oncol Biol Phys* 2012; **84**: 1151-1158 [PMID: 22516806 DOI: 10.1016/j.ijrobp.2012.01.083]

26   **Srivastava S**, Kedia S, Kumar S, Pratap Mouli V, Dhingra R, Sachdev V, Tiwari V, Kurrey L, Pradhan R, Ahuja V. Serum human trefoil factor 3 is a biomarker for mucosal healing in ulcerative colitis patients with minimal disease activity. *J Crohns Colitis* 2015; **9**: 575-579 [PMID: 25964429 DOI: 10.1093/ecco-jcc/jjv075]

27   **Babyatsky M**, Lin J, Yio X, Chen A, Zhang JY, Zheng Y, Twyman C, Bao X, Schwartz M, Thung S, Lawrence Werther J, Itzkowitz S. Trefoil factor-3 expression in human colon cancer liver metastasis. *Clin Exp Metastasis* 2009; **26**: 143-151 [PMID: 18979216 DOI: 10.1007/s10585-008-9224-9]

28   **Grønbaek H**, Vestergaard EM, Hey H, Nielsen JN, Nexø E. Serum trefoil factors in patients with inflammatory bowel disease. *Digestion* 2006; **74**: 33-39 [PMID: 17068395 DOI: 10.1159/000096591]

29   **Yio X**, Zhang JY, Babyatsky M, Chen A, Lin J, Fan QX, Werther JL, Itzkowitz S. Trefoil factor family-3 is associated with aggressive behavior of colon cancer cells. *Clin Exp Metastasis* 2005; **22**: 157-165 [PMID: 16086236 DOI: 10.1007/s10585-005-6615-z]

30   **Rivat C**, Rodrigues S, Bruyneel E, Piétu G, Robert A, Redeuilh G, Bracke M, Gespach C, Attoub S. Implication of STAT3 signaling in human colonic cancer cells during intestinal trefoil factor 3 (TFF3) -- and vascular endothelial growth factor-mediated cellular invasion and tumor growth. *Cancer Res* 2005; **65**: 195-202 [PMID: 15665295]

31   **Sun Z**, Liu H, Yang Z, Shao D, Zhang W, Ren Y, Sun B, Lin J, Xu M, Nie S. Intestinal trefoil factor activates the PI3K/Akt signaling pathway to protect gastric mucosal epithelium from damage. *Int J Oncol* 2014; **45**: 1123-1132 [PMID: 24990304 DOI: 10.3892/ijo.2014.2527]

32   **Fresno Vara JA**, Casado E, de Castro J, Cejas P, Belda-Iniesta C, González-Barón M. PI3K/Akt signalling pathway and cancer. *Cancer Treat Rev* 2004; **30**: 193-204 [PMID: 15023437 DOI: 10.1016/j.ctrv.2003.07.007]

33   **Blanchard C**, Durual S, Estienne M, Bouzakri K, Heim MH, Blin N, Cuber JC. IL-4 and IL-13 up-regulate intestinal trefoil factor expression: requirement for STAT6 and de novo protein synthesis. *J Immunol* 2004; **172**: 3775-3783 [PMID: 15004182]

34   **Teng X**, Xu LF, Zhou P, Sun HW, Sun M. Effects of trefoil peptide 3 on expression of TNF-alpha, TLR4, and NF-kappaB in trinitrobenzene sulphonic acid induced colitis mice. *Inflammation* 2009; **32**: 120-129 [PMID: 19238529 DOI: 10.1007/s10753-009-9110-x]

35   **Buda A**, Jepson MA, Pignatelli M. Regulatory function of trefoil peptides (TFF) on intestinal cell junctional complexes. *Cell Commun Adhes* 2012; **19**: 63-68 [PMID: 23181544 DOI: 10.3109/15419061.2012.748326]

ORIGINAL ARTICLE

Basic Study

Miniature magnetically anchored and controlled camera system for trocar-less laparoscopy

Ding-Hui Dong, Hao-Yang Zhu, Yu Luo, Hong-Ke Zhang, Jun-Xi Xiang, Fei Xue, Rong-Qian Wu, Yi Lv

Ding-Hui Dong, Hao-Yang Zhu, Yu Luo, Hong-Ke Zhang, Jun-Xi Xiang, Fei Xue, Rong-Qian Wu, Yi Lv, Department of Hepatobiliary Surgery, First Affiliated Hospital, Xi'an Jiaotong University, Xi'an 710061, Shaanxi Province, China

Abstract

AIM

To design a miniature magnetically anchored and controlled camera system to reduce the number of trocars which are required for laparoscopy.

METHODS

The system consists of a miniature magnetically anchored camera with a 30° downward angle, an external magnetically anchored unit, and a vision output device. The camera weighs 12 g, measures $\Phi 10.5$ mm × 55 mm and has two magnets, a vision model, a light source, and a metal hexagonal nut. To test the prototype, the camera was inserted through a 12-mm conventional trocar in an *ex vivo* real liver laparoscopic training system. A trocar-less laparoscopic cholecystectomy was performed 6 times using a 12-mm

and a 5-mm conventional trocar. In addition, the same procedure was performed in four canine models.

RESULTS
Both procedures were successfully performed using only two conventional laparoscopic trocars. The cholecystectomy was completed without any major complication in 42 min (38-45 min) *in vitro* and in 50 min (45-53 min) using an animal model. This camera was anchored and controlled by an external unit magnetically anchored on the abdominal wall. The camera could generate excellent image. with no instrument collisions.

CONCLUSION
The camera system we designed provides excellent optics and can be easily maneuvered. The number of conventional trocars is reduced without adding technical difficulties.

Key words: Trocar-less laparoscopy; Magnetically anchored and controlled camera; Minimally invasive surgery

Core tip: This study introduced a miniature magnetically anchored and controlled camera system. The miniature magnetically anchored camera is among the smallest size, and it can pass through a conventional 12-mm trocar. Magnetically anchored instruments are positioned intra-abdominally and stabilized through a coupling force to external magnets on the abdominal skin. In this way, the instruments do not share space with the trocar during surgery. By using this camera system, the number of trocars required for conventional laparoscopy could be reduced without adding technical difficulties.

INTRODUCTION

Laparoscopic surgery has significantly evolved as a conventional surgical procedure for smaller incisions and faster recovery since its emergence in the late 1980s[1,2]. It is a standard alternative practice to the traditional open operation in cholecystectomy, nephrectomy, and other procedures[3-5]. Conventional laparoscopic surgery requires 3 or more 5-10 mm trocars. The number of trocars is associated with postoperative pain, cosmesis, and the risk of bleeding or organ damage[6,7]. Minimizing the invasiveness of surgery is a fundamental driving force for surgeons and patients seeking new procedures with fewer trocars.

Single-site laparoscopy (SSL), represented by laparoendoscopic single-site (LESS) surgery and natural orifice transluminal endoscopic surgery, has recently gained more interest among minimally invasive laparoscopic surgeons[8-10]. SSL is superior to conventional multiport laparoscopy for cosmesis because the new procedure relies on a single port site that is limited to an inconspicuous position. In theory, the number of trocars is reduced in SSL. However, in reality, the incision length of the single port in SSL is much greater than that used in conventional multiport laparoscopy (approximately 25-30 mm *vs* 5-12 mm), which increases the risk of post-operative inflammation[11,12]. Moreover, because all of the ins-truments are restricted to a single trocar, SSL is also technically demanding, and the technical challenges are intrinsically linked to loss of triangulation and instrument conflicts[13-15]. As a result, the widespread adoption of SSL is limited.

In the hands of laparoscopic surgeons, laparoscopic surgery has relied on fewer conventional trocars and multiple instruments constrained in a single trocar to overcome the challenges faced by SSL. Therefore, our team attempted to perform trocar-less laparoscopy by developing a miniature magnetically anchored camera that can pass through a conventional laparoscopic trocar. The magnetically anchored and controlled instruments were first introduced by Caddedu in 2007 and were called magnetically anchored and guided systems[16]. Such instruments are positioned intra-abdominally and stabilized through a coupling force to external magnets on the abdominal skin. In this way, the instruments do not share space with the trocar during surgery.

In the present study, we present the initial deve-lopment of an MMAC weighing 12 g and measuring $\Phi 10.5$ mm $\times$ 55 mm. It has a 30° downward angle and can be inserted into the abdomen through a conventional 12 mm trocar. By using this miniature camera, the number of conventional trocars is reduced without adding more demanding technics. This camera provides excellent optics of the surgical space and can easily be maneuvered in the abdomen. With this camera, a trocar-less laparoscopic cholecystectomy using two conventional trocars was performed in an animal model.

MATERIALS AND METHODS

System composition
Similar to our previous work, the miniature magnetically anchored and controlled camera system consists of an MMAC with a 30° downward angle, an external magnetically anchored unit, and a vision output device.
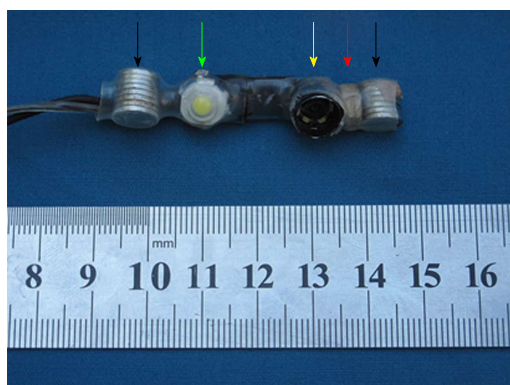
Figure 1 The miniature magnetically anchored camera with a 30° downward angle. It consists of inner magnets (black arrow), a light source (green arrow), a vision model (yellow arrow), and a metal hexagonal nut (red arrow).

The camera is the internal component and is inserted into the abdominal cavity through a conventional trocar. Its position in the abdomen is controlled by the external magnetically anchored unit *via* a magnetic force through the abdominal wall. On the basis of previous work, the external magnetically anchored unit is composed of 15 magnets (60 mm × 10 mm × 4 mm, NdFeB N50 permanent magnet). The external magnetically anchored unit regulates the magnetic force according to abdominal wall thickness. The vision output device displays an image captured by the miniature camera and supports power for the system.

Miniature magnetically anchored camera
The size of the camera is the most important part for trocar-less laparoscopy surgery. The MMAC measures Φ10.5 mm × 55 mm and is composed of two magnets (Φ8 mm × 7 mm, NdFeB N50 permanent magnet), a vision model, a metal hexagonal nut, and a light source (Figure 1). The vision model is produced by Audenson Technology Corporation (Shenzhen, China) and consists of a 1/5" 1024 × 768 pixel color CMOS camera and a 720-line TV contained in an 8 mm × 6 mm × 2.5 mm cuboid. The focal length of the camera lens is 6-8 cm. The metal hexagonal nut is fixed on the lateral surface of the vision model. The nut is used to achieve the 30° downward angle of the vision model. A 3 W hemispherical light-emitting diode (LED) (Juli Industrial Development Corporation, Shenzhen, China) is the light source. It provides high-color temperature (6000-6500 K) and luminous flux (200-220 LM) equal to that of xenon lamps used in conventional laparoscopy. The MMAC weighs approximately 12 g.

In vitro test
A laparoscopy training platform, called the *ex vivo* real-liver laparoscopic training system, was used as the bench test in this study (Figure 2A). It was composed of a special dummy and a laparoscope. The abdominal wall of the dummy was made of a material and a nonmagnetic metal support that mimics the elasticity and shape of a human pneumoperitoneum. A partial porcine liver was placed in the dummy for the laparoscopic cholecystectomy model.

In the bench test, a laparoscope was inserted through an umbilical trocar when necessary to visualize the camera performance. The trocar-less laparoscopic cholecystectomy was performed using a 12-mm trocar and a 5-mm conventional trocar. The 12-mm trocar was placed below the xiphoid, and the 5-mm trocar was placed at the right mid-clavicular line. The MMAC was inserted into the abdomen through the 12-mm conventional trocar and then coupled to the external magnetically anchored unit by gently depressing it. The camera was maneuvered into position using the external magnet. The conventional laparoscopic instruments (Hangzhou Kangji Medical Instrument Co., Ltd., Zhejiang, China) were inserted through the 12- and 5-mm trocars as needed during the cholecystectomy. The gallbladder was freed from its hepatic attachments, and the cystic duct was transected. After completing the procedure, the gallbladder was extracted from the xiphoid defect. The camera was removed by pulling cables after decoupling the external magnet. The procedure was performed 6 times. The performance of the MMAC was evaluated by manipulation, operative time, and the achievement of critical views.

In vivo test
After obtaining approval from the institutional animal care and use committee of our institution, the trocar-less laparoscopic cholecystectomy was performed in 4 male canines weighing a mean of 15 kg. The surgical instruments and techniques used were the same as for the *in vitro* test. Additionally, the abdominal cavity was insufflated with carbon dioxide to a pressure of 15 mm Hg. The surgeon manipulated the external magnet during the procedure to adjust the view of the MMAC. After the operation, the surgeon closed the incision with an interrupted absorbable suture. In addition to the indicators mentioned above, estimated blood loss, adverse events, and biosafety were used to assess the camera. Biosafety was evaluated by peritoneal specimens in the active area of the magnetic camera. They were harvested after the operation and examined using HE staining.

RESULTS
The trocar-less laparoscopic cholecystectomy using 12- and 5-mm conventional trocars was successfully performed *in vitro* in all 6 cases. The MMAC easily passed through the 12-mm conventional trocar, and only thin wires for powering and imaging were left in the trocar, which provided sufficient space for conventional laparoscopic instruments and did not cause instrument conflicts. The camera was anchored and controlled well by the external magnetically
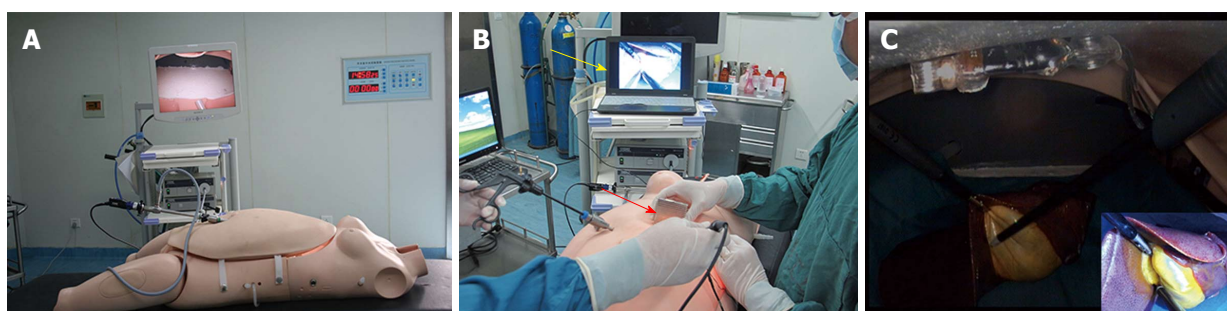
Figure 2 In vitro test. A: The bench test consists of a special mannequin and a laparoscope; B: External view image for trocar-less laparoscopic cholecystectomy *in vitro*: the external magnetically anchored unit (red arrow) and the vision output device (yellow arrow); C: Critical view image for trocar-less laparoscopic cholecystectomy *in vitro* (the picture-in-picture view is the image captured by the miniature magnetically anchored camera with its own light source).
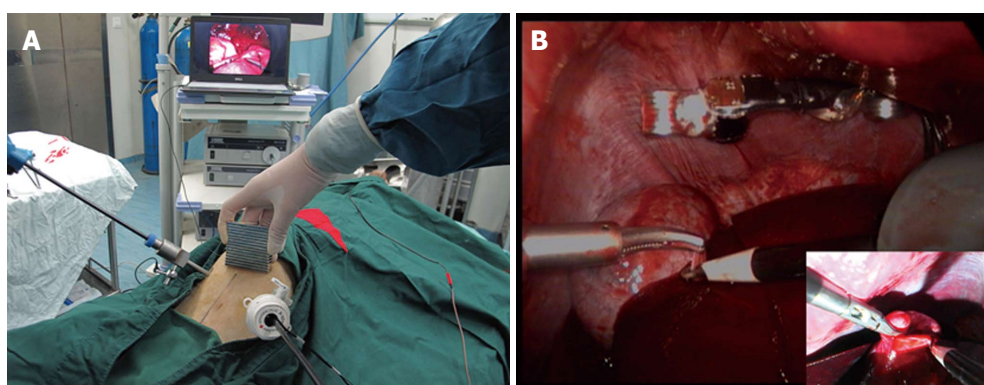
Figure 3 In vivo test. A: External view image for trocar-less laparoscopic cholecystectomy in a canine mode; B: Critical view image for trocar-less laparoscopic cholecystectomy in a canine model (the picture-in-picture view is the image captured by the miniature magnetically anchored camera with its own light source).

anchored unit on the dummy skin surface. Decoupling did not occur during the operation, even when the wires were used to pull out the camera. The camera's 30° downward angle enabled critical views of the procedure. The image generated by the camera was excellent because it was equipped with the high-color temperature LED and the focal length of the camera lens was appropriate for the height of the pneumoperitoneum. The mean operative time was 42 min (38-45 min) (Figure 2B and C).

After confirming the camera's feasibility for trocar-less laparoscopic cholecystectomy *in vitro*, similar procedures were also completed in 4 canines without adverse events, such as bile spillage. The mean estimated blood loss was 6 mL (3-10 mL). In the canine model, the camera was also inserted into the abdomen through a 12-mm conventional trocar and was easily "pulled up" to the thin abdominal wall (approximately 1 cm according to the preoperative ultrasound). The camera was manipulated into position by smoothly sliding the external magnetically anchored unit. Because of the 30° downward angle, the critical views were achieved well, and the image quality was as excellent as it was *in vitro*, with high resolution and sufficient lighting (Figure 3). Approximately once per operation, the camera had to be pulled out by the wires to clean fog off of the lens. There were no instrument collisions. The mean operative time was 50

min (45-53 min) *in vivo*. Hematoxylin-eosin staining showed no significant tissue damage at the muscle layer in the camera's active area, thus supporting its biosafety *in vivo* (Figure 4).

DISCUSSION

SSL was once considered a promising alternative approach to conventional laparoscopic surgery[9]. Unfortunately, all instruments were constrained in a single port, which was technically demanding, and the incision length impeded the widespread adoption of SSL for laparoscopic surgery[11-15]. The new approach must maintain the ergonomics of traditional laparoscopy and reduce the incision length. We have therefore investigated trocar-less laparoscopy, which relies on conventional laparoscopic trocars. For example, a laparoscopic cholecystectomy was performed using 2 conventional trocars in this study. Because trocar-less laparoscopy is predicated on conventional trocars, the surgical instruments for the new approach, such as the retractor and cautery device, are the same as those used for conventional laparoscopy. Trocar-less laparoscopy addresses the dilemma of SSL while offering additional benefits. It saves money and training time compared with SSL, which encourages its clinical adoption.

The new instrument's special positioning technology
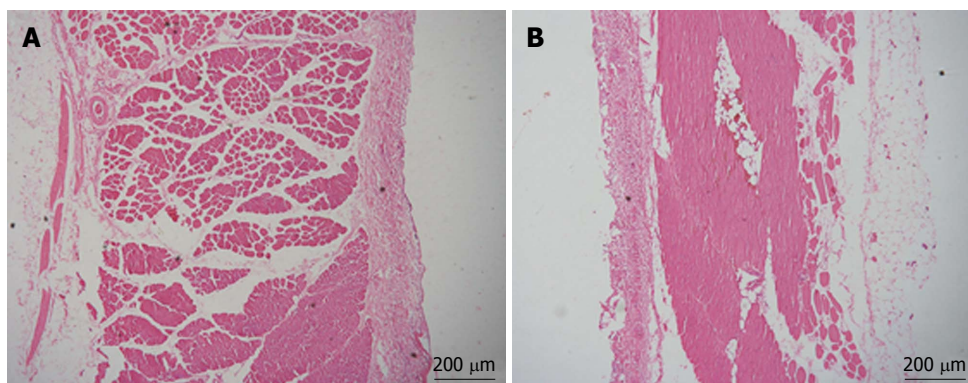
Figure 4 Pathologic assessment of abdominal wall. A: Hematoxylin-eosin (HE) staining of normal area; B: HE staining of active area.
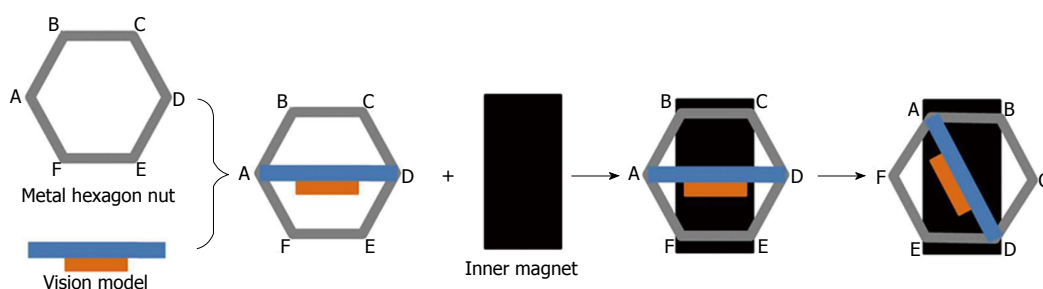
Figure 5 The method of achieving a 30° downward angle using the miniature magnetically anchored camera.

is the key to trocar-less laparoscopy and should address the following requirements: First, the new instrument should not occupy space in the trocar during surgery to avoid instrument conflicts. Second, the new instrument should use the entire insufflated abdomen to accommodate the required changes in position. Many positioning technologies have been attempted in trocar-less surgery, such as vacuum positioning, needle anchoring, and magnetic anchoring[16-18]. Magnetically anchoring is the most promising positioning technology because the coupling force is generated by internal and outer components without direct contact, thus allowing the instrument to be free from the trocar and enabling the full use of the insufflated abdomen. Other positioning technologies have deficiencies in reliable anchoring and convenient guidance.

Surgeons who perform minimally invasive laparoscopic procedures have significant interests in developing magnetically anchored and controlled instruments. Numerous instruments have been developed, such as cameras, retractors, dissectors, and even surgical robots[19-25]. Most of these instruments are above Φ20 mm because they aim to address instrument collisions in SSL, which are not suitable for trocar-less surgery that relies on conventional trocars. Our team has strongly advocated this promising technology in the hope of further advancing minimally invasive surgery. Our team previously developed a deployable magnetically anchored retractor that could pass through a conventional 12 mm trocar[26]. We also developed an MMAC measuring Φ11 mm × 50 mm aimed at reducing

the incision of SSL (unpublished data). However, the view angle of this camera is 90° downward, whereas conventional laparoscopy has a 30° downward angle. In our current work, we refined the camera's internal magnets and vision model to minimize its size. More significantly, the latest generation of the magnetically anchored camera achieves a 30° downward angle using a metal hexagonal nut, thus making the new camera suitable for trocar-less surgery. The 30° downward angle was achieved as follows: (1) the bottom of the vision model was fixed to point A and point D of the metal hexagonal nut; (2) the metal nut was attracted by an inner magnet (the inner magnet was vertical to the long axis of the camera) so the vision model would achieve a different downward angle by turning the nut; and (3) when L_{AE} was parallel to the long axis of the inner magnet, the vision model had a 30° downward angle (Figure 5). This is a simple but reliable method to achieve the 30° downward angle because the nut is hexagonal.

This is a small pilot study limited to a canine model. The new generation of cameras will be a vital advance only if the following problems are addressed: First, if the new camera can cooperate with other magnetically anchored and controlled instruments (such as the magnetically anchored retractor that we developed previously), the trocar-less laparoscopy performed with a single conventional trocar will be possible. Unfortunately, in our experience, multiple outer magnets cause magnet-to-magnet interference and operator hand-to-magnet collisions. The minimum

separation distance of outer magnets is considered to be 3 cm[16]. Future studies should focus on developing an external magnet platform to reconcile the collisions by keeping the outer magnets at an appropriate separation distance. Second, kinematic safety is still a blind area in magnetically anchored and controlled instruments. Although decoupling rarely occurs in current research because the latest camera is light and the outer magnet is sufficiently large, it is necessary to resolve the cause of decoupling to operate magnetically anchored and controlled instruments safely. Abdominal wall thickness has been regarded as a relative factor[27]. However, more issues need to be explored for the further development of magnetically anchored and controlled instruments.

In conclusion, we have successfully designed, manufactured, and implemented a new magnetically anchored and controlled camera system to perform trocar-less laparoscopy. The system consists of an MMAC with a 30° downward angle, a vision output device, and an external magnetically anchored unit. The miniature camera measures Φ10.5 mm × 55 mm and weighs 12 g. It can be inserted into a 12-mm conventional trocar and easily maneuvered in the abdomen. The image generated by the camera is excellent and sufficient to perform cholecystectomy. Pilot studies in canine models have demonstrated the feasibility of canine laparoscopic cholecystectomy using the MMAC with a 30° downward angle and only 2 conventional trocars. Future studies will aim to modify the current device and develop new magnetically anchored and controlled instruments to minimize the invasiveness of laparoscopic surgery further.

COMMENTS

Background
Laparoscopy is a promising minimally invasive method that is based on multiple trocars. Decreasing the number of trocars necessary for laparoscopy could further reduce surgical trauma and achieve a better cosmetic outcome. Caddedu first introduced magnetically anchored and controlled instruments in 2007. Such instruments could not share space with the trocar during surgery. However, previous magnetically anchored instruments were greater than Φ20 mm to address instrument collisions in single-site laparoscopy (SSL). This would not be suitable for conventional laparoscopy. Therefore, the authors designed a miniature magnetically anchored and controlled camera system that could pass through the conventional laparoscopic trocar. By using this camera system, the number of trocars required for conventional laparoscopy could be reduced without adding technical difficulties.

Research frontiers
A magnetically anchored instrument is the most promising positioning method for trocar-less laparoscopy because the coupling force is generated by internal and outer components without direct contact, thus allowing the instrument to be free from the trocar and enabling full use of the insufflated abdomen. However, previous magnetically anchored instruments are too large to pass through a conventional laparoscopic trocar.

Innovations and breakthroughs
In this study, the authors provided a miniature magnetically anchored camera measuring Φ10.5 mm × 55 mm, which was the first able to pass through the conventional 12 mm trocar. A trocar-less laparoscopic cholecystectomy using 2 conventional trocars was performed using this camera system. The authors developed an artful method to achieve the 30° downward angle of the camera by simply using a metal hexagon, which is crucial to obtain excellent intraoperative optics.

Applications
The miniature magnetically anchored and controlled camera system provided in this study could realize trocar-less laparoscopy by replacing the trocar used for the laparoscopy. Combined with other instruments, a laparoscopic cholecystectomy based on only one conventional trocar could be achieved in the future by using the camera system, which would be much less invasive than current SSL.

Terminology
Magnetically anchored instruments are positioned intra-abdominally and stabilized through a coupling force to external magnets on the abdominal skin. In this way, the instruments do not share space with the trocar during surgery. The miniature magnetically anchored camera is a type of magnetically anchored instrument used for providing intraoperative optics.

Peer-review
The research group presented an interesting concept. The miniature magnetically anchored and controlled camera system has drawn the interest of surgeons to conduct further research and make a judgment about it. It may be a promising method for trocar-less laparoscopy. This is a very interesting work.

REFERENCES

1   **Cooperman AM**. Laparoscopic cholecystectomy for severe acute, embedded, and gangrenous cholecystitis. *J Laparoendosc Surg* 1990; **1**: 37-40 [PMID: 2151856]

2   **Cuschieri A**, Dubois F, Mouiel J, Mouret P, Becker H, Buess G, Trede M, Troidl H. The European experience with laparoscopic cholecystectomy. *Am J Surg* 1991; **161**: 385-387 [PMID: 1825763]

3   **Sajid MS**, Khawaja AH, Sains P, Singh KK, Baig MK. A systematic review comparing laparoscopic vs open adhesiolysis in patients with adhesional small bowel obstruction. *Am J Surg* 2016; **212**: 138-150 [PMID: 27162071 DOI: 10.1016/j.amjsurg.2016.01.030]

4   **Taghavi S**, Ambur V, Jayarajan SN, Gaughan J, Toyoda Y, Dauer E, Sjoholm LO, Pathak A, Santora T, Goldberg AJ, Rappold J. Postoperative outcomes with cholecystectomy in lung transplant recipients. *Surgery* 2015; **158**: 373-378 [PMID: 25999250 DOI: 10.1016/j.surg.2015.02.021]

5   **Yu HY**, Hevelone ND, Lipsitz SR, Kowalczyk KJ, Hu JC. Use, costs and comparative effectiveness of robotic assisted, laparoscopic and open urological surgery. *J Urol* 2012; **187**: 1392-1398 [PMID: 22341274 DOI: 10.1016/j.juro.2011.11.089]

6   **Lowry PS**, Moon TD, D'Alessandro A, Nakada SY. Symptomatic port-site hernia associated with a non-bladed trocar after laparoscopic live-donor nephrectomy. *J Endourol* 2003; **17**: 493-494 [PMID: 14565880 DOI: 10.1089/089277903769013649]

7   **Salö M**, Järbur E, Hambraeus M, Ohlsson B, Stenström P, Arnbjörnsson E. Two-trocar appendectomy in children - description of technique and comparison with conventional laparoscopic appendectomy. *BMC Surg* 2016; **16**: 52 [PMID: 27491442 DOI: 10.1186/s12893-016-0170-1]

8   **Cai HH**, Liu MB, He YL. Treatment of Early Stage Endometrial Cancer by Transumbilical Laparoendoscopic Single-Site Surgery Versus Traditional Laparoscopic Surgery: A Comparison Study. *Medicine* (Baltimore) 2016; **95**: e3211 [PMID: 27057851 DOI: 10.1097/MD.0000000000003211]

9   **Hernandez J**, Ross S, Morton C, McFarlin K, Dahal S, Golkar F, Albrink M, Rosemurgy A. The learning curve of laparoendoscopic single-site (LESS) cholecystectomy: definable, short, and safe. *J Am Coll Surg* 2010; **211**: 652-657 [PMID: 20851645 DOI: 10.1016/j.jamcollsurg.2010.07.008]

10 **Hungness ES**, Sternbach JM, Teitelbaum EN, Kahrilas PJ, Pandolfino JE, Soper NJ. Per-oral Endoscopic Myotomy (POEM)

After the Learning Curve: Durable Long-term Results With a Low Complication Rate. *Ann Surg* 2016; **264**: 508-517 [PMID: 27513156 DOI: 10.1097/SLA.0000000000001870]

11   **Ishibashi S**, Takeuchi H, Fujii K, Shiraishi N, Adachi Y, Kitano S. Length of laparotomy incision and surgical stress assessed by serum IL-6 level. *Injury* 2006; **37**: 247-251 [PMID: 16434039 DOI: 10.1016/j.injury.2005.08.008]

12   **Watanabe J**, Ota M, Fujii S, Suwa H, Ishibe A, Endo I. Randomized clinical trial of single-incision versus multiport laparoscopic colectomy. *Br J Surg* 2016; **103**: 1276-1281 [PMID: 27507715 DOI: 10.1002/bjs.10212]

13   **Milas M**, Deveđija S, Trkulja V. Single incision versus standard multiport laparoscopic cholecystectomy: up-dated systematic review and meta-analysis of randomized trials. *Surgeon* 2014; **12**: 271-289 [PMID: 24529791 DOI: 10.1016/j.surge.2014.01.009]

14   **Rivas H**, Varela E, Scott D. Single-incision laparoscopic cholecystectomy: initial evaluation of a large series of patients. *Surg Endosc* 2010; **24**: 1403-1412 [PMID: 20035355 DOI: 10.1007/s00464-009-0786-7]

15   **Wang GJ**, Afaneh C, Aull M, Charlton M, Ramasamy R, Leeser DB, Kapur S, Del Pizzo JJ. Laparoendoscopic single site live donor nephrectomy: single institution report of initial 100 cases. *J Urol* 2011; **186**: 2333-2337 [PMID: 22014813 DOI: 10.1016/j.juro.2011.07.071]

16   **Park S**, Bergs RA, Eberhart R, Baker L, Fernandez R, Cadeddu JA. Trocar-less instrumentation for laparoscopy: magnetic positioning of intra-abdominal camera and retractor. *Ann Surg* 2007; **245**: 379-384 [PMID: 17435544 DOI: 10.1097/01.sla.0000232518.01447.c7]

17   **Hiroki C**, YI S, Yu WW. A Study on Wire-Wire Driven Abdominal Cavity Mobile Micro Robot. The 2010 IEEE/RSJ International Conference on Intelligent Robots and Systems. Taipei, Taiwan, 2010: 2816-2821

18   **Ohdaira T**, Endo K, Abe N, Yasuda Y. Usefulness in NOTES of an intra-abdominal antifogging wireless charge-coupled device (CCD) camera with pantograph-type needle unit for placement to the intra-abdominal wall. *Surg Endosc* 2010; **24**: 198-209 [PMID: 19533239 DOI: 10.1007/s00464-009-0554-8]

19   **Arain NA**, Cadeddu JA, Hogg DC, Bergs R, Fernandez R, Scott DJ. Magnetically anchored cautery dissector improves

triangulation, depth perception, and workload during single-site laparoscopic cholecystectomy. *J Gastrointest Surg* 2012; **16**: 1807-1813 [PMID: 22744636 DOI: 10.1007/s11605-012-1926-2]

20   **Arain NA**, Rondon L, Hogg DC, Cadeddu JA, Bergs R, Fernandez R, Scott DJ. Magnetically anchored camera and percutaneous instruments maintain triangulation and improve cosmesis compared with single-site and conventional laparoscopic cholecystectomy. *Surg Endosc* 2012; **26**: 3457-3466 [PMID: 22648118 DOI: 10.1007/s00464-012-2354-9]

21   **Terry BS**, Mills ZC, Schoen JA, Rentschler ME. Single-Port-Access Surgery with a Novel Magnet Camera System. IEEE transactions on biomedical engineering, 2012: 1187-1193

22   **Best SL**, Bergs R, Scott DJ, Fernandez R, Mashaud LB, Cadeddu JA. Solo surgeon laparo-endoscopic single site nephrectomy facilitated by new generation magnetically anchored and guided systems camera. *J Endourol* 2012; **26**: 214-218 [PMID: 22191662 DOI: 10.1089/end.2011.0143]

23   **Lehman AC**, Dumpert J, Wood NA, Redden L, Visty AQ, Farritor S, Varnell B, Oleynikov D. Natural orifice cholecystectomy using a miniature robot. *Surg Endosc* 2009; **23**: 260-266 [PMID: 19057960 DOI: 10.1007/s00464-008-0195-3]

24   **Raman JD**, Bergs RA, Fernandez R, Bagrodia A, Scott DJ, Tang SJ, Pearle MS, Cadeddu JA. Complete transvaginal NOTES nephrectomy using magnetically anchored instrumentation. *J Endourol* 2009; **23**: 367-371 [PMID: 19196056 DOI: 10.1089/end.2008.0220]

25   **Simi M**, Ciuti G, Tognarelli S, Valdastri P, Menciassi A, Dario P. Magnetic link design for a robotic laparoscopic camera. *J Appl Phys* 2010; **107**: 09B302 [DOI: 10.1063/1.3352581]

26   **Shang Y**, Guo H, Zhang D, Xue F, Yan X, Shi A, Dong D, Wang S, Ma F, Wang H, Li J, Liu X, Luo R, Wu R, Lv Y. An application research on a novel internal grasper platform and magnetic anchoring guide system (MAGS) in laparoscopic surgery. *Surg Endosc* 2017; **31**: 274-280 [PMID: 27177955 DOI: 10.1007/s00464-016-4968-9]

27   **Best SL**, Bergs R, Gedeon M, Paramo J, Fernandez R, Cadeddu JA, Scott DJ. Maximizing coupling strength of magnetically anchored surgical instruments: how thick can we go? *Surg Endosc* 2011; **25**: 153-159 [PMID: 20533063 DOI: 10.1007/s00464-010-1149-0]

ORIGINAL ARTICLE

Basic Study

Acanthopanax senticosus polysaccharides-induced intestinal tight junction injury alleviation *via* inhibition of NF-κB/MLCK pathway in a mouse endotoxemia model

Jie Han, Ji-Hong Li, Guang Bai, Guo-Shun Shen, Jing Chen, Jia-Nan Liu, Shuo Wang, Xian-Jun Liu

Jie Han, Guo-Shun Shen, Jing Chen, Jia-Nan Liu, Xian-Jun Liu, Key Laboratory of Zoonosis of Liaoning Province, College of Animal Science and Veterinary Medicine, Shenyang Agricultural University, Shenyang 110866, Liaoning Province, China

Ji-Hong Li, Department of Pharmacy, Affiliated Hospital, Liaoning University of Traditional Chinese Medicine, Shenyang 110032, Liaoning Province, China

Guang Bai, Department of Gastroenterology, Affiliated Hospital of Liaoning University of Traditional Chinese Medicine, Shenyang 110032, Liaoning Province, China

Shuo Wang, Testing and Analysis Center, Shenyang Agricultural University, Shenyang 110866, Liaoning Province, China

Abstract

AIM
To examine the effects of *Acanthopanax senticosus* polysaccharides (ASPS) on intestinal tight junction (TJ) disruption and nuclear factor-kappa B (NF-κB)/myosin light chain kinase (MLCK) activation in endotoxemia.

METHODS
BALB/C mice (6-8-weeks-old) received continuous intragastric gavage of ASPS for 7 d before injection of lipopolysaccharide (LPS), or received ASPS once after LPS injection. Blood and intestinal mucosal samples were collected 6 h after LPS challenge. Clinical symptoms, histological injury, intestinal permeability,

TJ ultrastructure, and TJ protein expression were determined.

RESULTS
Compared with mice in the LPS group, pretreatment with ASPS improved clinical and histological scores by 390.9% ($P < 0.05$) and 57.89% ($P < 0.05$), respectively, and gut permeability change in endotoxemic mice was shown by a 61.93% reduction in reduced leakage of fluorescein isothiocyanate-dextran 6 h after LPS injection ($P < 0.05$). ASPS pretreatment also prevented LPS-induced TJ ultrastructure breakdown supported by increased electron dense materials between adjoining cells, sustained redistribution and expression of occludin (0.597 ± 0.027 *vs* 0.103 ± 0.009, $P < 0.05$) and zonula occludens-1 (0.507 ± 0.032 *vs* 0.125 ± 0.019, $P < 0.05$), and suppressed activation of the NF-κB/MLCK pathway indicated by reduced expression of NF-κB, phospho-inhibitor kappa B-alpha, MLCK and phospho-myosin light-chain-2 by 16.06% ($P < 0.05$), 54.31% ($P < 0.05$), 66.10% ($P < 0.05$) and 64.82% ($P < 0.05$), respectively.

CONCLUSION
ASPS pretreatment may be associated with inhibition of the NF-κB/MLCK pathway and concomitant amelioration of LPS-induced TJ dysfunction of intestinal epithelium in endotoxemia.

Key words: *Acanthopanax senticosus* polysaccharide; Intestinal permeability; Tight junction; Nuclear factor-kappa B; Myosin light chain kinase

Core tip: *Acanthopanax senticosus* polysaccharides (ASPS) effectively protect against gastric tight junction (TJ) injury in sepsis. ASPS pretreatment significantly improved intestinal histological appearance and gut permeability, increased electron dense between adjoining cells, sustained the expression and redistribution of occludin and zonula occludens-1, suppressed the expression of nuclear factor-kappa B p65 (NF-κBp65) and phospho-inhibitor kappa B-alpha and myosin light chain kinase (MLCK), as well as phospho-myosin light-chain-2 in endotoxemia. These findings suggest that ASPS pretreatment may be associated with inhibition of the NF-κB/MLCK pathway and concomitant amelioration of gastric TJ dysfunction in the mouse model of endotoxemia.

INTRODUCTION
Sepsis and resulting organ system dysfunction are the most frequent causes of death in intensive care patients worldwide[1], and were identified to occur mainly in response to lipopolysaccharide (LPS) from Gram-negative bacteria and to develop rapidly into fatal systemic infections[2]. The gastrointestinal tract is involved in the initial response to the systemic inflammatory reaction[3]. Impaired intestinal barrier function or increased epithelial permeability may promote the translocation of bacteria and the entry of allergenic compounds from the gut into the body, increasing susceptibility to infections[4,5], and this process has been implicated in the development of sepsis and septic multiple organ dysfunction[6].

Tight junctions (TJs) and their associated proteins, such as zonula occludens (ZO), occludin and claudins, are critical in maintenance of the intact intestinal epithelial barrier[7], which can regulate the entry of nutrients, ions and water, while restricting the entry of luminal pathogens and antigenic molecules into the mucosa[8]. TJ breakdown occurs in polymicrobial sepsis when TJ proteins are remodeled due to interactions with external stimuli, such as pathogenic bacteria[9]. Signaling molecules, such as myosin light chain kinase (MLCK) have been implicated in the assembly and regulation of TJs *via* phosphorylation of myosin light chain (MLC)[10]. MLCK can be mediated according to transcriptional increase by nuclear factor-kappa B (NF-κB) in the inflammatory response, and thus results in TJ barrier breakdown[11]. *In vivo* and *in vitro* models have demonstrated that inhibition of MLCK[12] and NF-κB[13] can prevent the deleterious effects of LPS-induced sepsis and leads to TJ preservation.

In recent years, there has been growing interest in the development of new therapeutic strategies in sepsis. *Acanthopanax senticosus* (AS) has been widely used for thousands of years in China as a traditional herbal medicine to regulate hypoxia, fatigue and appetite loss without side effects[14,15]. Polysaccharides extracted from AS (ASPS) are major active ingredients with multiple pharmacologic and biological characteristics, including immune regulation[16] and anti-inflammation[17]. A recent *in vivo* study suggested that ASPS could exert positive effects on intestinal mucosal integrity and suppress NF-κB activation[18]. However, the mechanisms by which ASPS exert these effects on TJ disruption in a mouse model of endotoxemia have not yet been elucidated. In the present study, we determined the effects of ASPS on MLCK activation and TJ barrier breakdown in LPS-induced endotoxemia to evaluate whether the administration of ASPS alleviates endotoxemia-induced epithelial TJ breakdown by suppressing the NF-κB/MLCK signaling pathway.

MATERIALS AND METHODS
Preparation and analysis of ASPS
Details on the preparation of ASPS, which were taken

Table 1 Clinical scoring system

Variables	Score		
	0	1	2
Conjunctiva secretion	Closed eyes or opened with serious secretion	Opened eyes with moderate discharge	Normal eye without conjunctivitis
Stool consistency	Watery stool	Loose stool	Normal stool
Fur appearance	Rough and dull fur	Reduced grooming fur	Smooth and shiny fur
Stimulation activity	Lethargy and raising head after moderate stimulation	Inactive and reduced alert, < 2 steps after moderate stimulation	Normal action and reaction, > 2 steps after moderate stimulation

from the root of AS, using an ethanol precipitation method have been reported previously[18]. Proteins were removed by the Sevag method[19] and polysaccharide content after purification using the phenol-sulfuric acid method was 92.7%[20]. Analysis of monosaccharide composition in ASPS was by ion chromatography according to a previously described method[21], which showed that it is a heteropolysaccharide composed of glucose, galactose, arabinose, mannose, rhamnose and xylopyranose.

Experimental animals

Male BALB/C mice (Changsheng Life Sciences Co., Ltd., Changchun, China), weighing 20-25 g, aged 6-8 wk, were housed individually in a temperature (22 ± 2 ℃) and humidity (53% ± 2%) controlled room with a 12-h light/dark cycle and *ad libitum* access to chow and water. All animal experiments conformed to the guidelines on caring for and use of laboratory animals which were reviewed by the Animal Ethics Committee of College of Animal Science & Veterinary Medicine, Shenyang Agricultural University (Permit No. SYXK (Liao) 2011-0001).

Experimental protocols

Following acclimation for 1 wk, all animals were randomly assigned to 4 groups (7-8 mice per group): control, LPS, ASPS + LPS, and LPS + ASPS. Mice in the ASPS + LPS group were administered continuous intragastric gavage of ASPS dissolved in normal saline at the dose of 300 mg/kg daily for 7 d, and mice in the control, LPS, and LPS + ASPS groups were given an equivalent amount of normal saline. After 1 h of intragastric treatment on day 7, mice in the LPS, ASPS + LPS and LPS + ASPS groups were injected intraperitoneally with LPS from *Escherichia coli* serotype (055:B5; Sigma, St Louis, MO, United States) at 10 mg/kg dissolved in 1 mL normal saline, and the control group was given an equivalent amount of normal saline. The ASPS dose was determined in accordance with our previous study[17]. Mice in the LPS + ASPS group received 300 mg/kg ASPS intragastrically 30 min after LPS injection. All animals were anesthetized with pentobarbital sodium (60 mg/kg, intraperitoneally), killed by cervical dislocation and samples were collected 6 h after LPS treatment. All efforts were made to minimize animal suffering.

Clinical symptom score

Clinical symptom scores of severity of conjunctiva secretion, stool consistency, messy fur, and inactivity were determined at specified time points using a 3-point scale according to a method described previously[22] with slight modifications. The scoring system is presented in Table 1. Clinical symptoms in each mouse were evaluated at 2, 4 and 6 h after LPS injection and scored blindly by three independent researchers. The means of three assessments were obtained for grading.

Histopathological evaluation of the intestine

After sacrifice and excision of ileal and colonic segments near to the cecum for observation of intestinal macroscopic features, ileal segments measuring approximately 2-cm were stained with hematoxylin and eosin (HE) for morphological observation. The details of this process were as follows: intestinal segments were transferred into 4% paraformaldehyde and embedded in paraffin. Sections measuring 5-μm thick were sliced, deparaffinized, rehydrated and stained with HE to observe the degree of intestinal mucosal damage using a biomicroscope (Axio Scope A1; Zeiss, Oberkochen, Germany) and scored according to the method by Chiu, as follows: score of 0, normal mucosal villi without damage; 1, broadened subepithelial Gruenhagen's space at villous tip; 2, further extension of subepithelial space from the epithelial layer to the lamina propria; 3, detachment of less than half of the villous epithelium; 4, detachment of more than half of the villous epithelium and exposed villi with lamina propria; and 5, disintegration and detachment of the lamina propria. Five images in each slice were blindly assessed by three pathologists.

Determination of intestinal permeability

At 2, 4 and 6 h after LPS injection, 3 mice from each group were anesthetized with pentobarbital sodium and a midline laparotomy was performed to expose the intestinal tract. Lengths of distal ileum measuring 5 cm were isolated and ligated at both ends. A solution of 100 μL PBS containing 20 mg of 4-kDa fluorescein isothiocyanate (FITC)-dextran (Sigma) was injected into the lumen and then the midline skin was sutured. A 100 μL blood sample was collected *via* cardiac puncture 30 min after FITC-dextran injection and

was diluted with 1.9 mL of 50 mmol/L Tris-buffered saline (TBS) and centrifuged at 10000 × *g* for 10 min to obtain plasma. The concentration of FITC-dextran in plasma was assayed using a fluorescence spectrophotometer (970CRT; Shanghai Lengguang Technology Co, Shanghai, China) with excitation and emission wavelengths of 480 and 520 nm, respectively.

TJ transmission electron microscopy

After rinsing with cold PBS, distal ileal sections measuring 1 mm × 1 mm × 2 mm were cut on ice and immediately transferred into 4% glutaraldehyde to fix for 2 h, post-fixed with 1% osmium tetroxide, and embedded in Epon 812. Thin slices measuring 500 nm were cut and double stained with uranyl acetate and lead citrate, and then examined with an transmission electron microscope (TEM) (HT-7700; Hitachi, Tokyo, Japan) operated at 100 kV.

Immunofluorescence microscopy

Ileal segments were fixed with 4% paraformaldehyde and then cut into 3-μm thick slices. The slices were dewaxed and dehydrated with xylene and ethanol, respectively, and then incubated in 3% hydrogen peroxide and the antigens repaired in citrate buffer. The resulting tissue samples were blocked with 5% normal goat serum in PBS. After incubation with antibodies against occludin (1:100; Proteintech, Chicago, IL, United States), ZO-1 (1:100; Proteintech), and MLCK (1:200; Abcam, Cambridgeshire, United Kingdom) in 1% fetal bovine serum overnight at 4 ℃, the sections were washed and incubated with Cy3-conjugated secondary antibodies for 1 h. Sample images were obtained using a BX43 (Olympus, Tokyo, Japan) microscope.

Protein extraction from the nucleus and cytoplasm of intestinal mucosa

Protein extracts were prepared according to a previously described method[23] with some modifications. Ileal mucosa samples were collected near the cecocolonic junction and ground with liquid nitrogen. The powder was incubated on ice for 10 min with a buffer containing KCl at 10 mmol/L, HEPES at 10 mmol/L (pH 7.9), MgCl$_2$ at 1.5 mmol/L, dithiothreitol at 1 mmol/L and benzene methyl sulfuryl fluoride at 1 mmol/L, and then centrifuged at 5000 × *g* for 3 min. The precipitate was resuspended in this buffer and centrifuged again to obtain the supernatant as the cytoplasmic extract for protein expression assay of occludin, ZO-1, phospho-MLC2, and phospho-IκBa, and the resulting precipitate was lysed by incubation for 30 min in 0.2 mL buffer containing HEPES at 20 mmol/L, glycerol at 25%, NaCl at 420 mmol/L, MgCl$_2$ at 1.5 mmol/L and EDTA at 0.2 mmol/L. Following centrifugation at 12 000 for 15 min, the supernatant (nuclear extract) was obtained and the expression of NF-κB p65 and MLCK was analyzed. The extracted proteins were quantified

using the bicinchoninic acid assay and stored at -80 ℃ for subsequent assay.

Western blot assay

An equal amount of protein exact (20-40 μg) was electrophoresed on a 10% reducing polyacrylamide gel and transferred onto polyvinylidene difluoride membranes. Immunoblots were blocked with 3% bovine serum albumin (BSA) in TBS for 70 min at room temperature and incubated overnight at 4 ℃ with specific primary antibodies including rabbit anti-occludin (1:1000; Proteintech), rabbit anti-ZO-1 (1:1000; Proteintech), rabbit anti-NF-κB p65 (1:5000; Abcam), rabbit anti-MLCK (1:5000; Abcam), phospho-MLC2 (1:1000; Cell Signaling Technology, Danvers, MA, United States), and phospho-IκBα (1:1000; Cell Signaling Technology) in TBS and 0.05% Tween-20 containing 1% BSA.

Blots were washed and then incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibodies for 120 min at room temperature. The bands were detected by enhanced chemiluminescence and quantified (relative to β-actin expression) using Scion Image 4.03 analysis software.

Statistical analysis

Data were statistically analyzed by one-way analysis of variance (ANOVA) using IBM SPSS statistical software, version 22.0, and differences among the groups were compared using Duncan's multiple test. The results were expressed as mean ± SE, and a 5% level of probability was considered significant for all analyses.

RESULTS

Clinical symptom score and morphological and histopathologic evaluation of the intestine

The clinical symptoms and morphological and histopathologic changes following ASPS treatment were assessed in this model of endotoxemia induced by LPS challenge. The LPS group showed a pronounced decline in the clinical symptom score compared with the control group ($P < 0.05$). The clinical symptom score in mice pretreated with ASPS was significantly improved by 390.9% ($P < 0.05$) (Figure 1A), and showed less edema in the cecum and a thicker colon with more and larger stool pellets compared with the LPS-treated mice (Figure 1B).

The histological examination using HE staining showed marked damage characterized by atrophic villi with a discontinuous brush border and irregular epithelium in endotoxemic mice in the LPS group. As expected, these negative histologic changes in the LPS group were significantly alleviated by pretreatment with ASPS rather than subsequent administration of ASPS following LPS injection (Figure 1C). The intestinal histological score in the LPS group was significantly increased compared with the control group ($P < 0.05$).
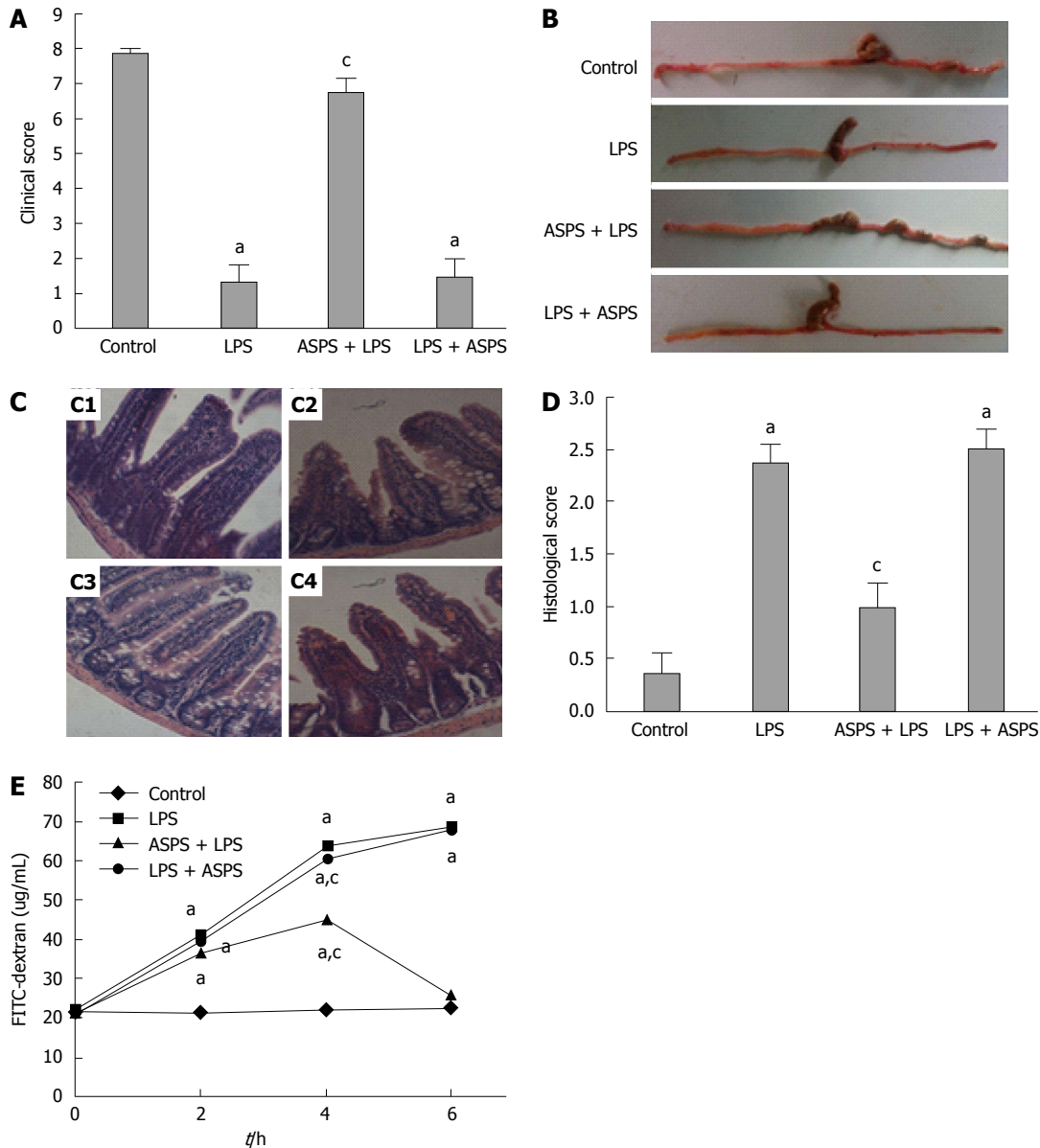
Figure 1 Effects of *acanthopanax senticosus* polysaccharides on clinical score, macroscopic features of distal ileum and colon, histological appearance and score of distal ileum in lipopolysaccharide-induced mice. A: Mice were assessed for clinical score at designated time points after lipopolysaccharide (LPS) challenge (n = 8); B: Representative photographs of the distal ileums and colons at 6 h after LPS injection (n = 8); C: Effects of acanthopanax senticosus polysaccharides (ASPS) on LPS-induced intestinal histopathologic changes. Ileum was processed for morphological and histopathologic evaluation at 6 h after LPS induction (n = 3). The representative photomicrographs of ileal segments stained with hematoxylin and eosin at 200 × magnification of C1, control group; C2, LPS group; C3, ASPS + LPS group; and C4, LPS + ASPS group; D: Intestinal histopathologic score was determined at 6 h after LPS challenge (n = 3); E: Effects of ASPS on LPS-induced increase in iliac mucosal permeability. The intestinal permeability of 4 kDa fluorescein isothiocyanate (FITC)-dextran in ileal pouch was measured at 2, 4 and 6 h after LPS administration (n = 8). [a]P < 0.05, *vs* the control group; [c]P < 0.05, *vs* the LPS group.

ASPS pretreatment markedly reversed the effect of LPS by 57.89% (P < 0.05). However, oral administration of ASPS following LPS injection did not reverse the damage induced by LPS (P > 0.05) (Figure 1D).

Intestinal permeability assay

At 2, 4 and 6 h after LPS administration, gut mucosal permeability was evaluated *ex vivo* by measuring the leakage of FITC-dextran from the intestinal epithelium into the systemic circulation. The concentration of FITC-dextran was significantly increased after LPS administration compared with the control group (P <

0.05). A marked reduction (61.93%) in the amount of FITC-dextran in the circulation was observed in the ASPS pretreatment group (P < 0.05) rather than post-treatment in the ASPS group (P > 0.05) (Figure 1E).

TJ protein location and expression and TJ ultrastructure

The localization and expression of occludin and ZO-1 proteins were evaluated by immunofluorescence to determine the influence of ASPS on TJ disruption induced by LPS. Mice in the LPS group exhibited less staining of occludin and ZO-1 in the ileum. Correspondingly, ASPS pretreatment attenuated the

redistribution of TJ proteins with the presence of continuous bands along the epithelial sheet. However, in the LPS + ASPS group, the loss of both proteins was not attenuated, and TJ distribution was similar to that in the LPS group (Figure 2A). Similarly, the expression of both proteins using immunoblotting was decreased in ileal epithelium in endotoxemic mice ($P < 0.05$). Pretreatment with ASPS partially up-regulated LPS-induced loss of occludin (0.597 ± 0.027 *vs* 0.103 ± 0.009, $P < 0.05$) and ZO-1 (0.507 ± 0.032 *vs* 0.125 ± 0.019, $P < 0.05$). In contrast, the administration of ASPS after LPS injection did not ameliorate the loss of these proteins ($P > 0.05$) (Figure 2B). The intact structure and electron dense materials between the adjoining cells observed in the control group decreased 6 h after LPS treatment. As expected, ASPS pretreatment significantly attenuated the negative changes induced by LPS induction. However, these pathologic changes were not reversed following the administration of ASPS after LPS injection (Figure 2C).

NF-κB/MLCK pathway response

Figure 3A shows the nuclear expression of NF-κB p65 and MLCK, and the cytoplasmic expression of phospho-IκBα and phospho-MLC2 in intestinal epithelium analyzed by western blotting in the 4 experimental groups. Furthermore, staining of MLCK in the distal ileal epithelium was shown by immunofluorescence to determine the distribution of MLCK (Figure 3B). The expression of NF-κB p65 and MLCK in the nucleus, and phospho-IκBα and phospho-MLC2 in the cytoplasm were markedly increased in LPS-challenged mice, which was concordant with localization of MLCK at the periphery of the cells ($P < 0.05$). ASPS pretreatment significantly reversed the effects of endotoxemia induced by LPS on the expression of these proteins 6 h after LPS challenge ($P < 0.05$). However, administration of ASPS following LPS injection did not improve these effects.

DISCUSSION

The gastrointestinal epithelium which forms a boundary effectively provides a selective permeable barrier that prevents pathogenic bacteria and their effectors entering the mucosal tissues from the intestinal lumen. This selective permeable barrier is achieved by intercellular TJ structures[8]. A TJ is a multi-protein complex comprised of the transmembrane proteins occludin, the claudin family proteins, as well as the cytoplasmic protein ZO-1, and forms a seal between adjacent intestinal epithelial cells[24]. However, opening of the TJ is primarily dependent on the composition and organization of these TJ proteins[6], which is not static but a highly dynamic structure that is constantly being remodeled due to interactions with pathogenic bacteria. These bacteria cause TJ damage and further increase intestinal permeability and the

systemic inflammatory response syndrome, which is characterized by a whole body inflammatory state and multiple organ failure[9]. Therapy is conceivable by regulating TJ integrity to trigger decreased permeability *via* the paracellular pathway.

Although the underlying mechanism by which intestinal TJ is damaged in endotoxemia is not fully elucidated, the altered localization of TJ proteins due to activation of the NF-κB/MLCK signaling pathway is believed to play a vital role in TJ disruption in intestinal inflammation. NF-κB is a transcription factor and has long been considered the central mediator of the inflammatory process, with the main heterodimer consisting of NF-κBp65 and regulating the genes involved in many aspects of the inflammatory response[25]. NF-κBp65 can be induced to undergo cytoplasmic-to-nuclear translocation when its inhibitory factor I-κB is phosphorylated and degraded in intestinal mucosa during endotoxemia[26], and can bind to the MLCK promoter region to cause MLCK-mediated MLC phosphorylation and concomitant remodeling of the localization of TJ proteins and functional opening by contracting actin-myosin filaments[27,28]. Thus, it is becoming increasingly evident that inhibiting activation of the NF-κB/MLCK signaling pathway may potentially lead to repair of the compromised intestinal TJ barrier in endotoxemia.

ASPS are widely used as therapy for immune regulation and anti-inflammation in China. ASPS have been demonstrated to ameliorate LPS-induced inflammatory response in piglets[15] and appear to have beneficial effects against LPS-induced intestinal mucosal injury and integrity loss in the mouse model of endotoxemia by suppressing over-activation of the NF-κB signaling pathway[17]. Although NF-κB and MLCK-mediated MLC phosphorylation are clearly involved in TJ regulation in inflammation, the beneficial effects of ASPS on intestinal TJ disruption in endotoxemia and whether this signaling pathway is involved in the opening of TJ following administration of ASPS are poorly elucidated.

In the current study, a well-documented mouse model of endotoxemia induced by LPS injection was successfully used. The mice appeared to have typical clinical symptoms characterized by watery stools, increased secretion, somnolence and inactivity, as well as histopathologic macroscopic and microscopic changes, including edematous and thin intestine, villus atrophy, and epithelial shedding. In addition, 6 h after injection of LPS was chosen as the sampling time, according to previous studies which had suggested that an acute intestinal inflammatory response was observed 3-6 h after LPS injection[29,30].

HE staining and the FITC-dextran assay of distal ileum showed that ASPS alleviated mucosal integrity loss in mice with endotoxemia, as demonstrated by an improvement in morphological appearance and a decline in the concentration of FITC-dextran in plasma.
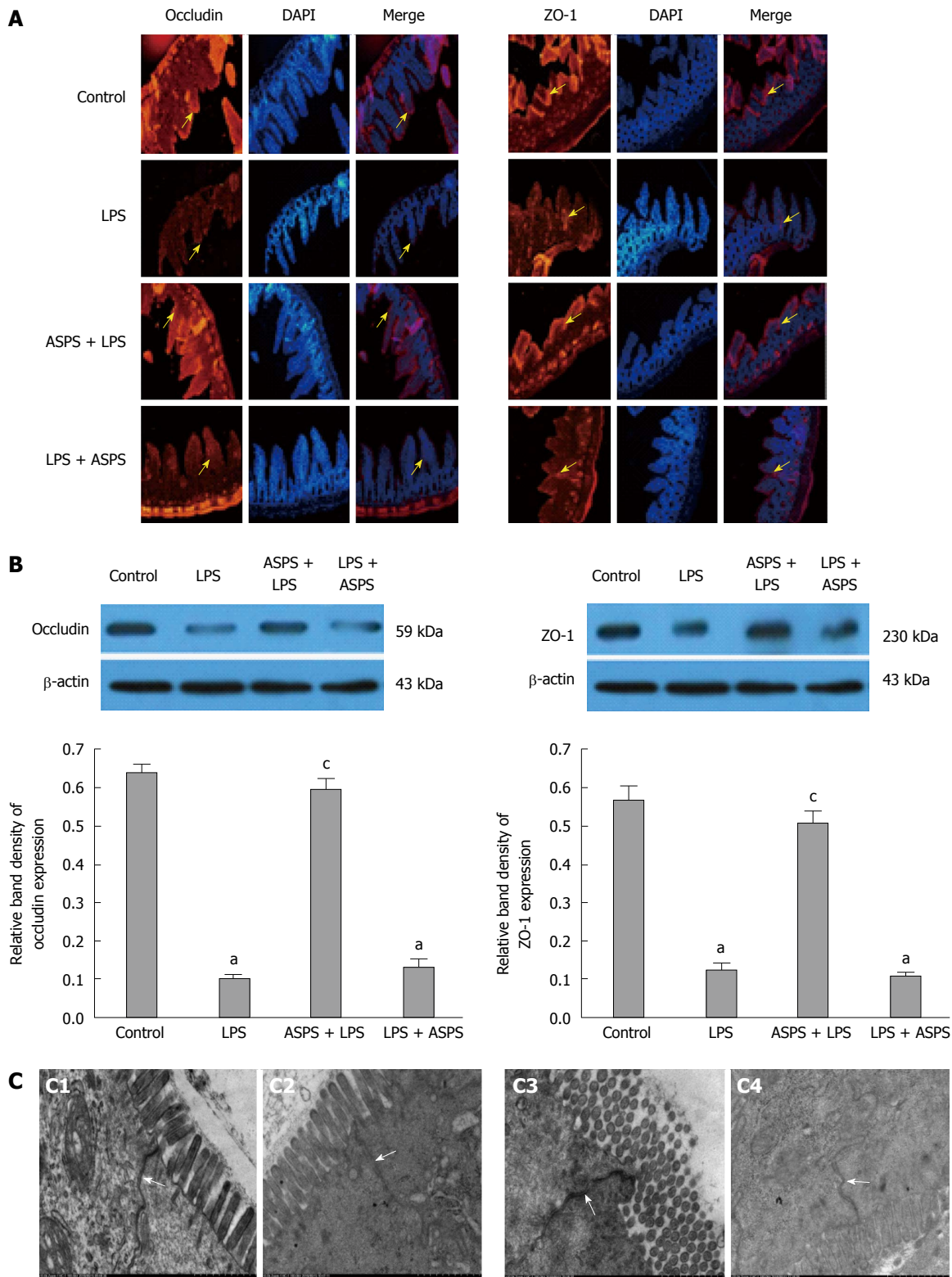
Figure 2 Localization and expression of tight junction proteins, and tight junction proteins ultrastructure in ileum were evaluated 6 h after lipopolysaccharide administration in mice of four groups. A: Effects of Acanthopanax senticosus polysaccharides (ASPS) on distribution of occludin and ZO-1. Staining of both proteins along the villous epithelium at a 200 × magnification (red fluorescence) were observed by immunofluorescence. Nuclei were stained by DAPI (blue fluorescence). Arrows indicate the location of tight junction (TJ) proteins staining; B: Effects of ASPS on intestinal TJ proteins expression of occludin and ZO-1 (*n* = 3). Protein samples were analyzed by western blotting, and β-actin was used as an internal control. The values are presented as mean ± SE. [a]*P* < 0.05, *vs* the control group; [c]*P* < 0.05, *vs* the LPS group; C: Effects of ASPS on intestinal TJ ultrastructure in ileum viewed under transmission electron microscope of C1, control group; C2, LPS group; C3, ASPS + LPS group; and C4, LPS + ASPS group. Arrows indicate the location of the TJ (scale bar = 1 μm).
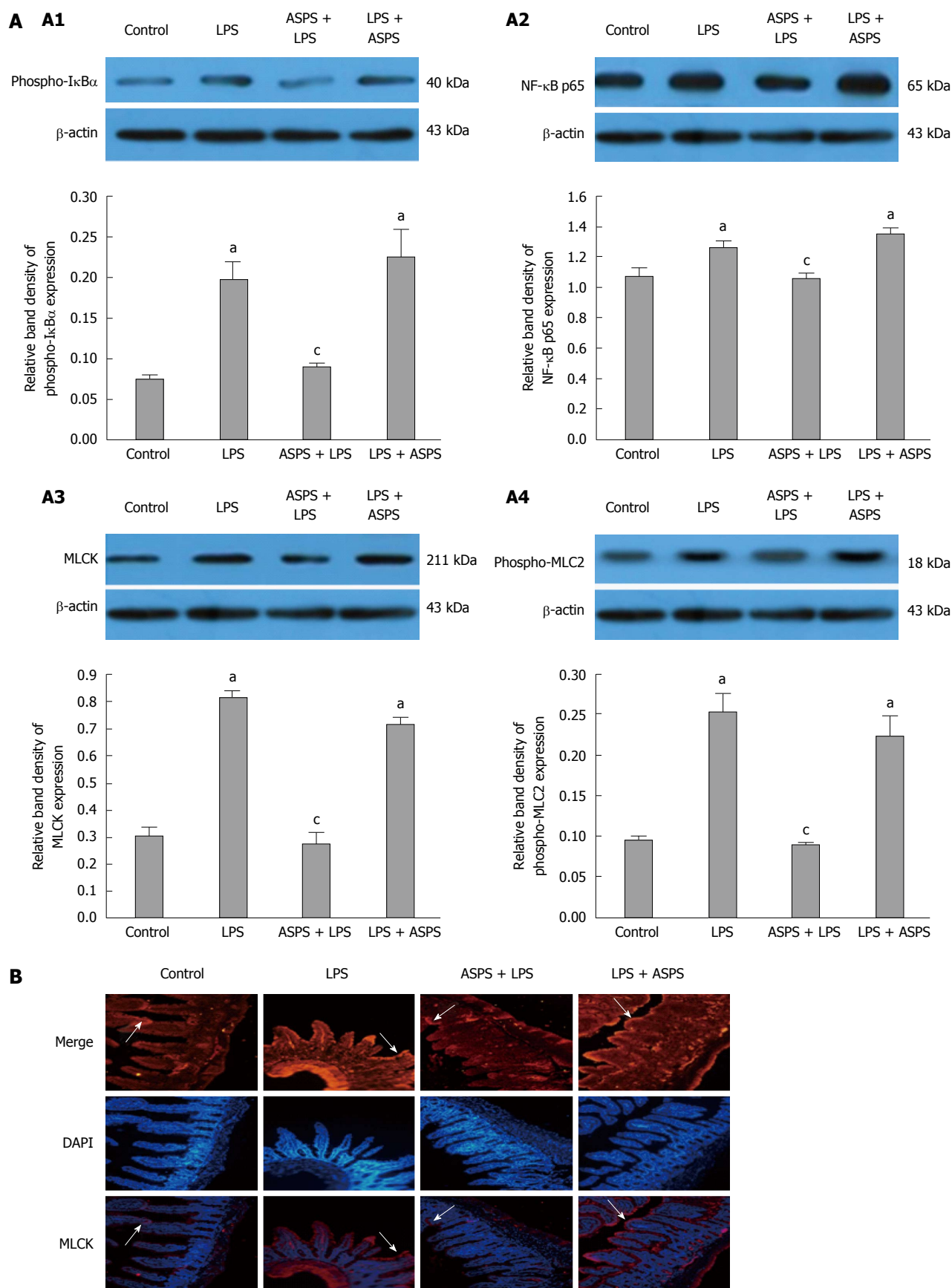
Figure 3 Protein expression of phospho-IκBα, nuclear factor-kappa B p65, myosin light chain kinase and phospho-MLC2 (A) and MLCK localization (B) in ileum epithelium. A: Protein expression of phospho-IκBα (A1), nuclear factor-kappa B (NF-κB) p65 (A2), myosin light chain kinase (MLCK) (A3) and phospho-MLC2 (A4) were analyzed by western blotting at 6 h after lipopolysaccharide (LPS) induction, and β-actin was used as internal control (*n* = 3). Data are shown as mean ± SE (*n* = 3). [a]*P* < 0.05, *vs* the control group; [c]*P* < 0.05, *vs* the LPS group; B: MLCK location was observed by immunofluorescence at 6 h after LPS administration at 200 × magnification (red fluorescence) (*n* = 3). Nuclei were stained by DAPI (blue fluorescence).

These findings were consistent with our previous results regarding improved intestinal integrity by ASPS in LPS-challenged mice. ASPS also prevented LPS-induced TJ ultrastructural breakdown, supported by increased electron dense materials between adjoining cells using TEM. In addition, ASPS pretreatment positively reversed the distribution and expression of occludin and ZO-1 in mice with endotoxemia. Collectively, these results indicate that pretreatment with oral ASPS may be a preventive option for decreasing TJ disruption in endotoxemia. However, our study on the effects of ASPS administration subsequent to LPS injection demonstrated their unavailability in endotoxemia.

Gut-associated systemic infection resulting in systemic diseases is associated with increased mucosal permeability[31]. TJ opening involved in permeability regulation is primarily dependent on MLCK-mediated MLC phosphorylation during the pathophysiology of endotoxemia. In order to determine the underlying mechanism involved in the beneficial effect of ASPS on TJ opening in endotoxemic mice, activity of the NF-κB/MLCK signaling pathway in intestinal epithelium was determined and the results showed that ASPS modulated the expression of NF-κBp65 and MLCK in the nucleus and phospho-IκBα and phospho-MLC2 in the cytoplasm. The results of our study demonstrated that ASPS pretreatment suppressed the activation of related signaling molecules of the NF-κB/MLCK pathway rather than post-administration, which is consistent with the results of attenuated TJ dysfunction and decreased intestinal permeability in endotoxemic mice. This may be attributed to the pharmacokinetic features of ASPS, although little is understood regarding these features. Interestingly, our recent work may provide some clues as to whether regulatory expression of TLR4 and EGF/EGFR occurred following pretreatment with ASPS[18,32]. We suggest that ASPS administration prior to endotoxemia functions *via* EGF/EGFR-dependent regulation of TLR4[33], whereby EGFR mediates intestinal epithelium growth and differentiation. More attention should be paid to the relationship between EGFR and TJ proteins. However, in the case of endotoxemia, ASPS are unavailable due to LPS combining with TLR4 to activate NF-κB rather than EGF/EGFR.

It is worth noting that our present study provides a new understanding of the influencing mechanism of ASPS on TJ damage in relation to the MLCK/NF-κB pathway. Further attention to other modulations between TJ damage and the protein kinase pathway, calcium ion pathway, G proteins and so on will allow the comprehensive identification of ASPS action. In addition, ASPS intake preceding any upcoming stressful and infectious conditions is likely to be applied in routine clinical practice. Further clinical research should be carried out to accumulate evidence to support treatment with ASPS.

In conclusion, the present study demonstrates that pretreatment with oral ASPS prior to the development of endotoxemia can mitigate intestinal epithelial TJ breakdown in the mouse model of endotoxemia. The underlying mechanism may be associated with inhibition of activation of the NF-κB/MLCK signaling pathway. These results suggest that ASPS may be a potential therapeutic strategy for intestinal permeability loss in sepsis.

COMMENTS

Background
Sepsis and subsequent organ system dysfunction are the most frequent causes of death in intensive care patients worldwide, and have been identified to have a close relationship with intestinal tight junction damage induced by systemic infections. However, it is unclear whether tight junction disruption and its modulatory nuclear factor-kappa B (NF-κB)/myosin light chain kinase (MLCK) signaling pathway are influenced by *Acanthopanax senticosus* polysaccharides (ASPS) in endotoxemia.

Research frontiers
Understanding and regulating intestinal epithelial barrier function *via* relevant inflammatory signaling pathways using a safe and effective substance is an important area of future research.

Innovations and breakthroughs
The present study demonstrates that ASPS pretreatment may be associated with inhibition of the NF-κB/MLCK pathway and concomitant amelioration of intestinal epithelium tight junction dysfunction in endotoxemia.

Applications
Further clinical research should be carried out to provide evidence to support treatment with ASPS, and ASPS intake preceding any upcoming stressful and infectious conditions should be applied in routine clinical practice.

Terminology
Acanthopanax senticosus polysaccharides - a major active extract isolated from *Acanthopanax senticosus*, which is a well-known shrub native to far eastern areas of Russia and northern regions of Japan, Korea and China. Tight junction - a multi-protein complex that forms a seal between adjacent intestinal epithelial cells.

Peer-review
Han *et al* try to understand the signaling pathway involved in the beneficial effects of ASPS against LPS-induced mouse intestinal injury, which is a logical follow-up of their recent article. Essentially, the paper pointed out that pretreatment of mice with ASPS inhibited the NF-κB/ MLCK pathway.

REFERENCES

1   **Angus DC**, Wax RS. Epidemiology of sepsis: an update. *Crit Care Med* 2001; **29**: S109-S116 [PMID: 11445744 DOI: 10.1097/00003 246-200107001-00035]
2   **Polderman KH**, Girbes AR. Drug intervention trials in sepsis: divergent results. *Lancet* 2004; **363**: 1721-1723 [PMID: 15158636 DOI: 10.1016/S0140-6736(04)16259-4]
3   **Hassoun HT**, Kone BC, Mercer DW, Moody FG, Weisbrodt NW, Moore FA. Post-injury multiple organ failure: the role of the gut. *Shock* 2001; **15**: 1-10 [PMID: 11198350 DOI: 10.1097/00024382-200115010-00001]
4   **Lee SH**. Intestinal permeability regulation by tight junction: implication on inflammatory bowel diseases. *Intest Res* 2015; **13**: 11-18 [PMID: 25691839 DOI: 10.5217/ir.2015.13.1.11]
5   **De-Souza DA**, Greene LJ. Intestinal permeability and systemic infections in critically ill patients: effect of glutamine. *Crit Care*

Med 2005; **33**: 1125-1135 [PMID: 15891348 DOI: 10.1097/01.CCM.0000162680.52397.97]

6   **Vogt A**, Reuken PA, Stengel S, Stallmach A, Bruns T. Dual-sugar tests of small intestinal permeability are poor predictors of bacterial infections and mortality in cirrhosis: A prospective study. *World J Gastroenterol* 2016; **22**: 3275-3284 [PMID: 27004006 DOI: 10.3748/wjg.v22.i11.3275]

7   **Suzuki T**. Regulation of intestinal epithelial permeability by tight junctions. *Cell Mol Life Sci* 2013; **70**: 631-659 [PMID: 22782113 DOI: 10.1007/s00018-012-1070-x]

8   **Ulluwishewa D**, Anderson RC, McNabb WC, Moughan PJ, Wells JM, Roy NC. Regulation of tight junction permeability by intestinal bacteria and dietary components. *J Nutr* 2011; **141**: 769-776 [PMID: 21430248 DOI: 10.3945/jn.110.135657]

9   **Li Q**, Zhang Q, Wang C, Liu X, Li N, Li J. Disruption of tight junctions during polymicrobial sepsis in vivo. *J Pathol* 2009; **218**: 210-221 [PMID: 19235836 DOI: 10.1002/path.2525]

10 **Yu M**, Yang S, Qiu Y, Chen G, Wang W, Xu C, Cai W, Sun L, Xiao W, Yang H. Par-3 modulates intestinal epithelial barrier function through regulating intracellular trafficking of occludin and myosin light chain phosphorylation. *J Gastroenterol* 2015; **50**: 1103-1113 [PMID: 25820151 DOI: 10.1007/s00535-015-1066-z]

11 **Ye D**, Ma I, Ma TY. Molecular mechanism of tumor necrosis factor-alpha modulation of intestinal epithelial tight junction barrier. *Am J Physiol Gastrointest Liver Physiol* 2006; **290**: G496-G504 [PMID: 16474009 DOI: 10.1152/ajpgi.00318.2005]

12 **Moriez R**, Salvador-Cartier C, Theodorou V, Fioramonti J, Eutamene H, Bueno L. Myosin light chain kinase is involved in lipopolysaccharide-induced disruption of colonic epithelial barrier and bacterial translocation in rats. *Am J Pathol* 2005; **167**: 1071-1079 [PMID: 16192642 DOI: 10.1016/S0002-9440(10)61196-0]

13 **Ye D**, Ma TY. Cellular and molecular mechanisms that mediate basal and tumour necrosis factor-alpha-induced regulation of myosin light chain kinase gene activity. *J Cell Mol Med* 2008; **12**: 1331-1346 [PMID: 18363837 DOI: 10.1111/j.1582-4934.2008.00302.x]

14 **Chen R**, Liu Z, Zhao J, Chen R, Meng F, Zhang M, Ge W. Antioxidant and immunobiological activity of water-soluble polysaccharide fractions purified from Acanthopanax senticosu. *Food Chem* 2011; **127**: 434-440 [PMID: 23140683 DOI: 10.1016/j.foodchem.2010.12.143]

15 **Wang C**, Liu LM, Song ZQ, Dong YZ, Du ZY, Ning ZC, Liu YY, Liu ZL. Survey of active components in commonly-used Chinese material medica injections and related Chinese material medica for cardiovascular disease. *Zhong Cao Yao* 2015; **46**: 2315-2328

16 **Han SB**, Yoon YD, Ahn HJ, Lee HS, Lee CW, Yoon WK, Park SK, Kim HM. Toll-like receptor-mediated activation of B cells and macrophages by polysaccharide isolated from cell culture of Acanthopanax senticosus. *Int Immunopharmacol* 2003; **3**: 1301-1312 [PMID: 12890428 DOI: 10.1016/S1567-5769(03)00118-8]

17 **Han J**, Bian L, Liu X, Zhang F, Zhang Y, Yu N. Effects of Acanthopanax senticosus Polysaccharide Supplementation on Growth Performance, Immunity, Blood Parameters and Expression of Pro-inflammatory Cytokines Genes in Challenged Weaned Piglets. *Asian-Australas J Anim Sci* 2014; **27**: 1035-1043 [PMID: 25050047 DOI: 10.5713/ajas.2013.13659]

18 **Han J**, Liu L, Yu N, Chen J, Liu B, Yang D, Shen G. Polysaccharides from Acanthopanax senticosus enhances intestinal integrity through inhibiting TLR4/NF-κB signaling pathways in lipopolysaccharide-challenged mice. *Anim Sci J* 2016; **87**: 1011-1018 [PMID: 26435041 DOI: 10.1111/asj.12528]

19 **Staub AM**. Removal of protein-sevag method. In: Whistler RL, Wolfrom ML. Methods in carbohydrate chemistry. New York: Academic Press, 1965: 5-6

20 **DuBois M**, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. *Anal Chem* 1956; **28**: 350-356 [DOI: 10.1021/ac60111a017]

21 **Ou YF**, Yin PH, Zhao L, Huang XS. Determination of monsaccharides in garlic polysaccharide using ion chromatography. *Spectrographic Lab* 2006; **23**: 629-632

22 **Kadl A**, Pontiller J, Exner M, Leitinger N. Single bolus injection of bilirubin improves the clinical outcome in a mouse model of endotoxemia. *Shock* 2007; **28**: 582-588 [PMID: 17577133 DOI: 10.1097/shk.0b013e31804d41dd]

23 **Gu L**, Li N, Gong J, Li Q, Zhu W, Li J. Berberine ameliorates intestinal epithelial tight-junction damage and down-regulates myosin light chain kinase pathways in a mouse model of endotoxinemia. *J Infect Dis* 2011; **203**: 1602-1612 [PMID: 21592990 DOI: 10.1093/infdis/jir147]

24 **Watts T**, Berti I, Sapone A, Gerarduzzi T, Not T, Zielke R, Fasano A. Role of the intestinal tight junction modulator zonulin in the pathogenesis of type I diabetes in BB diabetic-prone rats. *Proc Natl Acad Sci USA* 2005; **102**: 2916-2921 [PMID: 15710870 DOI: 10.1073/pnas.0500178102]

25 **Lawrence T**, Fong C. The resolution of inflammation: anti-inflammatory roles for NF-kappaB. *Int J Biochem Cell Biol* 2010; **42**: 519-523 [PMID: 20026420 DOI: 10.1016/j.biocel.2009.12.016]

26 **Arita Y**, Ito T, Oono T, Kawabe K, Hisano T, Takayanagi R. Lysophosphatidic acid induced nuclear translocation of nuclear factor-kappaB in Panc-1 cells by mobilizing cytosolic free calcium. *World J Gastroenterol* 2008; **14**: 4473-4479 [PMID: 18680225 DOI: 10.3748/wjg.14.4473]

27 **Lawrence T**. The nuclear factor NF-kappaB pathway in inflammation. *Cold Spring Harb Perspect Biol* 2009; **1**: a001651 [PMID: 20457564 DOI: 10.1101/cshperspect.a001651]

28 **Ma TY**, Boivin MA, Ye D, Pedram A, Said HM. Mechanism of TNF-{alpha} modulation of Caco-2 intestinal epithelial tight junction barrier: role of myosin light-chain kinase protein expression. *Am J Physiol Gastrointest Liver Physiol* 2005; **288**: G422-G430 [PMID: 15701621 DOI: 10.1152/ajpgi.00412.2004]

29 **Al-Sadi R**, Ye D, Dokladny K, Ma TY. Mechanism of IL-1beta-induced increase in intestinal epithelial tight junction permeability. *J Immunol* 2008; **180**: 5653-5661 [PMID: 18390750 DOI: 10.4049/jimmunol.180.8.5653]

30 **Mercer DW**, Smith GS, Cross JM, Russell DH, Chang L, Cacioppo J. Effects of lipopolysaccharide on intestinal injury; potential role of nitric oxide and lipid peroxidation. *J Surg Res* 1996; **63**: 185-192 [PMID: 8661195 DOI: 10.1006/jsre.1996.0245]

31 **Liu Y**, Huang J, Hou Y, Zhu H, Zhao S, Ding B, Yin Y, Yi G, Shi J, Fan W. Dietary arginine supplementation alleviates intestinal mucosal disruption induced by Escherichia coli lipopolysaccharide in weaned pigs. *Br J Nutr* 2008; **100**: 552-560 [PMID: 18275628 DOI: 10.1017/S0007114508911612]

32 **Han J**, Xu Y, Yang D, Yu N, Bai Z, Bian L. Effect of Polysaccharides from Acanthopanax senticosus on Intestinal Mucosal Barrier of Escherichia coli Lipopolysaccharide Challenged Mice. *Asian-Australas J Anim Sci* 2016; **29**: 134-141 [PMID: 26732337 DOI: 10.5713/ajas.15.0534]

33 **Liu K**, Anderson GP, Bozinovski S. DNA vector augments inflammation in epithelial cells via EGFR-dependent regulation of TLR4 and TLR2. *Am J Respir Cell Mol Biol* 2008; **39**: 305-311 [PMID: 18403779 DOI: 10.1165/rcmb.2007-0458OC]

ORIGINAL ARTICLE

Retrospective Cohort Study

Simultaneous occurrence of autoimmune pancreatitis and pancreatic cancer in patients resected for focal pancreatic mass

Peter Macinga, Adela Pulkertova, Lukas Bajer, Jana Maluskova, Martin Oliverius, Martin Smejkal, Maria Heczkova, Julius Spicak, Tomas Hucl

Peter Macinga, Adela Pulkertova, Lukas Bajer, Julius Spicak, Tomas Hucl, Department of Gastroenterology and Hepatology, Institute for Clinical and Experimental Medicine, Prague 140 21, Czech Republic

Jana Maluskova, Department of Pathology, Institute for Clinical and Experimental Medicine, Prague 140 21, Czech Republic

Martin Oliverius, Department of Transplant Surgery, Institute for Clinical and Experimental Medicine, Prague 140 21, Czech Republic

Martin Smejkal, Department of Radiodiagnostics and Interventional Radiology, Institute for Clinical and Experimental Medicine, Prague 140 21, Czech Republic

Maria Heczkova, Center for Experimental Medicine, Institute for Clinical and Experimental Medicine, Prague 140 21, Czech Republic

Abstract

AIM
To assess the occurrence of autoimmune pancreatitis (AIP) in pancreatic resections performed for focal pancreatic enlargement.

METHODS
We performed a retrospective analysis of medical records of all patients who underwent pancreatic resection for a focal pancreatic enlargement at our

tertiary center from January 2000 to July 2013. The indication for surgery was suspicion of a tumor based on clinical presentation, imaging findings and laboratory evaluations. The diagnosis of AIP was based on histology findings. An experienced pathologist specialized in pancreatic disease reviewed all the cases and confirmed the diagnosis in pancreatic resection specimens suggestive of AIP. The histological diagnosis of AIP was set according to the international consensus diagnostic criteria.

RESULTS
Two hundred ninety-five pancreatic resections were performed in 201 men and 94 women. AIP was diagnosed in 15 patients (5.1%, 12 men and 3 women) based on histology of the resected specimen. Six of them had AIP type 1, nine were diagnosed with AIP type 2. Pancreatic adenocarcinoma (PC) was also present in six patients with AIP (40%), all six were men. Patients with AIP + PC were significantly older (60.5 *vs* 49 years of age, $P = 0.045$), more likely to have been recently diagnosed with diabetes (67% *vs* 11%, $P = 0.09$), and had experienced greater weight loss (15.5 kg *vs* 8.5 kg, $P = 0.03$) than AIP patients without PC. AIP was not diagnosed in any patients prior to surgery; however, the diagnostic algorithm was not fully completed in every case.

CONCLUSION
The possible co-occurrence of PC and AIP suggests that preoperative diagnosis of AIP does not rule out simultaneous presence of PC.

Key words: Chronic pancreatitis; Pancreatic cancer; IgG4-related disease; Autoimmune pancreatitis; Malignancy

Core tip: In this retrospective study we confirmed that a considerable proportion of patients undergoing pancreatic resection for tumor suspicion have autoimmune pancreatitis. Furthermore, we show here the largest ever published group of patients with pancreatic cancer and autoimmune pancreatitis co-occurrence. The possible synchronous occurrence of autoimmune pancreatitis and pancreatic cancer implies major clinical consequences as the preoperative diagnosis of autoimmune pancreatitis might not rule out pancreatic cancer. Patients with autoimmune pancreatitis and patients with autoimmune pancreatitis and pancreatic cancer differed in age at presentation, presence of diabetes and the extent of weight loss.

INTRODUCTION

Autoimmune pancreatitis (AIP) is a rare clinical entity with an estimated prevalence of 0.82-2.2/100000 inhabitants in Japan[1,2]. The prevalence of this disease in Western countries remains to be determined. AIP is diagnosed in only about 6% of patients with idiopathic chronic pancreatitis[3]. Within this group, AIP is defined by specific clinical, laboratory, radiological, and histological findings[4]. Currently, two subtypes of AIP are recognized. Type 1, also known as lymphoplasmacytic sclerosing pancreatitis, is considered a pancreatic manifestation of IgG4-related sclerosing disease. Type 2, idiopathic duct-centric pancreatitis, is often associated with inflammatory bowel disease. Type 1 disease is characterized by sclerosing storiform fibrosis with a lymphoplasmatic infiltrate rich in IgG4-positive plasma cells, and elevated serum IgG4 levels[5]. Type 2 disease is characterized by disruption of the duct wall due to invasion by neutrophilic granulocytes, *i.e.,* granulocytic epithelial lesions, absence of IgG4-positive plasma cells, and no serum elevation of IgG4. These characteristic changes may result in diffuse swelling or focal enlargement of the organ. Patients with AIP often present with jaundice, abdominal pain and focal pancreatic enlargement. The lack of specific symptoms makes the diagnosis of AIP difficult.

Diagnostic algorithms from Japan, South Korea and United States were proposed in 2006[6-8]. The international consensus diagnostic criteria (ICDC) published in 2011, unify the previous diagnostic strategies while respecting regional differences in clinical practice[4]. The ICDC are based on evaluation of the pancreatic parenchyma by imaging (CT, MRI), the structure of the pancreatic duct, histology, serology, involvement of other organs, and response to corticosteroid therapy. A typical, although not the most common, imaging finding is diffuse enlargement of the pancreas. This may be accompanied by delayed enhancement (sausage-like pancreas or rim-like enhancement); however, often only segmental or focal enlargement of the pancreas is seen, especially in AIP type 2[9,10]. Consequently, differentiating pancreatic cancer (PC) from AIP can be difficult and requires demonstration of a combination of clinical, serological, morphological and histological features. Despite the availability of well-defined diagnostic criteria, 6%-8% of patients with a pancreatic mass undergo unnecessary resection prior to a finding of autoimmune pancreatitis[11].

The aim of our study was to determine the proportion of patients at our center with AIP among those who had a pancreatic resection for a pancreatic mass and to define the clinical characteristics of this
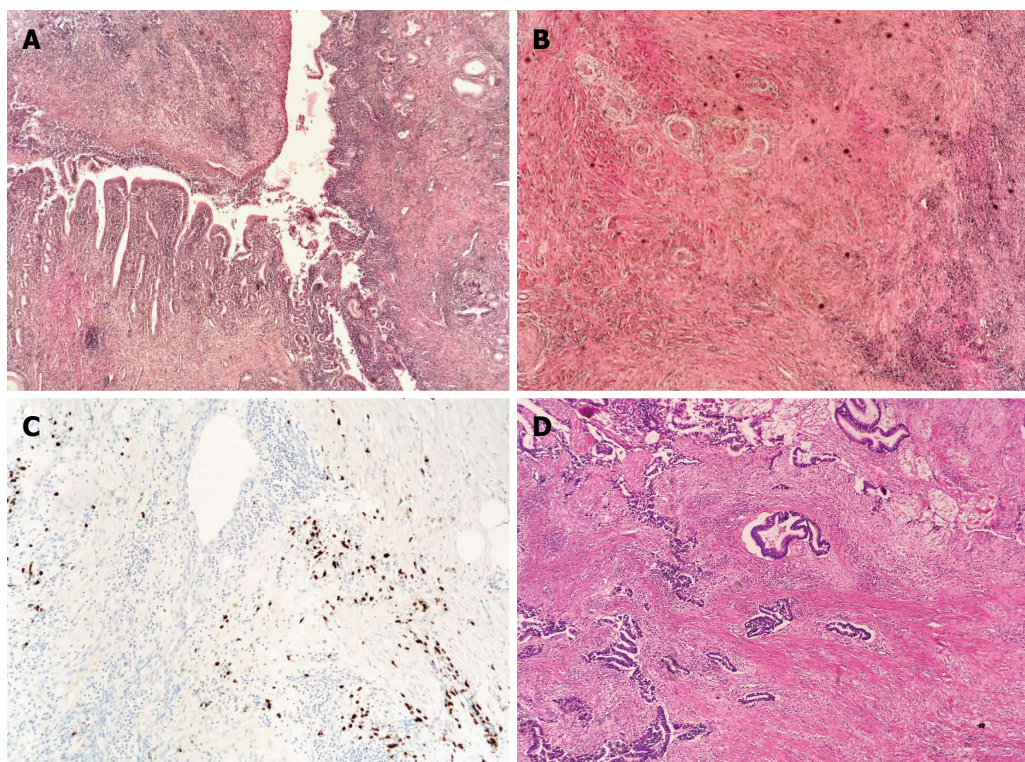
Figure 1 Histological findings in resected pancreatic tissue in a patient with synchronous presence of type 1 autoimmune pancreatitis and pancreatic cancer. A: Autoimmune pancreatitis (AIP), hematoxylin-eosin (HE) staining, original magnification × 40; B: AIP showing storiform fibrosis, HE staining, original magnification × 40; C: AIP with immunohistochemical staining of plasma cells for IgG4; D: Pancreatic cancer, HE staining, original magnification × 40.

subgroup.

MATERIALS AND METHODS

We retrospectively analyzed medical records of all patients who underwent pancreatic resection for a focal pancreatic enlargement at the Institute for Clinical and Experimental Medicine from January 2000 to July 2013. The indication for surgery was suspicion of a tumor based on clinical presentation, imaging findings and laboratory evaluations. Many patients were referred to our tertiary center for pancreatic surgery with a diagnostic workout already done in the referring hospital and with an established diagnosis of suspected pancreatic cancer.

The diagnosis of AIP was based on histology findings. An experienced pathologist (J.M.) specialized in pancreatic diseases (hundreds of PC and chronic pancreatitis cases reported) reviewed all the cases and confirmed the diagnosis in pancreatic resection specimens suggestive of AIP. The histological diagnosis of AIP was based on the ICDC criteria. In AIP type 1, the presence of storiform fibrosis, obliterative phlebitis, and abundant IgG4-positive plasma cells was required, granulocytic epithelial lesions were indicative of AIP type 2[4].

The Mann-Whitney U test was used for statistical analysis of quantitative data and the Fisher's exact test was used for qualitative data. A P-value of 0.05 was required for statistical significance. Data were analyzed by the center statistician using JMP 10 software (SAS Institute Inc., Cary, NC).

The study was performed according to Declaration of Helsinki including the changes accepted in Soul, South Korea, during the 59th WMA General Assembly.

RESULTS

During the study period, we performed a total of 295 pancreatic resections in 201 men (68%) and 94 women (32%) with a median age of 61 (36-78) years. Pathological examination of the resected specimens revealed AIP in 15 patients (5.1%); 12 men and 3 women with a median age of 57 (35-67) years. A diagnosis of AIP was considered, but not confirmed, in two of these patients prior to pancreatectomy. In 13 of those patients (87%), the indication for resection was preoperative focal enlargement in the pancreatic head; two patients had an expansion of the tail. Six patients (40%), all men with a median age of 53 (46-67) years, were diagnosed with AIP type 1. Nine patients (60%), six men and three women with a median age of 58 (35-64) years had pathological findings consistent with AIP type 2.

In six patients (40%) with AIP (two AIP type 1 and four AIP type 2), a PC was also present in the resected tissue (Figure 1A-D). In five patients the cancer was localized in the head of the pancreas and in one patient the pancreatic tail was affected. The characteristics of AIP patients with and without PC are

Table 1 Characteristics of patients with autoimmune pancreatitis and autoimmune pancreatitis + pancreatic cancer *n* (%)

	AIP without PC	AIP with PC	*P* value
Total	9 (60)	6 (40)	
AIP type 1	4 (44)	2 (33)	
AIP type 2	5 (56)	4 (67)	
Sex (males)	6 (67)	6 (100)	
Age	49 (35-64)	60.5 (54-67)	0.045
Smoking	5 (56)	4 (67)	
Recent onset of diabetes mellitus	1 (11)	4 (67)	0.090
History of another autoimmune disorder	4 (44)[1]	0	
History of pancreatic disease	5 (56)[2]	1 (17)[3]	
Jaundice	3 (33)	4 (67)	
Weight loss	6 (67)	6 (100)	
in kilograms	8.5 (3-12)	15.5 (8-50)	0.030
Location of lesion (head of the pancreas)	8 (89)	5 (83)	
Ca 19-9 (normal range 0-27 kU/L)	35.2 (2.5-300)	89.8 (19.8-110)	

[1] 1 × IgG4-related sclerosing cholangitis, 1 × IgG4-related sialadenitis, 1 × Crohn's disease, 1 × Autoimmune thyroiditis; [2] 2 × Chronic pancreatitis, 3 × Acute pancreatitis; [3] 1 × Chronic pancreatitis. Quantitative data are expressed as median (range), qualitative data as absolute values with percentages. AIP: Autoimmune pancreatitis; PC: Pancreatic cancer.

Table 2 Histopathology findings in patients with type 1 autoimmune pancreatitis + pancreatic cancer

Patient	Sex	Age	Periductal lymphoplasmacytic infiltrate without granulocytic infiltration	Obliterative phlebitis	Storiform fibrosis	IgG4-positive cells
2	M	67	Yes	Yes	Yes	47/HPF
6	M	61	Yes	Yes	Yes	58/HPF

HPF: High-power field.

Table 3 Histopathology findings in patients with type 2 autoimmune pancreatitis + pancreatic cancer

Patient	Sex	Age	Granulocytic infiltration of duct wall (GEL)	Granulocytic and lymphoplasmacytic acinar infiltrate	IgG4-positive cells
1	M	54	Yes		4/HPF
3	M	63	Yes		2/HPF
4	M	58	Yes		7/HPF
5	M	60	Yes		4/HPF

HPF: High-power field.

shown in Table 1. All patients with AIP and PC were men, and their median age was 60.5 (54-67) years. All patients with AIP + PC had a history of significant weight loss (median 15.5kg, range 8-50), which was greater than the weight loss present in the six AIP patients without PC (median 8.5kg, range 3-12, *P* = 0.03). Patients with AIP + PC were significantly older (median age 60.5 *vs* 49, *P* =0.045) and were more likely to have been diagnosed with recent-onset diabetes mellitus (within six months prior to resection) in the preoperative period (67% *vs* 11%, *P* = 0.09). History of smoking was similar in both groups (56% AIP patients *vs* 67% AIP + PC patients). There was not a statistically significant difference in the presence of jaundice between the groups. Histopathological findings in patients with AIP + PC are summarized in Tables 2 and 3.

Six patients with AIP had a history of pancreatic disease - three had chronic pancreatitis (two with AIP and one with AIP + PC), and three patients

with AIP alone had experienced an acute episode of pancreatitis of unspecified etiology. Four patients with AIP and none with AIP + PC had a history of other autoimmune diseases. Two patients with AIP type 1 had an involvement of other organs (IgG4-sclerosing cholangitis and sialadenitis) manifesting during postsurgical follow-up. One patient with AIP type 1 had autoimmune thyroiditis, and one patient with AIP type 2 had a history of Crohn's disease.

In eleven patients (seven with AIP and four with AIP + PC), a fine needle aspiration biopsy (FNAB) of the pancreatic lesion had been performed. Cytological examination of the aspirates from the AIP + PC patients was true positive in three and inconclusive in one. In those with AIP, the examination was true negative in four patients, false-positive in two, and inconclusive in one (Table 4).

Knowing the final histological diagnosis, we retrospectively evaluated the medical histories, imaging findings, and laboratory results of patients with

Table 4 Serum IgG4, imaging methods and fine needle aspiration biopsy results in patients with autoimmune pancreatitis and autoimmune pancreatitis + pancreatic cancer

	Sex	Age	Serum IgG4 (mg/dL)	CT	ERP	EUS	EUS-FNA
AIP type 1 + PC	M	67	N/A	A	N/A	N/A	N/A
	M	61	N/A	A	CBD stricture; no wirsungography	Susp M	Inconclusive
AIP type 1	M	46	81.5	L2	CBD stricture; no wirsungography	Ambigious	Negative
	M	57	81.5	A	CBD stricture; no wirsungography	N/A	N/A
	M	49	N/A	A	Unsuccesful attempt for wirsungography	Cystic tumour; signs of CHP	Inconclusive
	M	48	23.1	L2	N/A	Susp M	Negative
AIP type 2 + PC	M	54	NR	L2	N/A	Ambigious	Susp M
	M	63	NR	A	N/A	Ambigious	Susp M
	M	58	NR	A	Wirsungolithiasis	N/A	N/A
	M	60	NR	A	N/A	Ambigious	Susp M
AIP type 2	F	61	NR	L2	N/A	Susp M	Susp M
	F	64	NR	A	Dilated PD; mucous secretion	Susp MD-IPMN	Negative
	M	35	NR	L2	N/A	ambigious	Susp M
	F	47	NR	L2	N/A	ambigious	Negative
	M	53	NR	A	N/A	N/A	N/A

L2: Level 2 evidence of parenchymal imaging according to ICDC criteria; M: Male; F: Female; NR: Not relevant; N/A: Results not available or examination not done; A: Atypical-finding not suggestive of AIP; susp M: Findings suspected of malignancy; CHP: Chronic pancreatitis; CBD: Common bile duct; PD: Pancreatic duct; EUS: Endoscopic ultrasonography; EUS-FNA: Endoscopic ultrasound-guided fine needle aspiration biopsy; CT: Computed tomography; AIP: Autoimmune pancreatitis; PC: Pancreatic cancer.
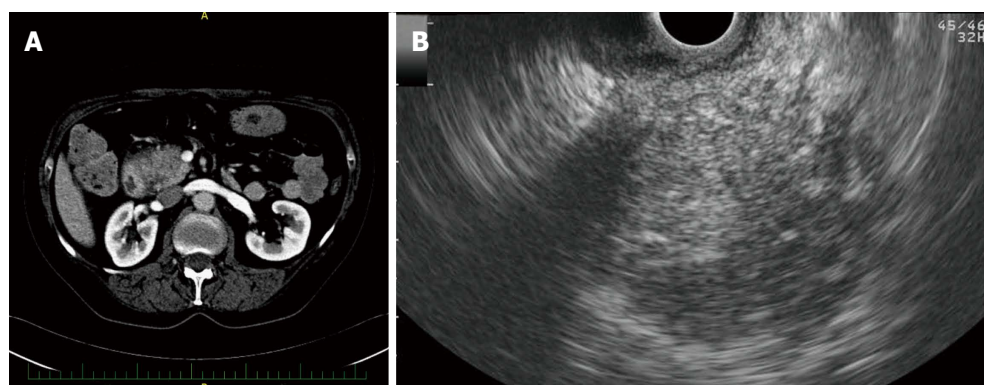
Figure 2 Imaging findings in a patient with autoimmune pancreatitis. A: Hypodense lesion in the pancreatic head on computed tomography; B: Hypoechoic lesion of the pancreatic head on endoscopic ultrasonography.

AIP + PC to assess the possibility of preoperative diagnosis of AIP using the current consensus criteria. None of the patients would have met the ICDC criteria preoperatively. Serum levels of IgG4 were not determined in the two patients with AIP type 1, and histology was not obtained in any of the AIP type 2 patients. Only one patient (with AIP type 2) had a CT finding suggestive of AIP, however malignant elements were found in the FNAB cytology. In the remaining five patients the CT findings would not have raised suspicion of AIP. Preoperative findings in both groups are shown in Table 4, Figures 2 and 3.

DISCUSSION

AIP and PC may present with similar manifestations, but have very different treatments. Typically, an older patient presents with abdominal pain and obstructive jaundice caused by a focal pancreatic lesion. If AIP is diagnosed, the mainstay of treatment is immunosuppression using corticosteroids, usually resulting in rapid regression of the expansion and alleviation of symptoms. This therapy spares the patient from a challenging surgical procedure associated with high morbidity and considerable mortality. On the other hand, if pancreatic cancer is the cause of symptoms, the only chance for survival is prompt surgical treatment. Such clinical cases represent a complex diagnostic dilemma. Precise differential diagnosis of AIP and PC is essential for the right treatment and prognosis of patients, but is sometimes extremely difficult, if not impossible,
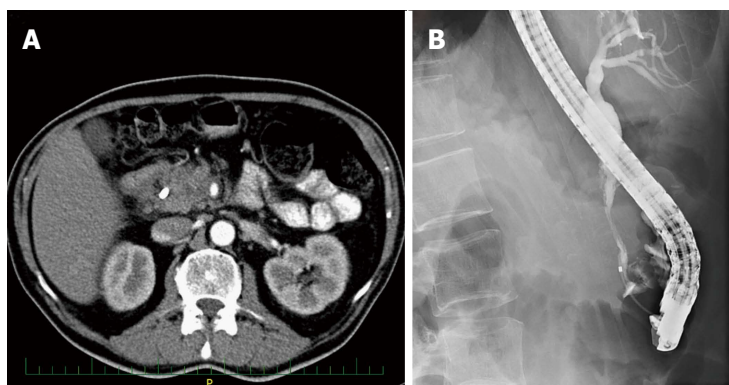
Figure 3 Imaging findings in a patient with autoimmune pancreatitis + pancreatic cancer. A: Hypodense lesion in the pancreatic head with a common bile duct (CBD) stent on computed tomography; B: Distal CBD stricture on endoscopic retrograde cholangio-pancreatography.

to determine. Serum markers of AIP (notably serum IgG4) are often helpful in diagnosis of both conditions[12]. However, IgG4 levels exceeding twice the upper limit of normal were found in a considerable proportion of patients with pancreatic cancer and thus this marker cannot be used alone to exclude the diagnosis of malignancy[13]. In cases where the presence of pancreatic cancer cannot be ruled out with certainty, pancreatic resection is indicated. The aim of our study was to evaluate the proportion of AIP in all patients undergoing resection for a suspected tumor. The finding of AIP in 5% of all resections in our patient series is in agreement with previously published data. The occurrence of AIP in patients resected for a suspected tumor has been shown to be around 6%-8%[11]. This high number reflects the similar presentation of both diseases and the difficult diagnostic algorithm of AIP.

The relatively high proportion of patients with type 2 AIP (60%) in our series might be explained by a higher prevalence of this subtype in our geographic region as well as more frequent presentation of type 2 AIP as a focal pancreatic lesion[14-16]. In addition, recognition of this subtype in the absence of a serological marker and extrapancreatic manifestations is more challenging. It can thus be assumed that this subtype will be found more often in patients with unrecognized AIP, and will not precisely match the characteristics of AIP patients in the general population.

An intriguing finding in our study is the high incidence of pancreatic adenocarcinoma in patients with AIP, which reached 40%. In our opinion, this represents the largest ever published group of patients with co-occurrence of PC and AIP. Pancreatic cancer in patients with AIP has so far been documented only in individual cases[13,17-20]. Recently, two patients with both AIP and PC were described in a study that examined serum IgG4 level in 106 patients with histologically confirmed pancreatic cancer[13]. None of our patients was diagnosed with AIP or had ever been given immunosuppressive therapy before surgery. A retrospective analysis of all available data revealed that our AIP patients would not have met the ICDC criteria

preoperatively. However, the diagnostic algorithm was not complete in any of them, as they were referred for surgery with an already established diagnosis of suspected pancreatic cancer. Nevertheless, it needs to be emphasized that some of our patients had been resected many years ago before AIP was thoroughly described and the ICDC criteria proposed.

Diagnosis of AIP accompanying PC based on histology is a major drawback of our study. We are aware that the nonspecific peritumoral pancreatitis adjacent to pancreatic neoplasms might share some histologic features with AIP type 1, *i.e.*, by abundance of IgG4+ plasma cells, venulitis or periductal inflammation[21]. However, distribution of IgG4+ plasma cells in nonspecific peritumoral pancreatitis was shown to be patchy, in contrary to diffuse infiltration which is described in AIP[22,23]. All cases of AIP + PC were reviewed by a pathologist specialized in pancreatic diseases. Only cases with diffuse distribution of IgG4+ plasma cells (density > 50/HPF) and with the presence of all morphologic features of AIP type 1 were included in the study. This cutoff was shown to provide an excellent specificity in distinguishing AIP type 1 and peritumoral pancreatitis[21]. In AIP type 2 the granulocytic epithelial lesions were nosognostic for the disease.

The relationship between AIP and PC is poorly understood although several different explanations were formulated. The first one considers AIP as a precursor for pancreatic cancer due to chronic inflammation which leads to harboring of mutations and, over time, to development of cancer. Chronic pancreatitis is a well-known risk factor of pancreatic cancer, increasing the risk of cancer development by as much as 13.3-fold[24]. The cumulative risk of developing pancreatic cancer in patients with chronic pancreatitis is estimated to be 4%[25]. A similar association in patients with AIP has not yet been demonstrated. However, in line with the case reports of pancreatic cancer in patients with AIP mentioned above, there are data that indirectly support this assumption. For example, Gupta *et al*[26] in a retrospective analysis of resected tissue of AIP patients, found a higher prevalence of premalignant lesions, *i.e.,* pancreatic intraepithelial neoplasia (PanIN

1-2), in patients with AIP compared with patients with otherwise nonspecified chronic pancreatitis. In addition, they noted development of pancreatic cancer in two of 84 patients with AIP during a prospective 49-mo follow-up period. The high frequency of K-ras mutations found in pancreatic tissue of patients with AIP further supports the association of the two diseases[27].

Higher incidence of pancreatic cancer has scarcely been reported in prospectively followed cohorts of patients with AIP[28]. However, population studies are usually limited by a small number of patients due to the low incidence of the disease and also by short follow-up periods. Furthermore, prospectively followed patients with AIP are usually adequately treated with immunosuppression, unlike patients with unrecognized AIP or with pancreatitis of other etiologies. In such a scenario, one might speculate that suppression of inflammatory activity may reduce the risk of malignancy development in a similar way to that seen in inflammatory bowel disease[29]. An increased incidence of pancreatic cancer would then be expected in untreated patients or in those with an insufficient response to immunosuppressive treatment. Duration of follow-up is also an important factor. If patients with chronic pancreatitis of other etiologies develop pancreatic cancer, then it is usually in the interval of one to two decades after chronic pancreatitis is diagnosed[25]. There are patients suffering for years from unrecognized AIP in the absence of cardinal symptoms such as jaundice or typical radiological findings. Recently published data suggest that up to one third of patients with AIP can develop signs typical of advanced chronic pancreatitis of other etiologies (*e.g.*, parenchymal atrophy or calcifications)[30,31].

Another consideration proposes AIP type 1 as a paraneoplastic phenomenon. This hypothesis is based on observation of a significantly higher incidence of malignancy in patients with IgG4-RD within the first year of follow-up compared to subsequent years[32,33]. The explanation would be that occult cancer may alter cell-mediated immunological responses and thus create an inflammatory environment favorable for onset of autoimmune disease - in this case AIP type 1 or any other IgG4-RD. However, these initial observations from Japanese authors were not further supported by western studies[34,35].

Despite the small number of patients in our study, we found three major differences between patients with AIP and AIP + PC, with one of them being statistically significant. The significantly higher age of patients with AIP + PC may be somewhat expected due to the mechanism of cancer development, presumed to be a long-term chronic inflammatory process. It is consistent with the concept of pancreatic cancer being a late complication of chronic pancreatitis, much like colorectal cancer being a late complication of ulcerative colitis. The higher proportion of recent onset diabetes in patients with AIP + PC compared with patients with AIP only is an interesting

finding. Diabetes mellitus has been reported in 42%-78% of patients at the onset of AIP, however it persisted in only 10% of the AIP patients following corticosteroid treatment of the acute inflammation[36]. In our study, recent-onset diabetes was present in only 11% patients with AIP. New onset diabetes mellitus as a symptom of pancreatic cancer has been documented in numerous studies[37]. However, its use in the differential diagnosis of cancer and chronic pancreatitis is difficult, because diabetes is a common complication of advanced chronic pancreatitis of non-autoimmune etiology. In our study only six patients without PC had weight loss as opposed to all patients with PC. Weight loss in patients with AIP and PC was much greater than in those who had AIP without PC (15.5 kg *vs* 8.5 kg, $P = 0.03$). Even though exocrine pancreatic insufficiency and weight loss are not uncommon in patients with AIP[38], a severe weight loss should raise suspicion for a possible presence of pancreatic cancer.

The possible synchronous occurrence of AIP and PC implies major clinical consequences. Our data indicate that distinguishing these two entities becomes even more challenging, as the preoperative diagnosis of AIP does not rule out pancreatic cancer. Even patients with an established diagnosis of AIP must thus be treated and followed with caution.

A shortcoming of our study, beyond its small size and retrospective nature, is the fact that the selection of patients was based on histological examination of resected tissue. Our hospital is a tertiary center that performs many resections as a service for regional gastroenterology facilities. Consequently, the opportunity to change the diagnostic algorithm, which is often not fully completed, is sometimes limited. Finally, the natural course of pancreatic cancer is so unfavorable that all our patients have already died. Consequently, they could not be revaluated.

Autoimmune pancreatitis and pancreatic cancer may have similar presentations and their distinction is often difficult. We evaluated all patients who underwent pancreatic resection for a focal pancreatic enlargement and found that a considerable proportion of the resected patients had autoimmune pancreatitis. Furthermore, we found that some patients with autoimmune pancreatitis also had pancreatic cancer, demonstrating the eventuality of synchronous presence of PC in patients with proven AIP. Our results show that those with AIP and cancer were older, more likely to have recent-onset diabetes and had a greater weight loss than those with AIP only. Definitive confirmation of these initial observations will require additional prospective studies with a larger number of patients.

COMMENTS

Background
Autoimmune pancreatitis (AIP) is a distinct form of chronic pancreatitis

characterized by specific clinical, laboratory, radiological and histological findings. AIP may mimic pancreatic cancer (PC), as it often presents with obstructive jaundice and focal pancreatic enlargement.

Research frontiers
Due to similar manifestation of AIP and PC, a lot of attention was given to the differentiation of the two conditions, as the precise differential diagnosis is essential for the right treatment and prognosis of patients. However, because the diagnosis of AIP is complex, many AIP patients undergo unnecessary surgery rather than immunosuppressive treatment. Chronic inflammatory process is a well-known risk factor of malignancy, as described in chronic pancreatitis and PC. A similar association in patients with AIP and PC has been suggested but not demonstrated. There are only a few cases of PC in AIP patients reported in the literature.

Innovations and breakthroughs
In the presented study, we show that a considerable proportion of patients undergoing pancreatic resection for a cancer suspicion may have AIP. However, we also showed that patients with AIP may have synchronous presence of pancreatic cancer. Those with AIP and PC were older, have been more often recently diagnosed with diabetes, and have experienced a greater weight loss than those without PC. The presented group of patients with PC and AIP co-occurrence is, to our knowledge, the largest ever published.

Applications
The possible synchronous occurrence of AIP and PC implies major consequences, as diagnosing AIP in a patient with focal pancreatic enlargement may not rule out the presence of pancreatic cancer. The knowledge of characteristics distinguishing the two groups of patients might aid in the differential diagnosis.

Terminology
Pancreatic cancer is usually an adenocarcinoma derived from pancreatic ductal cells; autoimmune pancreatitis is a rare chronic inflammatory disease of the pancreas defined by a combination of the following features: frequent presentation with obstructive jaundice accompanied with diffuse or focal organ swelling, rapid response to steroids, as well as by histological finding of lymphoplasmacytic infiltrate and fibrosis of the pancreas. Based on laboratory results, clinical profiling and histology, it is classified into type 1 and type 2.

Peer-review
"Simultaneous occurrence of autoimmune pancreatitis and pancreatic cancer in patients resected for focal pancreatic mass" is an interesting paper.

REFERENCES

1 **Nishimori I**, Tamakoshi A, Otsuki M. Prevalence of autoimmune pancreatitis in Japan from a nationwide survey in 2002. *J Gastroenterol* 2007; **42** Suppl 18: 6-8 [PMID: 17520216 DOI: 10.1007/s00535-007-2043-y]
2 **Kanno A**, Nishimori I, Masamune A, Kikuta K, Hirota M, Kuriyama S, Tsuji I, Shimosegawa T. Nationwide epidemiological survey of autoimmune pancreatitis in Japan. *Pancreas* 2012; **41**: 835-839 [PMID: 22466167 DOI: 10.1097/MPA.0b013e3182480c99]
3 **Pickartz T**, Mayerle J, Lerch MM. Autoimmune pancreatitis. *Nat Clin Pract Gastroenterol Hepatol* 2007; **4**: 314-323 [PMID: 17541445 DOI: 10.1038/ncpgasthep0837]
4 **Shimosegawa T**, Chari ST, Frulloni L, Kamisawa T, Kawa S, Mino-Kenudson M, Kim MH, Klöppel G, Lerch MM, Löhr M, Notohara K, Okazaki K, Schneider A, Zhang L. International consensus diagnostic criteria for autoimmune pancreatitis: guidelines of the International Association of Pancreatology. *Pancreas* 2011; **40**: 352-358 [PMID: 21412117 DOI: 10.1097/MPA.0b013e3182142fd2]
5 **Klöppel G**, Lüttges J, Löhr M, Zamboni G, Longnecker D. Autoimmune pancreatitis: pathological, clinical, and immunological features. *Pancreas* 2003; **27**: 14-19 [PMID: 12826900]
6 **Okazaki K**, Kawa S, Kamisawa T, Naruse S, Tanaka S, Nishimori I, Ohara H, Ito T, Kiriyama S, Inui K, Shimosegawa T, Koizumi M, Suda K, Shiratori K, Yamaguchi K, Yamaguchi T, Sugiyama M, Otsuki M. Clinical diagnostic criteria of autoimmune pancreatitis: revised proposal. *J Gastroenterol* 2006; **41**: 626-631 [PMID: 16932998 DOI: 10.1007/s00535-006-1868-0]
7 **Kim KP**, Kim MH, Kim JC, Lee SS, Seo DW, Lee SK. Diagnostic criteria for autoimmune chronic pancreatitis revisited. *World J Gastroenterol* 2006; **12**: 2487-2496 [PMID: 16688792 DOI: 10.3748/wjg.v12.i16.2487]
8 **Chari ST**, Smyrk TC, Levy MJ, Topazian MD, Takahashi N, Zhang L, Clain JE, Pearson RK, Petersen BT, Vege SS, Farnell MB. Diagnosis of autoimmune pancreatitis: the Mayo Clinic experience. *Clin Gastroenterol Hepatol* 2006; **4**: 1010-106; quiz 934 [PMID: 16843735]
9 **Sah RP**, Chari ST. Autoimmune pancreatitis: an update on classification, diagnosis, natural history and management. *Curr Gastroenterol Rep* 2012; **14**: 95-105 [PMID: 22350841 DOI: 10.1007/s11894-012-0246-8]
10 **Frulloni L**, Scattolini C, Falconi M, Zamboni G, Capelli P, Manfredi R, Graziani R, D'Onofrio M, Katsotourchi AM, Amodio A, Benini L, Vantini I. Autoimmune pancreatitis: differences between the focal and diffuse forms in 87 patients. *Am J Gastroenterol* 2009; **104**: 2288-2294 [PMID: 19568232 DOI: 10.1038/ajg.2009.327]
11 **Sugumar A**, Chari S. Autoimmune pancreatitis: an update. *Expert Rev Gastroenterol Hepatol* 2009; **3**: 197-204 [PMID: 19351289 DOI: 10.1586/egh.09.2]
12 **Morselli-Labate AM**, Pezzilli R. Usefulness of serum IgG4 in the diagnosis and follow up of autoimmune pancreatitis: A systematic literature review and meta-analysis. *J Gastroenterol Hepatol* 2009; **24**: 15-36 [PMID: 19067780 DOI: 10.1111/j.1440-1746.2008.05676.x]
13 **Bojková M**, Dítě P, Dvořáčková J, Novotný I, Floreánová K, Kianička B, Uvírová M, Martínek A. Immunoglobulin G4, autoimmune pancreatitis and pancreatic cancer. *Dig Dis* 2015; **33**: 86-90 [PMID: 25531501 DOI: 10.1159/000368337]
14 **Hart PA**, Kamisawa T, Brugge WR, Chung JB, Culver EL, Czakó L, Frulloni L, Go VL, Gress TM, Kim MH, Kawa S, Lee KT, Lerch MM, Liao WC, Löhr M, Okazaki K, Ryu JK, Schleinitz N, Shimizu K, Shimosegawa T, Soetikno R, Webster G, Yadav D, Zen Y, Chari ST. Long-term outcomes of autoimmune pancreatitis: a multicentre, international analysis. *Gut* 2013; **62**: 1771-1776 [PMID: 23232048 DOI: 10.1136/gutjnl-2012-303617]
15 **Sah RP**, Chari ST, Pannala R, Sugumar A, Clain JE, Levy MJ, Pearson RK, Smyrk TC, Petersen BT, Topazian MD, Takahashi N, Farnell MB, Vege SS. Differences in clinical profile and relapse rate of type 1 versus type 2 autoimmune pancreatitis. *Gastroenterology* 2010; **139**: 140-148; quiz e12-13 [PMID: 20353791 DOI: 10.1053/j.gastro.2010.03.054]
16 **Kamisawa T**, Chari ST, Giday SA, Kim MH, Chung JB, Lee KT, Werner J, Bergmann F, Lerch MM, Mayerle J, Pickartz T, Lohr M, Schneider A, Frulloni L, Webster GJ, Reddy DN, Liao WC, Wang HP, Okazaki K, Shimosegawa T, Kloeppel G, Go VL. Clinical profile of autoimmune pancreatitis and its histological subtypes: an international multicenter survey. *Pancreas* 2011; **40**: 809-814 [PMID: 21747310 DOI: 10.1097/MPA.0b013e3182258a15]
17 **Ghazale A**, Chari S. Is autoimmune pancreatitis a risk factor for pancreatic cancer? *Pancreas* 2007; **35**: 376 [PMID: 18090248 DOI: 10.1097/MPA.0b013e318073ccb8]
18 **Fukui T**, Mitsuyama T, Takaoka M, Uchida K, Matsushita M, Okazaki K. Pancreatic cancer associated with autoimmune pancreatitis in remission. *Intern Med* 2008; **47**: 151-155 [PMID: 18239323]
19 **Loos M**, Esposito I, Hedderich DM, Ludwig L, Fingerle A, Friess H, Klöppel G, Büchler P. Autoimmune pancreatitis complicated by carcinoma of the pancreatobiliary system: A case report and review of the literature. *Pancreas* 2011; **40**: 151-154

20   **Chandrasegaram MD**, Chiam SC, Nguyen NQ, Ruszkiewicz A, Chung A, Neo EL, Chen JW, Worthley CS, Brooke-Smith ME. A case of pancreatic cancer in the setting of autoimmune pancreatitis with nondiagnostic serum markers. *Case Rep Surg* 2013; **2013**: 809023 [PMID: 23781378 DOI: 10.1155/2013/809023]

21   **Dhall D**, Suriawinata AA, Tang LH, Shia J, Klimstra DS. Use of immunohistochemistry for IgG4 in the distinction of autoimmune pancreatitis from peritumoral pancreatitis. *Hum Pathol* 2010; **41**: 643-652 [PMID: 20149413 DOI: 10.1016/j.humpath.2009.10.019]

22   **Deshpande V**, Chicano S, Finkelberg D, Selig MK, Mino-Kenudson M, Brugge WR, Colvin RB, Lauwers GY. Autoimmune pancreatitis: a systemic immune complex mediated disease. *Am J Surg Pathol* 2006; **30**: 1537-1545 [PMID: 17122509 DOI: 10.1097/01.pas.0000213331.09864.2c]

23   **Zhang L**, Notohara K, Levy MJ, Chari ST, Smyrk TC. IgG4-positive plasma cell infiltration in the diagnosis of autoimmune pancreatitis. *Mod Pathol* 2007; **20**: 23-28 [PMID: 16980948 DOI: 10.1038/modpathol.3800689]

24   **Lowenfels AB**, Maisonneuve P, Cavallini G, Ammann RW, Lankisch PG, Andersen JR, Dimagno EP, Andrén-Sandberg A, Domellöf L. Pancreatitis and the risk of pancreatic cancer. International Pancreatitis Study Group. *N Engl J Med* 1993; **328**: 1433-1437 [PMID: 8479461 DOI: 10.1056/NEJM199305203282001]

25   **Raimondi S**, Lowenfels AB, Morselli-Labate AM, Maisonneuve P, Pezzilli R. Pancreatic cancer in chronic pancreatitis; aetiology, incidence, and early detection. *Best Pract Res Clin Gastroenterol* 2010; **24**: 349-358 [PMID: 20510834 DOI: 10.1016/j.bpg.2010.02.007]

26   **Gupta R**, Khosroshahi A, Shinagare S, Fernandez C, Ferrone C, Lauwers GY, Stone JH, Deshpande V. Does autoimmune pancreatitis increase the risk of pancreatic carcinoma?: a retrospective analysis of pancreatic resections. *Pancreas* 2013; **42**: 506-510 [PMID: 23271394 DOI: 10.1097/MPA.0b013e31826bef91]

27   **Kamisawa T**, Tsuruta K, Okamoto A, Horiguchi S, Hayashi Y, Yun X, Yamaguchi T, Sasaki T. Frequent and significant K-ras mutation in the pancreas, the bile duct, and the gallbladder in autoimmune pancreatitis. *Pancreas* 2009; **38**: 890-895 [PMID: 19752775 DOI: 10.1097/MPA.0b013e3181b65a1c]

28   **Ikeura T**, Miyoshi H, Uchida K, Fukui T, Shimatani M, Fukui Y, Sumimoto K, Matsushita M, Takaoka M, Okazaki K. Relationship between autoimmune pancreatitis and pancreatic cancer: a single-center experience. *Pancreatology* 2014; **14**: 373-379 [PMID: 25278307 DOI: 10.1016/j.pan.2014.04.029]

29   **Velayos FS**, Terdiman JP, Walsh JM. Effect of 5-aminosalicylate use on colorectal cancer and dysplasia risk: a systematic review and metaanalysis of observational studies. *Am J Gastroenterol* 2005; **100**: 1345-1353 [PMID: 15929768]

30   **Maire F**, Le Baleur Y, Rebours V, Vullierme MP, Couvelard A, Voitot H, Sauvanet A, Hentic O, Lévy P, Ruszniewski P, Hammel P. Outcome of patients with type 1 or 2 autoimmune pancreatitis. *Am J Gastroenterol* 2011; **106**: 151-156 [PMID: 20736934 DOI: 10.1038/ajg.2010.314]

31   **Maruyama M**, Watanabe T, Kanai K, Oguchi T, Asano J, Ito T, Ozaki Y, Muraki T, Hamano H, Arakura N, Kawa S. Autoimmune pancreatitis can develop into chronic pancreatitis. *Orphanet J Rare Dis* 2014; **9**: 77 [PMID: 24884922 DOI: 10.1186/1750-1172-9-77]

32   **Shiokawa M**, Kodama Y, Yoshimura K, Kawanami C, Mimura J, Yamashita Y, Asada M, Kikuyama M, Okabe Y, Inokuma T, Ohana M, Kokuryu H, Takeda K, Tsuji Y, Minami R, Sakuma Y, Kuriyama K, Ota Y, Tanabe W, Maruno T, Kurita A, Sawai Y, Uza N, Watanabe T, Haga H, Chiba T. Risk of cancer in patients with autoimmune pancreatitis. *Am J Gastroenterol* 2013; **108**: 610-617 [PMID: 23318486 DOI: 10.1038/ajg.2012.465]

33   **Asano J**, Watanabe T, Oguchi T, Kanai K, Maruyama M, Ito T, Muraki T, Hamano H, Arakura N, Matsumoto A, Kawa S. Association Between Immunoglobulin G4-related Disease and Malignancy within 12 Years after Diagnosis: An Analysis after Longterm Followup. *J Rheumatol* 2015; **42**: 2135-2142 [PMID: 26472416 DOI: 10.3899/jrheum.150436]

34   **Hart PA**, Law RJ, Dierkhising RA, Smyrk TC, Takahashi N, Chari ST. Risk of cancer in autoimmune pancreatitis: a case-control study and review of the literature. *Pancreas* 2014; **43**: 417-421 [PMID: 24622072 DOI: 10.1097/MPA.0000000000000053]

35   **Huggett MT**, Culver EL, Kumar M, Hurst JM, Rodriguez-Justo M, Chapman MH, Johnson GJ, Pereira SP, Chapman RW, Webster GJ, Barnes E. Type 1 autoimmune pancreatitis and IgG4-related sclerosing cholangitis is associated with extrapancreatic organ failure, malignancy, and mortality in a prospective UK cohort. *Am J Gastroenterol* 2014; **109**: 1675-1683 [PMID: 25155229 DOI: 10.1038/ajg.2014.223]

36   **Nishimori I**, Tamakoshi A, Kawa S, Tanaka S, Takeuchi K, Kamisawa T, Saisho H, Hirano K, Okamura K, Yanagawa N, Otsuki M. Influence of steroid therapy on the course of diabetes mellitus in patients with autoimmune pancreatitis: findings from a nationwide survey in Japan. *Pancreas* 2006; **32**: 244-248 [PMID: 16628078 DOI: 10.1097/01.mpa.0000202950.02988.07]

37   **Pannala R**, Basu A, Petersen GM, Chari ST. New-onset diabetes: a potential clue to the early diagnosis of pancreatic cancer. *Lancet Oncol* 2009; **10**: 88-95 [PMID: 19111249 DOI: 10.1016/S1470-2045(08)70337-1]

38   **Buijs J**, Cahen DL, van Heerde MJ, Rauws EA, de Buy Wenniger LJ, Hansen BE, Biermann K, Verheij J, Vleggaar FP, Brink MA, Beuers UH, van Buuren HR, Bruno MJ. The Long-Term Impact of Autoimmune Pancreatitis on Pancreatic Function, Quality of Life, and Life Expectancy. *Pancreas* 2015; **44**: 1065-1071 [PMID: 26355549 DOI: 10.1097/MPA.0000000000000451]

Retrospective Cohort Study

Endosonographic surveillance of 1-3 cm gastric submucosal tumors originating from muscularis propria

Ming-Luen Hu, Keng-Liang Wu, Chi-Sin Changchien, Seng-Kee Chuah, Yi-Chun Chiu

Ming-Luen Hu, Keng-Liang Wu, Chi-Sin Changchien, Seng-Kee Chuah, Yi-Chun Chiu, Division of Hepato-Gastroenterology, Department of Internal Medicine, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, Kaohsiung 833, Taiwan

Correspondence to: Yi-Chun Chiu, MD, Division of Hepato-Gastroenterology, Department of Internal Medicine, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, 123 Ta-Pei Road, Niao-Song District, Kaohsiung 833, Taiwan. chiuku@ms14.hinet.net
Telephone: +886-7-7317123
Fax: +886-7-7322402

Abstract

AIM
To observe the natural course of 1-3 cm gastric submucosal tumors originating from the muscularis propria (SMTMPs).

METHODS
By reviewing the computerized medical records over a period of 14 years (2000-2013), patients with 1-3 cm gastric SMTMPs who underwent at least two endoscopic ultrasound (EUS) examinations were enrolled. Tumor progression was defined as a $\geq$ 1.2 times enlargement in tumor diameter observed during EUS surveillance. All patients were divided into stationary and progressive subgroups and further analyzed. We also reviewed the patients in the progressive subgroup again in 2016.

RESULTS
A total of 88 patients were studied, including 25 in the progressive subgroup. The mean time of EUS surveillance was 24.6 mo in the stationary subgroup and 30.7 mo in the progressive subgroup. Risk factors for tumor progression included larger tumor size and irregular border. Initial tumor size > 14.0 mm may be considered a cut-off size for predicting tumor progression. Seventeen patients underwent surgery, of whom 13 had gastrointestinal stromal tumors (GISTs) and 4 had leiomyomas. Tumor progression was found only in patients with GISTs. All of the tumors exhibited benign behaviors without metastasis until 2016.

CONCLUSION
Most 1-3 cm gastric SMTMPs (71.6%) are indolent. Tumor progression was found only in GISTs, and it is a good predictor for differentiating GISTs from leiomyomas. Predictors of tumor progression include

larger tumor size (> 14.0 mm) and irregular border.

Core tip: Most gastric submucosal tumors originating from muscularis proprias (SMTMPs) are gastrointestinal stromal tumors (GISTs) or leiomyomas. GISTs have a malignant potential but leiomyomas are benign. We enrolled patients with 1-3 cm gastric SMTMPs and under endoscopic ultrasound surveillance over a period of 14 years between 2000 and 2013 to observe the natural behaviors of such tumors. We also reviewed the patients with progressive tumors again in 2016.

INTRODUCTION

Due to advances in endoscopy and its widespread use, detection of submucosal tumors (SMTs) of the gastrointestinal (GI) tract is not uncommon. In the evaluation of SMTs of the GI tract, endoscopic ultrasound (EUS) is a useful tool for identifying the tumor's layer of origin, measuring its size, providing the details of tumor echotexture, and differentiating it from external compression[1]. Among SMTs in the stomach, gastrointestinal stromal tumors (GISTs) are the most common[2]. When EUS reveals a hypoechoic submucosal tumor originating from the muscularis propria (SMTMP) in the stomach, GIST is considered first followed by leiomyoma[3-9]. Because all GISTs have a malignant potential and leiomyomas have a benign nature, tissue acquisition is often recommended for such tumors. At present, EUS-guided fine needle aspiration (EUS-FNA) is a feasible method. However, the diagnostic rate may be limited when the tumor is smaller or the tumor location is difficult to approach[10-12].

Based on the National Institute of Health Consensus, tumor size and mitotic activity are the two most important factors for predicting malignant potential of a GIST[13]. Obviously, tissue obtained by EUS-FNA can demonstrate GISTs only but cannot provide further information regarding mitotic activity. EUS features suggestive of a malignant GIST include larger tumor size, heterogeneous hypoechotexure, irregular tumor border, and internal cystic or calcified changes[8,14,15]. At present, a GIST > 3 cm is considered to have higher malignant potential and is recommended for surgical resection[16]. As for GISTs < 1 cm, they are frequently considered to harbor a low risk of malignancy and tissue acquisition in these cases is controversial[17]. Notably, GISTs in the stomach are often indolent and rapid progression is uncommon. It should be considered whether all the myogenic submucosal tumors in the stomach are necessary for pathologic demonstration to differentiate GISTs from leiomyomas, especially in 1-3 cm tumors. Until now, associated discussions regarding the natural course and management of 1-3 cm gastric SMTMPs are limited. Here, we reviewed computerized medical records over a period of 14 years from our institution to study the natural behaviors of such tumors.

MATERIALS AND METHODS

Patient selection
All the patients who underwent at least two EUS examinations to follow gastric SMTMP during a period of 14 years between January 2000 and December 2013 were retrospectively reviewed using the computerized medical record system of Kaohsiung Chang Gung Memorial Hospital, a tertiary medical center in Kaohsiung City in Taiwan.

EUS modality and examination
In all patients, EUS was performed using a miniprobe with a 12 MHz radial scan (Olympus UM-2R, Tokyo, Japan). When EUS showed a myogenic tumor with hypoechoic echotexture originating from the muscularis propria in the stomach, it was regarded as a gastric GIST first or leiomyoma. We used the maximal tumor diameter as tumor size. The intervals of EUS follow-up were not defined, mainly depending upon the clinician's discretion.

Inclusion and exclusion criteria
If the tumor size exceeded 3 cm, we recommended FNA or surgical resection. When a tumor was < 1 cm, we considered it to be benign. Therefore, we excluded the patients with an initial tumor size larger than 3 cm or persistently smaller than 1 cm. We also excluded the patients who underwent EUS only once without subsequent follow-up. We also enrolled the patients whose small tumors subsequently grew to 1 cm or more during surveillance. Therefore, only the patients with 1-3 cm myogenic tumors under EUS surveillance were enrolled in this study.

Pathological classification to predict malignant potential of GISTs
If a patient underwent surgery to remove a GIST, the pathology of GIST was classified into "very low risk", "low risk", "intermediate risk", or "high risk" using tumor size and mitotic count based on the National Institute of Health consensus[13].

Data collection and analysis
We defined a ratio of follow-up tumor size to initial
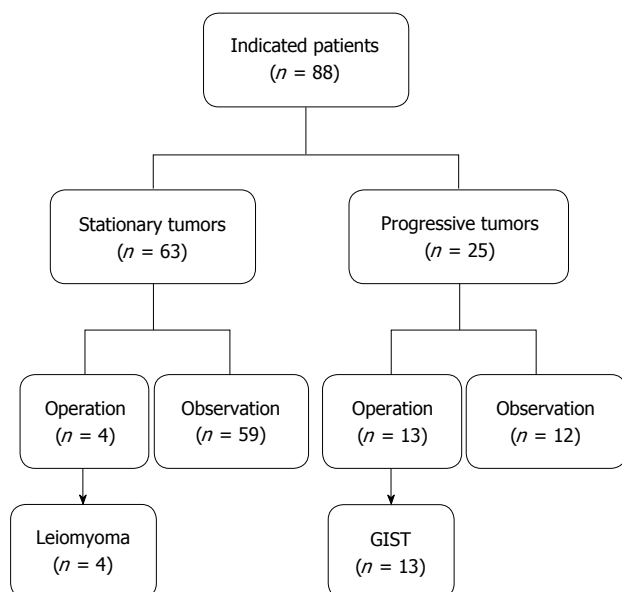
Figure 1 Flow chart of management of 88 indicated patients with submucosal tumors originating from the muscularis propria in the stomach. EUS: Endoscopic ultrasound; GIST: Gastrointestinal stromal tumor.

tumor size $\geqslant$ 1.2 as tumor progression based on the Response Evaluation Criteria in Solid Tumor (RECIST)[18]. Patients were then divided into a progressive subgroup and a stationary subgroup. Baseline characteristics of each subgroup, initial tumor size, echotexture, border and location of myogenic tumors, the number of surveillance procedures, and the interval and duration of EUS were recorded and further analyzed.

Second review for patients with progressive tumors
We followed the patients in the progressive subgroup again in 2016 by medical record review and phone call contact.

Statistical analysis
Continuous variables were analyzed using the Mann Whitney U test and categorical variables analyzed using the Pearson χ^2 test. The sensitivity and specificity of various tumor sizes were analyzed using a receiver operating characteristic (ROC) curve, and the optimal cutoff value was determined. All statistical analyses were performed using SPSS statistical software (SPSS for Windows, version 13; SPSS Inc., IL). A P-value < 0.05 was considered statistically significant.

RESULTS
During the 14 years between 2000 and 2013, 6755 EUS procedures were performed by four endosono-graphers. Of these, 1725 EUS results were associated with gastric SMTMPs. Based on the inclusion and exclusion criteria, 88 patients (44 males and 44 females) were identified and enrolled in the study. The initial patient age was 57.1 ± 11.0 years (mean ± SD) and the initial tumor size was 14.7 ± 4.9 mm.
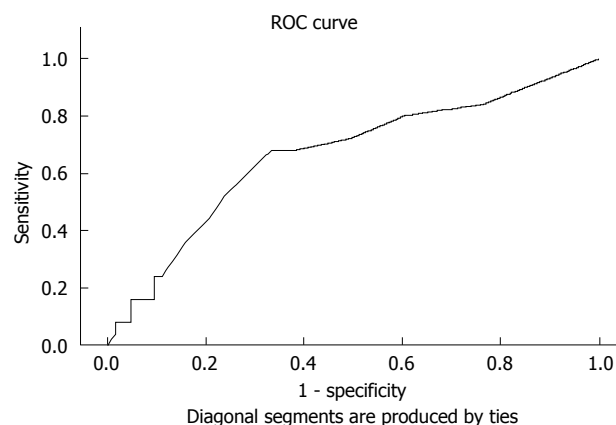
Figure 2 Receiver operating characteristic curve analysis of tumor size for predicting potential tumor progression. Initial tumor size of 1.4 cm was determined as the optimal cut-off size, with a sensitivity of 68.0%, a specificity of 66.7%, and an accuracy of 67.0%.
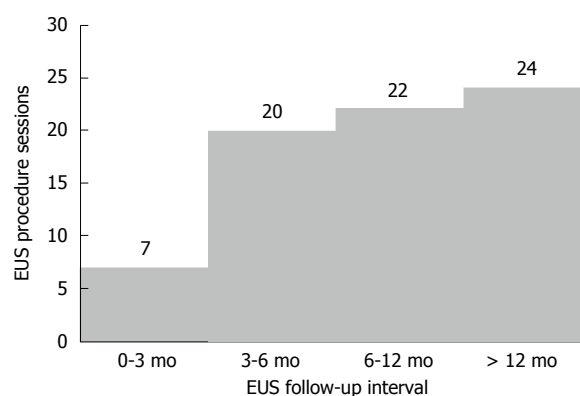
Figure 3 Intervals of endoscopic ultrasound follow-up in 25 patients with 1-3 cm gastric submucosal tumors originating from the muscularis propria in tumor progression. EUS: Endoscopic ultrasound.

Both the duration and interval of EUS surveillance ranged from 1.1 mo to 144.9 mo. The number of EUS surveillance procedures ranged from 2 to 9. Of the 88 patients, 25 (28.4%) were in the progressive subgroup and 63 (71.6%) in the stationary subgroup (Figure 1). The basic characteristics and EUS findings in each subgroup are shown in Table 1. By comparing the progressive and stationary subgroups, initially larger tumor size and irregular tumor border were identified to be predictors of tumor progression. Regarding initial tumor size, we performed an ROC curve analysis to determine the optimal cut-off size for predicting potential tumor progression. We found 1.4 cm to be the optimal cut-off tumor size associated with tumor progression, with a sensitivity of 68.0%, a specificity of 66.7%, and an accuracy of 67.0 % (Figure 2). The interval of EUS surveillance in the progressive subgroup is shown in Figure 3. The interval of most EUS examinations was $\geqslant$ 3 mo (66/73 = 90.4%). A total of 17 patients underwent surgery. Of these, 13 patients from the progressive subgroup were confirmed to have GISTs and 4 patients from
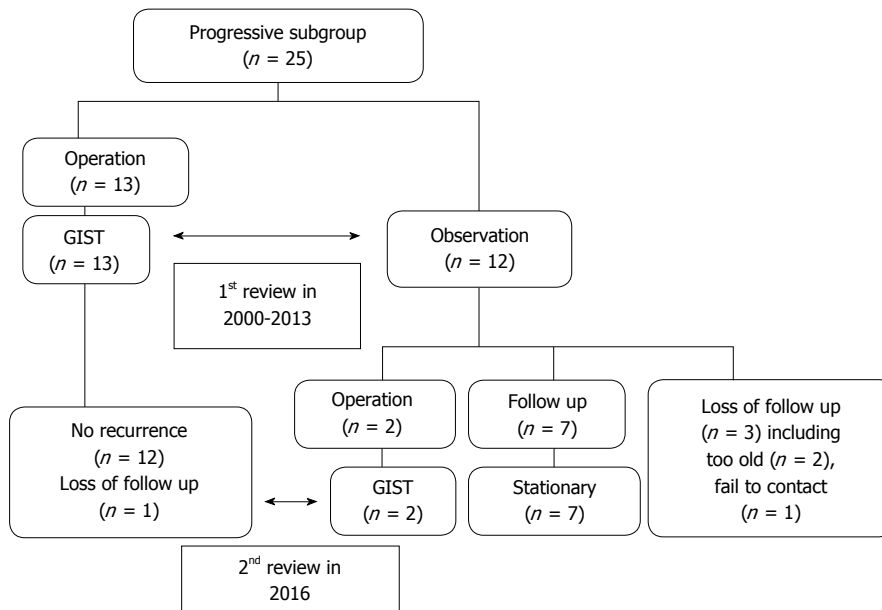
Figure 4 Flow chart of patients in the progressive subgroup. These patients were reviewed twice; the first was based on medical records in 2013 and the second was performed by phone calls as well as based on medical records in 2016.

Table 1 Basic characteristics and endoscopic ultrasound findings in 88 patients with suspected gastrointestinal stromal tumors in the stomach			
Basic characteristic or EUS finding	Stationary group $n = 63$	Progressive group $n = 25$	*P* value
Age (mean ± SD, yr)	57.4 ± 10.6	56.4 ± 12.4	0.690
Sex (M/F)	35/28	9/16	0.100
Location			0.650
Cardia	16	5	
Fundus	16	8	
Body	24	11	
Antrum	7	1	
EUS tumor size and echotexture			
Initial tumor size (mean ± SD, mm)	13.9 ± 4.5	16.6 ± 5.5	0.020
Homogeneous/ heterogeneous hypoechoicity	44/19	12/13	0.060
Smooth/irregular tumor border	56/7	15/10	0.002
With/without internal cystic change or calcification	8/55	4/21	0.680
EUS surveillance			
Surveillance duration (mean ± SD, mo)	24.6 ± 20.3	30.7 ± 21.7	0.220

EUS: Endoscopic ultrasound.

the stationary subgroup were confirmed to have leiomyomas. Basic characteristics and EUS findings for patients with confirmed GISTs and leiomyomas are shown in Tables 2-4. CD117 was positive in all 13 patients with confirmed GISTs (100%), whereas CD34 was positive in 11 (84.6%). Pathology results for confirmed cases suggested 4 GISTs with a very low malignant potential, 6 with a low potential, 2 with an intermediate potential, and 1 with a high potential. No patient was found to have malignant transformation or distant metastasis during surveillance. Notably, tumor progression (tumor enlargement $\geq$ 1.2 times) was only shown in the cases with GISTs. Among another 12 patients in the progressive subgroup, we followed them until 2016. Two patients eventually underwent surgery due to gradually enlarged tumors and were confirmed to have GISTs with a low malignant potential. Two patients refused EUS surveillance due to old age (> 80 years). Seven patients who took regular follow-ups remained condition stable without tumor metastasis. One patient was lost to follow-up. The flow chart of these 12 patients in the progressive subgroup is shown in Figure 4.

DISCUSSION

GISTs are the most common mesenchymal tumors in the GI tract. Pathologically, most GISTs are composed of spindle cells and epithelioid cells which are derived from interstitial cells of Cajal[19-21]. Most GISTs (approximately 65%) occur in the stomach, followed by 30%-35% in the small intestine and 5%-10% in the colon. About 95% of GISTs are characterized by the positive expression of c-kit receptor tyrosine kinase (CD117), whereas approximately 60%-70% of the tumors are positive for CD34[22-24]. Most gastric GISTs are asymptomatic and are detected incidentally as submucosal tumors during endoscopy. Therefore, the real incidence of GISTs in the stomach remains unclear. EUS is the most common modality for the evaluation of submucosal tumors. A suspected GIST is a hypoechoic and myogenic tumor originating mostly from the muscularis propria and occasionally from the muscularis mucosae. Similar to GISTs in terms of

Table 2 Basic characteristics and endoscopic ultrasound findings in13 patients with confirmed gastrointestinal stromal tumors in the stomach

Case	Age (yr)/ sex	Location	Heterogeneous hypoechoic echotexture	Irregular border	Internal cystic change or calcification	Initial size (*I*, mm)	Final size (*F*, mm)	Tumor progression (*F/I* $\geq$ 1.2)	Surveillance procedures	Surveillance duration (mo)	Malignant potential
1	41/F	Body	+	-	-	15	23	+	4	82.1	Very low
2	67/F	Fundus	+	-	+	15	23	+	5	66.5	Very low
3	50/F	Cardia	-	+	-	16	20	+	4	22.8	Very low
4	70/M	Body	-	-	-	15	20	+	8	37.9	Very low
5	57/F	Cardia	+	+	-	28	50	+	3	19.3	Low
6	46/M	Fundus	+	+	-	30	35	+	2	3.4	Low
7	55/F	Antrum	-	-	-	18	23	+	2	63.0	Low
8	69/F	Body	-	-	-	21	28	+	2	3.7	Low
9	49/M	Body	+	+	-	24	30	+	3	47.9	Low
10	61/F	Fundus	+	+	-	24	33	+	6	41.9	Low
11	54/M	Body	+	+	-	21	28	+	5	32.1	Intermediate
12	59/F	Body	+	+	-	18	23	+	2	5.5	Intermediate
13	60/F	Fundus	+	+	-	30	51	+	2	31.3	High

Table 3 Basic characteristics and endoscopic ultrasound findings in 4 patients with confirmed leiomyomas in the stomach

Case	Age (yr)/ sex	Location	Heterogeneous hypoechoic echotexture	Irregular border	Internal cystic change or calcification	Initial size (*I*, mm)	Final size (*F*, mm)	Tumor progression (*F/I* $\geq$ 1.2)	Surveillance procedures	Surveillance duration (mo)
1	69/F	Body	-	-	-	10	10	-	2	3.5
2	52/M	Fundus	-	-	-	10	9	-	2	3.7
3	64/F	Antrum	+	-	-	13	13	-	3	21.3
4	50/M	Cardia	+	+	+	18	20	-	2	3.0

Table 4 Comparison of basic characteristics and endoscopic ultrasound findings between patients with gastrointestinal stromal tumors and leiomyomas by the Mann -Whitney *U* test

Basic characteristic or EUS finding	GIST *n* = 13	Leiomyoma *n* = 4	*P* value
Age (median, range, yr)	57 (41-70)	58 (50-69)	0.785
Sex (M/F)	4/9	2/2	0.482
Location			0.868
Cardia	2	1	
Fundus	4	1	
Body	6	1	
Antrum	1	1	
EUS tumor size and echotexture			
Initial tumor size (median, mm)	21	11.5	0.015
Final tumor size (median, mm)	28	11.5	0.003
Homogeneous/heterogeneous hypoechoicity	4/9	2/2	0.482
Smooth/ irregular tumor border	5/8	0/4	0.682
With/without internal cystic change or calcification	1/12	0/4	0.567
EUS surveillance			
Surveillance duration (median, range, mo)	31.3 (3.1-81.0)	3.6 (3.0-21.4)	0.023
Surveillance procedure (median, range, times)	3 (2-8)	2 (2-3)	0.163
Tumor progression	13	0	< 0.001

GISTs: Gastrointestinal stromal tumors; EUS: Endoscopic ultrasound.

EUS findings, leiomyomas are also tumors of muscular origin. Unlike GISTs, leiomyomas are negative for CD117 and CD34, but positive for smooth muscle actin (SMA) and desmin on immunohistochemical staining. Moreover, leiomyomas are completely benign.

Recent studies have demonstrated that all GISTs have a malignant potential. Therefore, suspected GISTs should be confirmed histologically and managed accordingly. However, GISTs often behave differently at different locations. A GIST in the stomach is often more indolent than a GIST with a similar size and mitotic count located in another GI tract site[25]. Therefore, EUS surveillance alone is feasible for a small suspected GIST in the stomach that does not require immediate tissue proof or resection[2,26].

Most GISTs < 1 cm harbor a very low malignant potential, while GISTs $\geq$ 3 cm with irregular tumor borders, heterogeneous hypoechogenicity, and internal

cystic or calcified changes suggest a higher malignant potential. All leiomyomas are benign. Therefore, we were interested in the natural course of 1-3 cm SMTMPs in the stomach. To evaluate tumor growth, we calculated the ratio of follow-up tumor size to initial tumor size on EUS and defined the ratio of ≥ 1.20 as tumor progression based on RECIST. Among 88 patients with 1-3 cm gastric myogenic tumors, we found that most tumors were indolent and tumor progression was detected in 25 (28.4%) patients. No patients suffered from major complications such as tumor bleeding, obstruction, perforation or malignant transformation during surveillance. A total of 19 (17 + 2) patients underwent surgery. Of these, 15 patients had GISTs and 4 patients had leiomyomas. Notably, tumor progression (tumor enlargement ≥ 1.2 times) was found only in GISTs but not in leiomyomas. Therefore, tumor progression may be a good predictor for differentiating GISTs from leiomyomas. Moreover, we found that larger tumors with irregular margins showed a tendency toward progressive change and should be monitored more closely. From the ROC curve analysis, we found 1.4 cm to be the optimal cut-off tumor size associated with tumor progression. The same 1.4 cm cut-off size was reported by Fang *et al*[27] in their study, which is similar to that reported by Lachter *et al*[28] who found tumor size larger than 1.7cm to be indicative of tumor progression. Tumors with heterogeneous hypoechotexture showed no statistical significance for predicting tumor progression (P = 0.06) in our study, but the finding is limited by our small number of cases and requires clarification in a larger study. Regarding the appropriate interval of EUS surveillance, it is difficult to conclude how often a suspected gastric GIST should be followed since malignant GISTs were not detected during surveillance in our study. Although an evidences-based optimal EUS surveillance policy remains lacking for small GISTs, yearly EUS follow-up for small sized GISTs (< 3 cm) should be considered from a study of Prachayakul *et al*[26] in 2012. At present, a guideline from European society of medical oncology recommended that an interval of 3 mo in the first follow-up and then annual EUS surveillance may be optimal for small suspected GISTs if no tumor growth occurs during surveillance[29]. In this review of 1725 EUS surveillances for gastric submucosal tumors from the 14 years of medical records, we found that most 1-3 cm SMTMPs in the stomach were indolent with only 28.4% of patients experiencing tumor progression (tumor enlargement ≥ 1.2 times). EUS surveillance is optimal for small gastric myogenic submucosal tumors without immediately obtaining tissue. Tumor progression is a good predictor for differentiating GISTs from leiomyomas. Risk factors for tumor progression include larger tumor and irregular borders. Initial tumor size > 14.0 mm may be considered a cut-off size for predicting tumor progression.

COMMENTS

Background

Most gastric submucosal tumors originating from muscularis propria (SMTMPs) are gastrointestinal stromal tumors (GISTs) and leiomyomas. Leiomyoma is benign but GIST has a malignant potential. Surgery is recommended if GISTs larger than 3 cm. Endoscopic ultrasound (EUS) fine needle aspiration is helpful to differentiate between GISTs and leiomyomas, but sometimes it is difficult to obtain tissue and cannot provide mitotic activity of GISTs.

Research frontiers

Because studies regarding the natural behaviors of 1-3 cm gastric SMTMPs are limited, the authors made a retrospective study by reviewing the past 14 years of computerized medical records in a tertiary medical center between 2000 and 2013.

Innovations and breakthroughs

Most gastric SMTMPs are indolent from our study. Risk factors for tumor progression include larger tumor size and irregular border.

Applications

Initial tumor size > 14.0 mm may be considered a cut-off size for predicting tumor progression. Therefore, a gastric SMTMP with irregular border or ≥ 14.0 mm in size should be observed closely and treated accordingly.

Terminology

GISTs are the common submucosal tumors arising from the muscularis propria in the stomach and have a malignant potential though the behavior of most tumors is indolent. EUS is a useful tool to detect submucosal tumors of the gastrointestinal tract.

Peer-review

This study provides important information (long term surveillance, EUS surveillance interval, a cut-off value of tumor size of > 14.0 mm) in the management of gastric small SMTMPs.

REFERENCES

1 **Boyce GA**, Sivak MV, Rösch T, Classen M, Fleischer DE, Boyce HW, Lightdale CJ, Botet JF, Hawes RH, Lehman GA. Evaluation of submucosal upper gastrointestinal tract lesions by endoscopic ultrasound. *Gastrointest Endosc* 1991; **37**: 449-454 [PMID: 1916167 DOI: 10.1016/s0016-5107(91)70778-5]

2 **Miettinen M**, Lasota J. Gastrointestinal stromal tumors (GISTs): definition, occurrence, pathology, differential diagnosis and molecular genetics. *Pol J Pathol* 2003; **54**: 3-24 [PMID: 12817876]

3 **Yasuda K**, Nakajima M, Yoshida S, Kiyota K, Kawai K. The diagnosis of submucosal tumors of the stomach by endoscopic ultrasonography. *Gastrointest Endosc* 1989; **35**: 10-15 [PMID: 2646166 DOI: 10.1016./s0016-5107(89)72678-x]

4 **Caletti G**, Zani L, Bolondi L, Brocchi E, Rollo V, Barbara L. Endoscopic ultrasonography in the diagnosis of gastric submucosal tumor. *Gastrointest Endosc* 1989; **35**: 413-418 [PMID: 2676689 DOI: 10.1016/s0016-5107(89)72846-7]

5 **Yasuda K**, Cho E, Nakajima M, Kawai K. Diagnosis of

submucosal lesions of the upper gastrointestinal tract by endoscopic ultrasonography. *Gastrointest Endosc* 1990; **36**: S17-S20 [PMID: 2184080 DOI: 10.1016/s0016-5107(90)71010-3]

6   **Buscarini E**, Stasi MD, Rossi S, Silva M, Giangregorio F, Adriano Z, Buscarini L. Endosonographic diagnosis of submucosal upper gastrointestinal tract lesions and large fold gastropathies by catheter ultrasound probe. *Gastrointest Endosc* 1999; **49**: 184-191 [PMID: 9925696 DOI: 10.1016/s0016-5107(99)70484-0]

7   **Hizawa K**, Matsumoto T, Kouzuki T, Suekane H, Esaki M, Fujishima M. Cystic submucosal tumors in the gastrointestinal tract: endosonographic findings and endoscopic removal. *Endoscopy* 2000; **32**: 712-714 [PMID: 10989996 DOI: 10.1055/s-2000-9025]

8   **Rösch T**, Kapfer B, Will U, Baronius W, Strobel M, Lorenz R, Ulm K. Accuracy of endoscopic ultrasonography in upper gastrointestinal submucosal lesions: a prospective multicenter study. *Scand J Gastroenterol* 2002; **37**: 856-862 [PMID: 12190103]

9   **Shim CS**, Jung IS. Endoscopic removal of submucosal tumors: preprocedure diagnosis, technical options, and results. *Endoscopy* 2005; **37**: 646-654 [PMID: 16010609 DOI: 10.1055/s-2005-861477]

10   **Sepe PS**, Moparty B, Pitman MB, Saltzman JR, Brugge WR. EUS-guided FNA for the diagnosis of GI stromal cell tumors: sensitivity and cytologic yield. *Gastrointest Endosc* 2009; **70**: 254-261 [PMID: 19482280 DOI: 10.1016/j.gie.2008.11.038]

11   **Ando N**, Goto H, Niwa Y, Hirooka Y, Ohmiya N, Nagasaka T, Hayakawa T. The diagnosis of GI stromal tumors with EUS-guided fine needle aspiration with immunohistochemical analysis. *Gastrointest Endosc* 2002; **55**: 37-43 [PMID: 11756912 DOI: 10.1067/mge.2002.120323]

12   **Nishida T**, Hirota S, Yanagisawa A, Sugino Y, Minami M, Yamamura Y, Otani Y, Shimada Y, Takahashi F, Kubota T. Clinical practice guidelines for gastrointestinal stromal tumor (GIST) in Japan: English version. *Int J Clin Oncol* 2008; **13**: 416-430 [PMID: 18946752 DOI: 10.1007/s10147-008-0798-7]

13   **Fletcher CD**, Berman JJ, Corless C, Gorstein F, Lasota J, Longley BJ, Miettinen M, O'Leary TJ, Remotti H, Rubin BP, Shmookler B, Sobin LH, Weiss SW. Diagnosis of gastrointestinal stromal tumors: A consensus approach. *Hum Pathol* 2002; **33**: 459-465 [PMID: 12094370 DOI: 10.1053/hupa.2002.123545]

14   **Chak A**, Canto MI, Rösch T, Dittler HJ, Hawes RH, Tio TL, Lightdale CJ, Boyce HW, Scheiman J, Carpenter SL, Van Dam J, Kochman ML, Sivak MV. Endosonographic differentiation of benign and malignant stromal cell tumors. *Gastrointest Endosc* 1997; **45**: 468-473 [PMID: 9199902 DOI: 10.1016/s0016-5107(97)70175-5]

15   **Palazzo L**, Landi B, Cellier C, Cuillerier E, Roseau G, Barbier JP. Endosonographic features predictive of benign and malignant gastrointestinal stromal cell tumours. *Gut* 2000; **46**: 88-92 [PMID: 10601061 DOI: 10.1136/gut.46.1.88]

16   **American Gastroenterological Association Institute**. American Gastroenterological Association Institute medical position statement on the management of gastric subepithelial masses. *Gastroenterology* 2006; **130**: 2215-2216 [PMID: 16762643 DOI: 10.1053/j.gastro.2006.04.032]

17   **Bennett JJ**, Rubino MS. Gastrointestinal stromal tumors of the stomach. *Surg Oncol Clin N Am* 2012; **21**: 21-33 [PMID: 22098829 DOI: 10.1016/j.soc.2011.09.008]

18   **Therasse P**, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, Verweij J, Van Glabbeke M, van Oosterom AT, Christian MC, Gwyther SG. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. *J Natl Cancer Inst* 2000; **92**: 205-216 [PMID: 10655437]

19   **Pidhorecky I**, Cheney RT, Kraybill WG, Gibbs JF. Gastrointestinal stromal tumors: current diagnosis, biologic behavior, and management. *Ann Surg Oncol* 2000; **7**: 705-712 [PMID: 11034250 DOI: 10.1017/s10434-000-0705-6]

20   **Sarlomo-Rikala M**, Kovatich AJ, Barusevicius A, Miettinen M. CD117: a sensitive marker for gastrointestinal stromal tumors that is more specific than CD34. *Mod Pathol* 1998; **11**: 728-734 [PMID: 9720500]

21   **Miettinen M**, Sobin LH, Sarlomo-Rikala M. Immunohistochemical spectrum of GISTs at different sites and their differential diagnosis with a reference to CD117 (KIT). *Mod Pathol* 2000; **13**: 1134-1142 [PMID: 11048809 DOI: 10.1038/modpathol.3880210]

22   **Demetri GD**, Benjamin RS, Blanke CD, Blay JY, Casali P, Choi H, Corless CL, Debiec-Rychter M, DeMatteo RP, Ettinger DS, Fisher GA, Fletcher CD, Gronchi A, Hohenberger P, Hughes M, Joensuu H, Judson I, Le Cesne A, Maki RG, Morse M, Pappo AS, Pisters PW, Raut CP, Reichardt P, Tyler DS, Van den Abbeele AD, von Mehren M, Wayne JD, Zalcberg J. NCCN Task Force report: management of patients with gastrointestinal stromal tumor (GIST)--update of the NCCN clinical practice guidelines. *J Natl Compr Canc Netw* 2007; **5** Suppl 2: S1-29; quiz S30 [PMID: 17624289]

23   **Miettinen M**, Lasota J. Gastrointestinal stromal tumors--definition, clinical, histological, immunohistochemical, and molecular genetic features and differential diagnosis. *Virchows Arch* 2001; **438**: 1-12 [PMID: 11213830 DOI: 10.1007/s004280000338]

24   **Miettinen M**, El-Rifai W, H L Sobin L, Lasota J. Evaluation of malignancy and prognosis of gastrointestinal stromal tumors: a review. *Hum Pathol* 2002; **33**: 478-483 [PMID: 12094372 DOI: 10.1053/hupa.2002.124123]

25   **Tashiro T**, Hasegawa T, Omatsu M, Sekine S, Shimoda T, Katai H. Gastrointestinal stromal tumour of the stomach showing lymph node metastases. *Histopathology* 2005; **47**: 438-439 [PMID: 16178904 DOI: 10.1111/j1365-2559.2005.02133.x]

26   **Prachayakul V**, Aswakul P, Pongprasobchai S, Pausawasdi N, Akaraviputh T, Sriprayoon T, Methasate A, Kachintorn U. Clinical characteristics, endosonographic findings and etiologies of gastroduodenal subepithelial lesions: a Thai referral single center study. *J Med Assoc Thai* 2012; **95** Suppl 2: S61-S67 [PMID: 22574531]

27   **Fang YJ**, Cheng TY, Sun MS, Yang CS, Chen JH, Liao WC, Wang HP. Suggested cutoff tumor size for management of small EUS-suspected gastric gastrointestinal stromal tumors. *J Formos Med Assoc* 2012; **111**: 88-93 [PMID: 22370287 DOI: 10.1016/j.jfma.2011.01.002]

28   **Lachter J**, Bishara N, Rahimi E, Shiller M, Cohen H, Reshef R. EUS clarifies the natural history and ideal management of GISTs. *Hepatogastroenterology* 2008; **55**: 1653-1656 [PMID: 19102362]

29   **Puchalski CM**. Spirituality in the cancer trajectory. *Ann Oncol* 2012; **23** Suppl 3: 49-55 [PMID: 22628416 DOI: 10.1093/annonc/mds088]

ORIGINAL ARTICLE

Retrospective Cohort Study

Effect of liver cirrhosis on long-term outcomes after acute respiratory failure: A population-based study

Chih-Cheng Lai, Chung-Han Ho, Kuo-Chen Cheng, Chien-Ming Chao, Chin-Ming Chen, Willy Chou

Chih-Cheng Lai, Chien-Ming Chao, Department of Intensive Care Medicine, Chi Mei Medical Center, Liouying, Tainan 736, Taiwan

Chung-Han Ho, Departments of Medical Research, Chi Mei Medical Center, Tainan 710, Taiwan

Kuo-Chen Cheng, Internal Medicine, Chi Mei Medical Center, Tainan 710, Taiwan

Kuo-Chen Cheng, Department of Safety, Health and Environment, Chung Hwa University of Medical Technology, Tainan 717, Taiwan

Chin-Ming Chen, Department of Intensive Care Medicine, Chi Mei Medical Center, Tainan 710, Taiwan

Chin-Ming Chen, Willy Chou, Department of Recreation and Health-Care Management, Chia Nan University of Pharmacy and Science, Tainan 717, Taiwan

Author contributions: Chen CM is the guarantor of this manuscript; Lai CC, Ho CH, Cheng KC, Chao CM and Chou W contributed to the conception and design of the study; Ho CH analyzed and interpreted the data; Lai CC and Chen CM drafted the manuscript.

Institutional review board statement: The study was approved by the Institutional Review Board (IRB 10409-E04) at Chi Mei Medical Center.

Informed consent statement: Because the data used in this study have been deidentified and released to the public for research purposes, the need for informed consent from enrolled patients was waived by the Institutional Review Board at Chi Mei Medical Center.

Conflict-of-interest statement: All authors declared there is no conflict of interest.

Data sharing statement: The original anonymous dataset is available on request from the corresponding author at chencm3383@yahoo.com.tw.

Abstract

AIM
To assessed the effect of liver cirrhosis (LC) on the poorly understood long-term mortality risk after first-ever mechanical ventilation (1-MV) for acute respiratory failure.

METHODS
All patients in Taiwan given a 1-MV between 1997 and 2013 were identified in Taiwan's Longitudinal Health Insurance Database 2000. Each patient with LC was individually matched, using a propensity-score method, to two patients without LC. The primary outcome was death after a 1-MV.

RESULTS
A total of 16653 patients were enrolled: 5551 LC-positive

(LC[Pos]) patients, including 1732 with cryptogenic LCs and 11102 LC-negative (LC[Neg]) controls. LC[Pos] patients had more organ failures and were more likely to be admitted to medical department than were LC[Neg] controls. LC[Pos] patients had a significantly lower survival rate (AHR = 1.38, 95%CI: 1.32-1.44). Moreover, the mortality risk was significantly higher for patients with non-cryptogenic LC than for patients with cryptogenic LC (AHR = 1.43, 95%CI: 1.32-1.54) and patients without LC (AHR = 1.56, 95%CI: 1.32-1.54). However, there was no significant difference between patients with cryptogenic and without LC (HR = 1.05, 95%CI: 0.98-1.12).

CONCLUSION
LC, especially non-cryptogenic LC, significantly increases the risk of death after a 1-MV.

Key words: Liver cirrhosis; Mechanical ventilation; Outcome

Core tip: Liver cirrhosis, especially non-cryptogenic liver cirrhosis, significantly increases the risk of death after acute respiratory failure.

INTRODUCTION

The burden of liver cirrhosis (LC) is increasing worldwide because of increases in alcohol abuse and in hepatitis B and C virus infections[1,2]. In France, the prevalence of LC was estimated to be 0.3%, and in the United Kingdom and Sweden, the annual incidence was 14.55-15.3 per 100000 population[3]. Furthermore, its associated morbidity and mortality are also gradually increasing. LC has become the 14th most common cause of death in adults worldwide: it caused 1.03 million deaths per year[4]. In Europe, LC is the fourth most common cause of death: 170000 deaths per year[3]. Chronic liver disease and cirrhosis is the ninth most common cause of death in Taiwan and, the overall incidence rate of death was 30.2 per 100000 per-years (42526 deaths per 140814448 person-years) from chronic liver disease and cirrhosis between 2000 and 2011[5].

Several major complications, such as variceal bleeding, ascites, spontaneous bacterial peritonitis, hepatorenal syndrome, and hepatopulmonary syn-drome, can develop in patients with decompensated LC. Because acute organ failure occurs in cirrhotic patients, they might require admission to an Intensive Care Unit (ICU). Several studies[6-12] have investigated the outcome of patients with LC in the ICU; three found that the mortality rate of this group ranged from 36% to 86%[6,7,11]. Other studies[9,13,14] reported that organ failures in critical cirrhotic patients were associated with poor outcomes. One recent study[9] said that using mechanical ventilation (MV) when admitting a patient with advanced cirrhosis was an independent risk factor of mortality. In fact, acute respiratory failure that requires invasive MV is one of the most common clinical causes of ICU admission. However, only one study[12] has assessed the prognosis of critical cirrhotic patients who require MV. Moreover, no study has specifically analyzed the effect of LC on the outcome of patients who require MV. Therefore, we investigated the long-term outcomes of patients with LC who underwent their first-ever MV (1-MV).

In addition to viral hepatitis- and alcohol-related LC, cryptogenic cirrhosis, which is defined as LC that cannot be explained by conventional clinical, laboratory, or histological findings[15,16], is becoming increasingly prevalent in Asia[17-19]. The clinical manifestations and outcomes of LC and cryptogenic LC are different[20]. Thus, we also investigated whether the effects of non-cryptogenic LC and cryptogenic LC on the patients requiring 1-MV are different.

MATERIALS AND METHODS

Data source
This study used Taiwan's National Health Insurance Research Database (NHIRD). Taiwan's NHI is a single-payer compulsory system that enrolls more than 23 million of the country's legal residents; more than 99.7% of the population is covered. The NHIRD uses the International Classification of Diseases, Ninth Revision, Clinical Modification (ICD-9-CM) diagnostic and procedure codes to provide detailed healthcare services information on the clinical visits for each insured beneficiary. We used the Longitudinal Health Insurance Database 2000 (LHID2000) which contains 1 million subjects who randomly selected NHI beneficiaries (about 4.34% of the total population) from the year 2000 Registry of Beneficiaries of the NHIR. The LHID2000 are representative of the demographic distribution of Taiwanese population and provides data on outpatient and inpatient medical care, diagnoses, surgical procedures, and prescribed medications on a longitudinal cohort from 1996 to 2013. The study was approved by the Institutional Review Board (IRB 10409-E04) at Chi Mei Medical Center. Because the data used in this study have been deidentified and released to the public for research purposes, the need for informed consent from enrolled patients was waived.
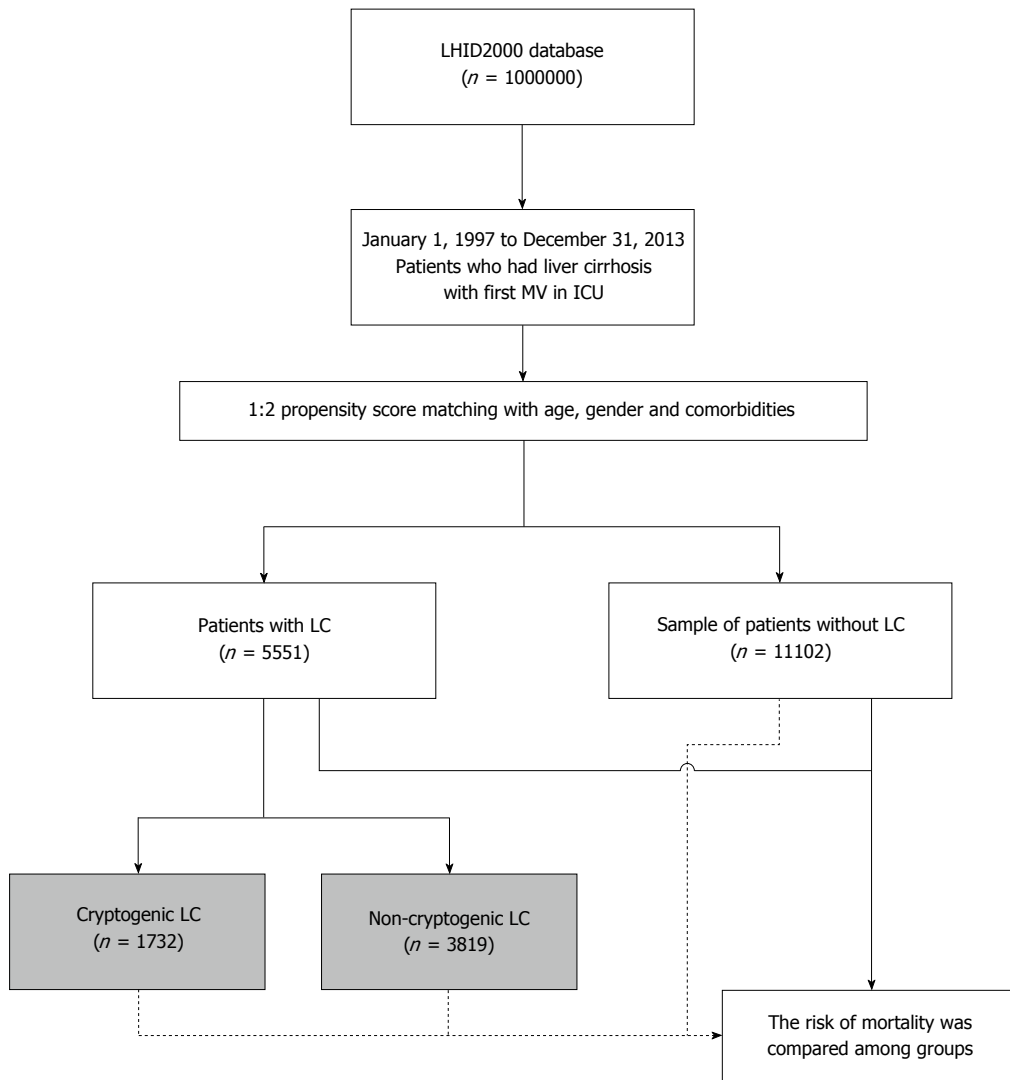
Figure 1 Algorithm of patient enrollment.

Patient selection and definition

We enrolled all inpatients with a 1-MV for acute respiratory failure (ARF) during their first hospitalization between 1997 and 2013 (n = 58383). Based on a recent study that used the LHID2000[21], our inclusion criteria for patients with LC (LC[Pos]) (ICD-9-CM codes 571.2, 571.5, and 571.6) were three outpatient visits in one year in which LC was diagnosed, or one inpatient admission for LC. Patients who were diagnosed with LC after a 1-MV were excluded (n = 1013). Each enrolled LC[Pos] patient (n = 5551, including 1732 with cryptogenic LC) was then, using propensity score matching, individually matched to two controls without LC (LC[Neg]) (Figure 1). The propensity score, *i.e.,* the probability of having LC, was estimated using a logistic regression model conditional on the covariates of age at times of 1-MV, gender, and individual comorbidities: diabetes mellitus (DM), hypertension (HTN), coronary artery disease (CAD), chronic obstructive pulmonary disease (COPD), cancer, stroke, and congestive heart failure

(CHF)[21]. In addition, we recorded other liver diseases: hepatitis B virus (HBV) (ICD-9-CM codes 070.2, 070.3, and V02.61), hepatitis C virus (HCV) (070.41, 070.44, 070.51, 070.54, V02.62, and 070.7), and cryptogenic LC, which was defined as LC without a history of HBV, HCV, alcohol drinking, autoimmune disease, hemachromatosis, Wilson's disease, and alpha-1 antitrypsin deficiency. All of the cryptogenic LC patients had received prior examinations of abdominal echography, and associated laboratory examinations, such as hepatitis B and hepatitis C markers, autoimmune tests. The characteristics of the two groups (LC[Pos] and LC[Neg]) were balanced after the propensity score matching.

Endpoint

The primary endpoint of the study was mortality after 1-MV. Patients were followed from the index admission date until death or the end of 2013. The secondary aim was to identify the risk factors for all-cause mortality after a 1-MV. We hypothesized that mortality is higher

Table 1 Demographic information of LC[Pos] and LC[Neg] patients n (%)

Variables	LC[Pos] patients (n = 5551)	LC[Neg] patients (n = 11102)	P value
Gender			0.99
Male	3655 (65.84)	7311 (65.85)	
Female	1896 (34.16)	3791 (34.15)	
Age group (yr)			0.59
< 50	1259 (22.68)	2417 (21.77)	
50-64	1441 (25.96)	2899 (26.11)	
65-79	1914 (34.48)	3902 (35.15)	
≥ 80	937 (16.88)	1884 (16.97)	
Department			< 0.01[a]
Surgical	542 (9.76)	1684 (15.17)	
Medical	5009 (90.24)	9418 (84.83)	
Number of organ failures			< 0.01[a]
0	3404 (61.32)	8433 (75.96)	
1	1791 (32.26)	2415 (21.75)	
≥ 2	356 (6.41)	254 (2.29)	
Comorbidity			
DM	2048 (36.89)	4082 (36.77)	0.87
HTN	2454 (44.21)	4895 (44.09)	0.89
CAD	1077 (19.40)	2144 (19.31)	0.89
ESRD	627 (11.30)	1287 (11.59)	0.57
COPD	1133 (20.41)	2277 (20.51)	0.88
Cancer	1362 (24.54)	2739 (24.67)	0.85
Stroke	980 (17.65)	1944 (17.51)	0.82
CHF	784 (14.12)	1547 (13.93)	0.74
HBV	14 (0.25)	28 (0.25)	1.00
HCV	21 (0.38)	35 (0.32)	0.51
Cryptogenic LC[Pos]	1732 (31.20)		

[a]$P < 0.05$. LC[Pos]: Liver cirrhosis-positive; LC[Neg]: Liver Cirrhosis-negative; DM: Diabetes mellitus; HTN: Hypertension; CAD: Cardiovascular disease; ESRD: End-stage renal disease; COPD: Chronic obstructive airway disease; CHF: Congestive heart failure; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

Table 2 Adjusted hazard ratios for mortality in patients after their 1st-ever mechanical ventilation n (%)

No. of deaths	LC[Pos] patients (n = 5551)	LC[Neg] patients (n = 11102)	Adjusted hazard ratio (95%CI)
Overall	3747 (67.50)	5902 (53.16)	1.38 (1.32-1.44)[a]
Age (yr)			
< 50	763 (13.75)	744 (6.70)	1.96 (1.76-2.18)[a]
50-64	911 (16.41)	1369 (12.33)	1.40 (1.29-1.53)[a]
65-79	1368 (24.64)	2458 (22.14)	1.24 (1.16-1.32)[a]
≥ 80	705 (12.70)	1331 (11.99)	1.41 (1.04-1.25)[a]
Gender			
Male	2464 (44.39)	3770 (33.96)	1.42 (1.35-1.49)[a]
Female	1283 (23.11)	2132 (19.20)	1.30 (1.21-1.39)[a]
Department			
Surgical	255 (4.59)	595 (5.36)	1.32 (1.14-1.54)[a]
Medical	3492 (62.91)	5307 (47.80)	1.37 (1.31-1.43)[a]
Number of organ failures			
0	2074 (37.36)	4015 (36.16)	1.40 (1.33-1.47)[a]
1	1379 (24.84)	1691 (15.23)	1.26 (1.17-1.35)[a]
≥ 2	294 (5.30)	196 (1.77)	1.16 (0.96-1.41)
Comorbidity			
DM	1374 (24.75)	2432 (21.91)	1.19 (1.12-1.28)[a]
HTN	1583 (28.52)	2851 (25.68)	1.15 (1.08-1.22)[a]
CAD	716 (12.90)	1276 (11.49)	1.17 (1.07-1.28)[a]
ESRD	488 (8.79)	920 (8.29)	1.19 (1.06-1.33)[a]
COPD	789 (14.21)	1459 (13.14)	1.15 (1.05-1.26)[a]
Cancer	972 (17.51)	1668 (15.02)	1.31 (1.21-1.42)[a]
Stroke	680 (12.25)	1261 (11.36)	1.16 (1.06-1.28)[a]
CHF	563 (10.14)	1002 (9.03)	1.21 (1.09-1.34)[a]
HBV	7 (0.13)	12 (0.11)	0.54 (0.11-2.57)
HCV	11 (0.20)	20 (0.18)	0.43 (0.14-1.28)

The model was adjusted for age, gender, length of hospital stay, length of 1st mechanical ventilation, length of intensive care unit stay, treatment department, number of organ failures, and the listed comorbidities. [a]$P < 0.05$. LC[Pos]: Liver cirrhosis-positive; LC[Neg]: Liver cirrhosis-negative; DM: Diabetes mellitus; HTN: Hypertension; CAD: Cardiovascular disease; ESRD: End-stage renal disease; COPD: Chronic obstructive pulmonary disease; CHF: Congestive heart failure; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

in LC[Pos] patients than in LC[Neg] patients who require MV. The demographic and clinical characteristics of age, gender, department to which admitted, number of organ failures, and comorbidities were used to estimate the mortality risk.

Statistical analysis

Differences in baseline characteristics between groups were evaluated using Pearson's χ^2 test for categorical variables. The actuarial survival rate of the two groups was determined using the Kaplan-Meier method, and a log-rank test was used to compare the difference between the two survival curves. The effect of LC on the mortality risk after 1-MV was assessed using a Cox proportional hazards regression model. Covariates included in the Cox model were age, gender, department to which admitted, number of organ failures, and comorbidities. The proportional hazards assumption was verified using plots of natural log transformed (ln) (survival function) *vs* ln (time). Significance was set at $P < 0.05$. SAS 9.4 for Windows (SAS Institute, Cary, NC, United States) was used for all analyses.

RESULTS

We enrolled 16653 patients: 5551 LC[Pos] patients and 11102 LC[Neg] controls (Table 1). LC[Pos] patients had more organ failures, were more likely to be admitted to a medical department, and had a higher mortality rate than did LC[Neg] controls.

Overall, LC[Pos] patients had a higher risk of death than did LC[Neg] patients (adjusted hazard ratio (AHR) = 1.38; 95%CI: 1.32-1.44). The AHR was higher (1.96; 95%CI: 1.76-2.18) for patients < 50 years old than for patients in other age groups. In addition, both men and women admitted by medical and surgical departments, patients with ≤ 1 organ failure, and patients with comorbid DM, HTN, CAD, ESRD, COPD, cancer, stroke, or CHF had significantly ($P < 0.05$) higher AHRs (Table 2). In contrast, there were no significant differences for patients with ≥ two organ failures, HBV, or HCV.

Kaplan-Meier survival curves showed that mortality in patients with non-cryptogenic LC after

Table 3 Hazard ratio of mortality risk for patients with non-cryptogenic liver cirrhosis, cryptogenic liver cirrhosis, and liver cirrhosis[Neg] patients after 1st-ever mechanical ventilation, stratified by gender and age group

	LC[Neg]	Cryptogenic LC[Pos]	Non-cryptogenic LC[pos]
Overall	1.00 (ref.)	1.05 (0.98-1.12)	1.56 (1.49-1.63)[a]
Patients with LC only		1.00 (ref.)	1.43 (1.32-1.54)[a]
Males only			
Overall	1.00 (ref.)	1.06 (0.97-1.16)	1.58 (1.49-1.67)[a]
Patients with LC only		1.00 (ref.)	1.40 (1.27-1.55)[a]
Females only			
Overall	1.00 (ref.)	1.02 (0.92-1.13)	1.52 (1.40-1.64)[a]
Patients with LC only		1.00 (ref.)	1.48 (1.31-1.67)[a]
Age group: < 50			
Overall	1.00 (ref.)	1.31 (1.07-1.60)[a]	2.17 (1.94-2.43)[a]
Patients with LC only		1.00 (ref.)	1.68 (1.36-2.08)[a]
Age group: 50-64			
Overall	1.00 (ref.)	0.96 (0.82-1.13)	1.59 (1.44-1.74)[a]
Patients with LC only		1.00 (ref.)	1.70 (1.42-2.03)[a]
Age group: 65-79			
Overall	1.00 (ref.)	1.07 (0.96-1.18)	1.35 (1.25-1.46)[a]
Patients with LC only		1.00 (ref.)	1.27 (1.13-1.43)[a]
Age group: ≥ 80			
Overall	1.00 (ref.)	0.92 (0.80-1.06)	1.31 (1.17-1.45)[a]
Patients with LC only		1.00 (ref.)	1.40 (1.19-1.64)[a]

The model was adjusted for age, gender, length of hospital stay, length of 1st mechanical ventilation, length of Intensive Care Unit stay, treatment department, number of organ failures, and the listed comorbidities. [a]$P < 0.05$. (ref.): Reference value; LC[Pos]: Liver cirrhosis-positive; LC[Neg]: Liver cirrhosis-negative.

Figure 2 Kaplan-Meier survival curves of patients with non-cryptogenic liver cirrhosis, patients with cryptogenic liver cirrhosis, and patients without liver cirrhosis after a 1st-ever mechanical ventilation.

1-MV precipitously declined early on and ran parallel thereafter (Figure 2); although the starting point was lower, the trajectory had not changed. In addition, the patients with cryptogenic LC had a higher mortality rate than did LC[Neg] patients, but lower than did patients with non-cryptogenic LC. The absolute survival rate also showed that LC[Neg] patients had the highest 1-, 3-, 5-, and 10-year survival rates, followed by the patients with cryptogenic LC.

Overall, the risk of mortality was significantly higher for patients with non-cryptogenic LC than for patients with cryptogenic LC and for LC[Neg] patients (Table 3). The risk differences in mortality between the patients with non-cryptogenic LC and LC[Neg] patients were significant across the subgroups for men and for women as well as across age groups. The mortality risk was higher (AHR = 2.17, 95%CI: 1.94-2.43) for patients < 50 years old than for patients ≥ 50 years old.

DISCUSSION

This is the first study that investigates (1) the effect of LC on the outcomes of the patients after 1-MV; and (2) the different effects of non-cryptogenic LC and cryptogenic LC on this specific group. We have several significant findings.

First, after adjusting for possible confounding factors, we found that LC itself was significantly associated with poor patient outcomes after a 1-MV (AHR = 1.38, 95%CI: 1.32-1.44). Although other studies have shown the grave outcomes of patients critically ill with LC[9,12,22,23] and one[24] reported that the overall in-hospital mortality rate of patients with LC in their Acute Physiology and Chronic Health Evaluation Ⅲ (APACHE Ⅲ)-matched group was higher than that in the LC[Neg] group (73.6% *vs* 57.5%, $P = 0.026$), the present study is the first one to show the negative effects of LC on the outcomes of critically ill patients who require MV. Moreover, we found that this kind of significant association was apparent only for patients with non-cryptogenic LC (AHR = 1.56, 95%CI: 1.49-1.63), but not for cryptogenic patients (AHR = 1.05, 95%CI: 0.98-1.12). All of these findings indicate that LC, especially non-cryptogenic LC, is associated with poor outcomes for critically ill patients who require MV.

Second, we found that non-cryptogenic LC was significantly associated with worse outcomes in patients after a 1-MV than cryptogenic LC (AHR = 1.43, 95%CI: 1.32-1.54). In contrast, one retrospective Malaysian cohort study[20] reported, after comparing the clinical outcomes in 94 cases cryptogenic LC and 207 cases of non-cryptogenic LC, cases that there was no significant difference in mortality between these two groups; however, the sample in that study was relatively small. A Japanese study[25], which compared 68 patients with cirrhotic non-alcoholic steatohepatitis (NASH) and 69 with HCV-induced LC, found that the 5-year survival rates and liver-related mortality were not significantly different in the two groups. A Sri Lankan study[26] of 306 alcoholic LC[Pos] and 243 cryptogenic LC[Pos] patients also found that survival rates were not significantly different between the two groups. The difference between the present study and these three Asian studies can be explained by different study designs and patient populations. Our study focused only on the mortality of patients after a 1-MV, and we used all-cause mortality for outcome analysis. However, additional large-scale studies are warranted

to determine whether the effects of LC and cryptogenic LC on different specific groups are different.

Third, we also investigated the negative effects of LC on the outcomes of patients (stratified by age and gender) after a 1-MV. We found that all LC[Pos] patients had higher mortality risks, but that only non-cryptogenic LC[Pos] patients had significantly higher AHRs regardless of age group and gender. The < 50 years old group had the highest AHR for mortality of all age groups. Thus, our findings suggest that we should pay more attention to developing methods to reduce the negative effects of LC for these younger high-risk patients. However, additional case-control studies are needed to confirm such a relationship. We also found that AHRs for mortality were not significantly different between male and female LC[Pos] patients after a 1-MV. Two recent studies[27,28] in Taiwan reported that in-hospital mortality was significantly more highly associated with men than with women, but an American study[29] reported the opposite. Differences in our findings might be attributable to our having enrolled only LC[Pos] patients, unlike the study populations of these other studies.

Our study has some strengths. It is a large population-based analysis of the effect of LC on patients given a 1-MV. NHIRD includes data on over 99% of all residents in Taiwan, therefore, it allows large-scale and longitudinal follow-up epidemiological studies and health services research. In addition, this kind of nationwide study design largely reduces the effect of referral bias, which is often seen in critical care studies. This investigation should provide robust data on the characteristics and effects of critical cirrhotic patients requiring MV in Taiwan.

Limits of the study

Our study also has some limitations. First, because our study relies on administrative databases rather than on actual patient charts for all diagnoses, including comorbidities, and on the claims data and ICD-9-CM diagnosis codes, some of the diagnoses might be incorrect. Alcoholic and NASH were the two major cause of LC. However, this study is using the NHIRD database, which cannot provide history of alcoholic using and the diagnosis of NASH. Therefore, we cannot make sure the diagnosis of alcoholic LC and analysis the effect of alcoholic LC. Besides, there are no images or lab data to support the diagnoses, our conclusions cannot be totally convincing. Nonetheless, the Taiwan NHI Bureau randomly reviews patient charts and interviews patients to verify the accuracy of the coding. Hospitals with outlier charges or practices might be audited and subsequently heavily penalized for malpractice or discrepancies. Therefore, the potential risk for bias based on coding practices can be minimized. Second, because the NHIRD does not contain data that differentiate disease severities, we were unable to take into account the illness severity

scores of cirrhotic patients who required MV; thus, we included the number of organ failures as a proxy for severity. Although we found LC[Pos] with MOF had higher risk of death than without MOF, the difference did not reach statistical significance. It may be due to the limited case number. Further larger scale study may be warranted to investigate this issue. Third, as in all observational studies, our study might contain some residual confounding, which prevents us from arriving at conclusions about causality but only correlations between risk factors and mortality. Moreover, the primary reasons for admitting these LC[Pos] patients with a 1-MV are unknown, as are additional details about the severity of their LC. Finally, the enrolled patients were selected from a heterogeneous general population, which more than likely makes generalizing our conclusions too arbitrary. However, given the large magnitude of the observed effects in this study, these limitations are unlikely to have compromised the results. Further investigation about the cause of death using other databank is required.

In conclusion, LC, especially non-cryptogenic LC, significantly increases the risk of mortality after a 1-MV. The greatest negative effect of LC was on patients < 50 years old.

COMMENTS

Background

In addition to viral hepatitis- and alcohol-related liver cirrhosis (LC), cryptogenic cirrhosis, which is defined as LC that cannot be explained by conventional clinical, laboratory, or histological findings, is becoming increasingly prevalent in Asia. The clinical manifestations and outcomes of LC and cryptogenic LC are different, especially for patients using mechanical ventilation (MV). Thus, the authors investigated the long-term outcomes of patients with LC who underwent their first-ever MV (1-MV), and also compared the different impact of 1-MV on the patients with non-cryptogenic LC or cryptogenic LC.

Research frontiers

Multiple organ failures in critical cirrhotic patients were associated with poor outcomes. The use of MV for a patient with advanced cirrhosis was an independent risk factor of mortality. However, no large study has specifically analyzed the effect of LC on the long-term outcome of patients who underwent their 1-MV.

Innovations and breakthroughs

A total of 16653 patients were enrolled. LC patients had a significantly lower survival rate (AHR = 1.38) after their 1-MV. Moreover, the mortality risk was significantly higher for patients with non-cryptogenic LC than for patients with cryptogenic LC (AHR = 1.43) and patients without LC (AHR = 1.56). However, there was no significant difference between patients with cryptogenic and without LC (AHR = 1.05, 95%CI: 0.98-1.12). The risk differences in mortality between the patients with non-cryptogenic LC and patients without LC were significant across the subgroups for men and for women as well as across age groups. The mortality risk was higher (AHR = 2.17) for patients < 50 years old than for patients ⩾ 50 years old.

Applications

After adjusting for possible confounding factors, we found that LC itself was significantly associated with poor patient outcomes after a 1-MV. Non-cryptogenic LC was also significantly associated with worse outcomes in patients after a 1-MV than cryptogenic LC and patients without LC. The < 50

years old group had the highest AHR for mortality of all age groups. Thus, our findings suggest that we should pay more attention to developing methods to reduce the negative effects of LC for these younger high-risk patients.

Terminology
LC, especially non-cryptogenic LC, significantly increases the risk of death after a 1-MV.

Peer-review
Very good work has been performed by Lai CC *et al* comparing the effect of LC on the poorly understood long-term mortality risk after a 1-MV for acute respiratory failure. Congratulation to the authors for adding valuable data for LC, especially non-cryptogenic LC, significantly increases the risk of death after a 1-MV.

REFERENCES

1   **Schuppan D**, Afdhal NH. Liver cirrhosis. *Lancet* 2008; **371**: 838-851 [PMID: 18328931 DOI: 10.1016/S0140-6736(08)60383-9]

2   **Tsochatzis EA**, Bosch J, Burroughs AK. Liver cirrhosis. *Lancet* 2014; **383**: 1749-1761 [PMID: 24480518 DOI: 10.1016/S0140-6736(14)60121-5]

3   **Blachier M**, Leleu H, Peck-Radosavljevic M, Valla DC, Roudot-Thoraval F. The burden of liver disease in Europe: a review of available epidemiological data. *J Hepatol* 2013; **58**: 593-608 [PMID: 23419824 DOI: 10.1016/j.jhep.2012.12.005]

4   **Lozano R**, Naghavi M, Foreman K, Lim S, Shibuya K, Aboyans V, Abraham J, Adair T, Aggarwal R, Ahn SY, Alvarado M, Anderson HR, Anderson LM, Andrews KG, Atkinson C, Baddour LM, Barker-Collo S, Bartels DH, Bell ML, Benjamin EJ, Bennett D, Bhalla K, Bikbov B, Bin Abdulhak A, Birbeck G, Blyth F, Bolliger I, Boufous S, Bucello C, Burch M, Burney P, Carapetis J, Chen H, Chou D, Chugh SS, Coffeng LE, Colan SD, Colquhoun S, Colson KE, Condon J, Connor MD, Cooper LT, Corriere M, Cortinovis M, de Vaccaro KC, Couser W, Cowie BC, Criqui MH, Cross M, Dabhadkar KC, Dahodwala N, De Leo D, Degenhardt L, Delossantos A, Denenberg J, Des Jarlais DC, Dharmaratne SD, Dorsey ER, Driscoll T, Duber H, Ebel B, Erwin PJ, Espindola P, Ezzati M, Feigin V, Flaxman AD, Forouzanfar MH, Fowkes FG, Franklin R, Fransen M, Freeman MK, Gabriel SE, Gakidou E, Gaspari F, Gillum RF, Gonzalez-Medina D, Halasa YA, Haring D, Harrison JE, Havmoeller R, Hay RJ, Hoen B, Hotez PJ, Hoy D, Jacobsen KH, James SL, Jasrasaria R, Jayaraman S, Johns N, Karthikeyan G, Kassebaum N, Keren A, Khoo JP, Knowlton LM, Kobusingye O, Koranteng A, Krishnamurthi R, Lipnick M, Lipshultz SE, Ohno SL, Mabweijano J, MacIntyre MF, Mallinger L, March L, Marks GB, Marks R, Matsumori A, Matzopoulos R, Mayosi BM, McAnulty JH, McDermott MM, McGrath J, Mensah GA, Merriman TR, Michaud C, Miller M, Miller TR, Mock C, Mocumbi AO, Mokdad AA, Moran A, Mulholland K, Nair MN, Naldi L, Narayan KM, Nasseri K, Norman P, O'Donnell M, Omer SB, Ortblad K, Osborne R, Ozgediz D, Pahari B, Pandian JD, Rivero AP, Padilla RP, Perez-Ruiz F, Perico N, Phillips D, Pierce K, Pope CA 3rd, Porrini E, Pourmalek F, Raju M, Ranganathan D, Rehm JT, Rein DB, Remuzzi G, Rivara FP, Roberts T, De León FR, Rosenfeld LC, Rushton L, Sacco RL, Salomon JA, Sampson U, Sanman E, Schwebel DC, Segui-Gomez M, Shepard DS, Singh D, Singleton J, Sliwa K, Smith E, Steer A, Taylor JA, Thomas B, Tleyjeh IM, Towbin JA, Truelsen T, Undurraga EA, Venketasubramanian N, Vijayakumar L, Vos T, Wagner GR, Wang M, Wang W, Watt K, Weinstock MA, Weintraub R, Wilkinson JD, Woolf AD, Wulf S, Yeh PH, Yip P, Zabetian A, Zheng ZJ, Lopez AD, Murray CJ, AlMazroa MA, Memish ZA. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet* 2012; **380**: 2095-2128 [PMID: 23245604 DOI: 10.1016/S0140-6736(12)61728-0]

5   **Chiang CJ**, Yang YW, Chen JD, You SL, Yang HI, Lee MH, Lai MS, Chen CJ. Significant reduction in end-stage liver diseases burden through the national viral hepatitis therapy program in Taiwan. *Hepatology* 2015; **61**: 1154-1162 [PMID: 25476749 DOI: 10.1002/hep.27630]

6   **Aggarwal A**, Ong JP, Younossi ZM, Nelson DR, Hoffman-Hogg L, Arroliga AC. Predictors of mortality and resource utilization in cirrhotic patients admitted to the medical ICU. *Chest* 2001; **119**: 1489-1497 [PMID: 11348958]

7   **Chen YC**, Tsai MH, Ho YP, Hsu CW, Lin HH, Fang JT, Huang CC, Chen PC. Comparison of the severity of illness scoring systems for critically ill cirrhotic patients with renal failure. *Clin Nephrol* 2004; **61**: 111-118 [PMID: 14989630]

8   **Jalan R**, Stadlbauer V, Sen S, Cheshire L, Chang YM, Mookerjee RP. Role of predisposition, injury, response and organ failure in the prognosis of patients with acute-on-chronic liver failure: a prospective cohort study. *Crit Care* 2012; **16**: R227 [PMID: 23186071 DOI: 10.1186/cc11882]

9   **Levesque E**, Hoti E, Azoulay D, Ichaï P, Habouchi H, Castaing D, Samuel D, Saliba F. Prospective evaluation of the prognostic scores for cirrhotic patients admitted to an intensive care unit. *J Hepatol* 2012; **56**: 95-102 [PMID: 21835136 DOI: 10.1016/j.jhep.2011.06.024]

10 **Shawcross DL**, Austin MJ, Abeles RD, McPhail MJ, Yeoman AD, Taylor NJ, Portal AJ, Jamil K, Auzinger G, Sizer E, Bernal W, Wendon JA. The impact of organ dysfunction in cirrhosis: survival at a cost? *J Hepatol* 2012; **56**: 1054-1062 [PMID: 22245890 DOI: 10.1016/j.jhep.2011.12.014]

11 **Shellman RG**, Fulkerson WJ, DeLong E, Piantadosi CA. Prognosis of patients with cirrhosis and chronic liver disease admitted to the medical intensive care unit. *Crit Care Med* 1988; **16**: 671-678 [PMID: 3371043]

12 **Levesque E**, Saliba F, Ichaï P, Samuel D. Outcome of patients with cirrhosis requiring mechanical ventilation in ICU. *J Hepatol* 2014; **60**: 570-578 [PMID: 24280294 DOI: 10.1016/j.jhep.2013.11.012]

13 **Saliba F**, Ichai P, Levesque E, Samuel D. Cirrhotic patients in the ICU: prognostic markers and outcome. *Curr Opin Crit Care* 2013; **19**: 154-160 [PMID: 23426137 DOI: 10.1097/MCC.0b013e32835f0c17]

14 **Jalan R**, Saliba F, Pavesi M, Amoros A, Moreau R, Ginès P, Levesque E, Durand F, Angeli P, Caraceni P, Hopf C, Alessandria C, Rodriguez E, Solis-Muñoz P, Laleman W, Trebicka J, Zeuzem S, Gustot T, Mookerjee R, Elkrief L, Soriano G, Cordoba J, Morando F, Gerbes A, Agarwal B, Samuel D, Bernardi M, Arroyo V. Development and validation of a prognostic score to predict mortality in patients with acute-on-chronic liver failure. *J Hepatol* 2014; **61**: 1038-1047 [PMID: 24950482 DOI: 10.1016/j.jhep.2014.06.012]

15 **Angulo P**. Nonalcoholic fatty liver disease. *N Engl J Med* 2002; **346**: 1221-1231 [PMID: 11961152 DOI: 10.1056/NEJMra011775]

16 **Tarantino G**, Finelli C. What about non-alcoholic fatty liver disease as a new criterion to define metabolic syndrome? *World J Gastroenterol* 2013; **19**: 3375-3384 [PMID: 23801829 DOI: 10.3748/wjg.v19.i22.3375]

17 **Clark JM**, Diehl AM. Nonalcoholic fatty liver disease: an underrecognized cause of cryptogenic cirrhosis. *JAMA* 2003; **289**: 3000-3004 [PMID: 12799409 DOI: 10.1001/jama.289.22.3000]

18 **Chitturi S**, Farrell GC, George J. Non-alcoholic steatohepatitis in the Asia-Pacific region: future shock? *J Gastroenterol Hepatol* 2004; **19**: 368-374 [PMID: 15012772]

19 **Dassanayake AS**, Kasturiratne A, Rajindrajith S, Kalubowila U, Chakrawarthi S, De Silva AP, Makaya M, Mizoue T, Kato N, Wickremasinghe AR, de Silva HJ. Prevalence and risk factors for non-alcoholic fatty liver disease among adults in an urban Sri Lankan population. *J Gastroenterol Hepatol* 2009; **24**: 1284-1288 [PMID: 19476560 DOI: 10.1111/j.1440-1746.2009.05831.x]

20 **Mohammed OK**, Mahadeva S. Clinical outcomes of cryptogenic compared with non-cryptogenic cirrhosis: A retrospective cohort study. *J Gastroenterol Hepatol* 2015; **30**: 1423-1428 [PMID: 25867030 DOI: 10.1111/jgh.12978]

21 **Cheng CY**, Ho CH, Wang CC, Liang FW, Wang JJ, Chio CC,

Chang CH, Kuo JR. One-Year Mortality after Traumatic Brain Injury in Liver Cirrhosis Patients--A Ten-Year Population-Based Study. *Medicine* (Baltimore) 2015; **94**: e1468 [PMID: 26448001 DOI: 10.1097/MD.0000000000001468]

22   **Cholongitas E**, Senzolo M, Patch D, Shaw S, Hui C, Burroughs AK. Review article: scoring systems for assessing prognosis in critically ill adult cirrhotics. *Aliment Pharmacol Ther* 2006; **24**: 453-464 [PMID: 16886911 DOI: 10.1111/j.1365-2036.2006.02998.x]

23   **Tsai MH**, Chen YC, Ho YP, Fang JT, Lien JM, Chiu CT, Liu NJ, Chen PC. Organ system failure scoring system can predict hospital mortality in critically ill cirrhotic patients. *J Clin Gastroenterol* 2003; **37**: 251-257 [PMID: 12960725]

24   **Fu CM**, Chang CH, Fan PC, Tsai MH, Lin SM, Kao KC, Tian YC, Hung CC, Fang JT, Yang CW, Chen YC. Prognosis of critically ill cirrhotic versus non-cirrhotic patients: a comprehensive score-matched study. *BMC Anesthesiol* 2014; **14**: 123 [PMID: 25580088 DOI: 10.1186/1471-2253-14-123]

25   **Yatsuji S**, Hashimoto E, Tobari M, Taniai M, Tokushige K, Shiratori K. Clinical features and outcomes of cirrhosis due to non-alcoholic steatohepatitis compared with cirrhosis caused by chronic hepatitis C. *J Gastroenterol Hepatol* 2009; **24**: 248-254 [PMID: 19032450 DOI: 10.1111/j.1440-1746.2008.05640.x]

26   **Senanayake SM**, Niriella MA, Weerasinghe SK, Kasturiratne A, de Alwis JP, de Silva AP, Dassanayake AS, de Silva HJ. Survival of patients with alcoholic and cryptogenic cirrhosis without liver transplantation: a single center retrospective study. *BMC Res Notes* 2012; **5**: 663 [PMID: 23198995 DOI: 10.1186/1756-0500-5-663]

27   **Chen CJ**, Shi HY, Lee KT, Huang TY. In-hospital mortality prediction in patients receiving mechanical ventilation in Taiwan. *Am J Crit Care* 2013; **22**: 506-513 [PMID: 24186822 DOI: 10.4037/ajcc2013950]

28   **Chen CM**, Lai CC, Cheng KC, Weng SF, Liu WL, Shen HN. Effect of end-stage renal disease on long-term survival after a first-ever mechanical ventilation: a population-based study. *Crit Care* 2015; **19**: 354 [PMID: 26423892 DOI: 10.1186/s13054-015-1071-x]

29   **Kollef MH**, O'Brien JD, Silver P. The impact of gender on outcome from mechanical ventilation. *Chest* 1997; **111**: 434-441 [PMID: 9041993]

ORIGINAL ARTICLE

Retrospective Study

possible role of soluble fibrin monomer complex after gastroenterological surgery

Masatoshi Kochi, Manabu Shimomura, Takao Hinoi, Hiroyuki Egi, Kazuaki Tanabe, Yasuyo Ishizaki, Tomohiro Adachi, Hirotaka Tashiro, Hideki Ohdan

Masatoshi Kochi, Hiroyuki Egi, Kazuaki Tanabe, Tomohiro Adachi, Hideki Ohdan, Department of Gastroenterological and Transplant Surgery, Applied Life Sciences, Institute of Biomedical and Health Sciences, Hiroshima University, Hiroshima city, Hiroshima 734-8551, Japan

Manabu Shimomura, Department of Surgery, National Hospital Organization Higashihiroshima Medical Center, Higashihiroshima City, Hiroshima 739-0041, Japan

Takao Hinoi, Hirotaka Tashiro, Department of Surgery, Institute for Clinical Research, National Hospital Organization Kure Medical Center and Chu-goku Cancer Center, Kure City, Hiroshima 737-0023, Japan

Yasuyo Ishizaki, Department of Surgery, National Hospital Organization Hiroshima-nishi Medical Center, Otake City, Hiroshima 739-0696, Japan

Author contributions: Shimomura M designed the study; Kochi M and Shimomura M analyzed and interpreted the data; Hinoi T, Egi H, Tanabe K, Ishizaki Y and Adachi T participated in this clinical study and treated the patients; Kochi M and Shimomura M wrote the paper; Ohdan H is the chief clinical investigator; all authors read and approved the final manuscript.

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

Informed consent statement: All patients gave informed consent prior to study enrolment.

Conflict-of-interest statement: The authors have no potential conflicts of interest to disclose.

Data sharing statement: No additional data are available.

Abstract

AIM

To examine the role of soluble fibrin monomer complex (SFMC) in the prediction of hypercoagulable state after gastroenterological surgery.

METHODS

We collected data on the clinical risk factors and fibrin-related makers from patients who underwent gastroenterological surgery at Hiroshima University Hospital between April 1, 2014 and March 31, 2015. We investigated the clinical significance of SFMC, which is known to reflect the early plasmatic activation of coagulation, in the view of these fibrin related markers.

RESULTS

A total of 123 patients were included in the present study. There were no patients with symptomatic VTE. Thirty-five (28%) patients received postoperative anticoagulant therapy. In the multivariate analysis, a high SFMC level on POD 1 was independently associated with D-dimer elevation on POD 7 (OR = 4.31, 95%CI: 1.10-18.30, *P* = 0.03). The cutoff SFMC level was 3.8 μg/mL (AUC = 0.78, sensitivity, 63%, specificity, 89%). The D-dimer level on POD 7 was significantly reduced in high-SFMC patients who received anticoagulant therapy in comparison to high-SFMC patients who did not.

CONCLUSION

The SFMC on POD 1 strongly predicted the hypercoagulable state after gastroenterological surgery than the clinical risk factors and the other fibrin related markers.

Key words: Hypercoagulable state; Gastroenterological surgery; Soluble fibrin monomer complex; Venous thromboembolism; Anticoagulant therapy

Core tip: We found that the plasma level of soluble fibrin monomer complex (SFMC) on POD 1 was more strongly associated with D-dimer elevation on POD 7 than were the clinical risk factors or other fibrin-related markers in 123 cases after gastroenterological surgery, suggesting the possible role of SFMC in the prediction of a hypercoagulable state and subsequent venous thromboembolism. The present study also demonstrated the possibility that the plasma levels of SFMC could be used as an indication for anticoagulant therapy in patients who have undergone gastroenterological surgery.

INTRODUCTION

Venous thromboembolism (VTE) remains a significant complication after gastroenterological surgery. Thrombosis is sometimes fatal and can worsen a patient's quality of life[1]. VTE is an important, potentially preventable condition that has the potential to increase the rates of morbidity and mortality[2,3].

The current American College of Chest Physicians (ACCP 2012) guideline recommends pharmacological prophylaxis with low-molecular weight heparin or low-dose unfractionated heparin in addition to mechanical prophylaxis such as elastic stockings and intermittent pneumatic compression (IPC) for general and abdominal-pelvic surgery patients who are at high risk for VTE (approximately 6.0%)[4,5]. The Caprini score is widely accepted for selecting patients with a high clinical risk for VTE (score ≥ 5); however, the majority of patients who undergo gastroenterological surgery for malignant tumors are considered to be high risk. Although postoperative anticoagulant therapy is regarded as important for preventing VTE, it is not routinely used after gastroenterological surgery, mainly because it is associated with bleeding complications and epidural hematoma after epidural anesthesia.

The risk of VTE varies according to the thrombotic risk factors of individual patients; these include age, sex, obesity, cancer, familial history, infection, heart disease, respiratory disease, hormone treatment and poor functional status[1,4,6]. Thus, in order to confirm a suspected VTE event after gastroenterological surgery, it is important to develop a diagnostic marker with high sensitivity and specificity. The establishment of a marker that can identify patients who are at high risk for VTE will help to minimize the disadvantages associated with anticoagulant therapy and unnecessary radiography.

Soluble fibrin monomer complex (SFMC) appears in the bloodstream during the extremely early stage of blood coagulation. Thrombin cleaves fibrinopeptides from a fibrinogen molecule, and yields a fibrin monomer. When fibrin monomers are produced in the presence of fibrinogens, two fibrinogen molecules and one fibrin monomer create a soluble complex known as SFMC (Figure 1). SFMC reflects the plasmatic activation of coagulation and fibrinolysis[7,8]. However, there is little known about the clinical significance of SFMC after gastroenterological surgery.

The aim of the present study was to examine the possible role of the plasma level of SFMC in the prediction of hypercoagulable state and the subsequent VTE after gastroenterological surgery and to assess whether it can be used to indicate postoperative anticoagulant therapy.

MATERIALS AND METHODS

We retrospectively collected data related to the clinical risk factors for VTE and fibrin-related makers from 135 consecutive patients who had undergone gastroenterological surgery due to a diagnosed malignance or to treat a general abdominal disorder at Hiroshima University Hospital between April 1, 2014, and March 31, 2015. The levels of D-dimer, fibrin degradation products (FDP), SFMC, and thrombin antithrombin complex (TAT) (fibrin-related markers) were measured at four time points in the perioperative period (before and 1, 3, and 7 d after surgery). Twelve patients were excluded from the study due to missing fibrin-related marker data.
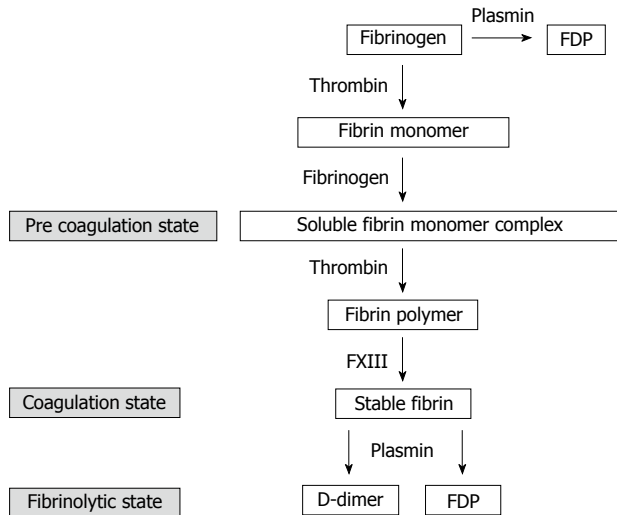
Figure 1 Schema of soluble fibrin monomer complex. Soluble fibrin monomer complex (SFMC) appears in the bloodstream during the extremely early stage of blood coagulation, and reflects the plasmatic activation of coagulation and fibrinolysis.

Symptomatic VTE did not occur in this study population. The D-dimer level on POD 7 reflected the hypercoagulable state after surgery and it have previously demonstrated the association of the presence of VTE; therefore, the D-dimer level on POD 7 was used as the main outcome in this study.

VTE prevention in the perioperative period
Mechanical prophylaxis against VTE, including the postoperative use of elastic stockings (ESs) and IPC was routinely applied in all cases. In the present study, unfractionated heparin (*via* continuous infusion unfractionated heparin for one week at a dose that maintained the APTT at 1.5 to 2 times the reference value) was administrated for the patients who was preoperatively medicated by anticoagulant therapy. Pharmacological prophylaxis was administered to the patients at high clinical risk of VTE, as determined by the original risk classification based on the Caprini score and the Japanese VTE guidelines. The safety and validity of this risk classification were demonstrated in the previous article[9]. Pharmacological prophylaxis was administrated by low molecular weight heparin: Enoxaparin sodium [*via* subcutaneous injection, two times a day, with enoxaparin sodium (2000 IU) for one week]. Thus, postoperative anticoagulant therapy was administrated in 35 patients (28%). Unless contraindicated, anticoagulant therapy initiated from 24 h after surgery to one week after surgery.

Post-operative pain control in patients receiving anticoagulant therapy was achieved *via* intravenous anesthesia (as a substitute for epidural anesthesia).

Plasma sample analyses
The levels of D-dimer (LIAS AUTO® D-dimer NEO, Sysmex, Kobe, Japan), SFMC (AUTO LIA® FM, Sysmex,

Kobe, Japan), and FDP (LIAS AUTO® P-FDP, Sysmex, Kobe, Japan) were measured by the latex agglutination method using a commercial immunoassay kit (LIAS AUTO® D-dimer NEO, Sysmex, Kobe, Japan). All tests were performed on a Sysmex CS5100 analyzer (Sysmex, Kobe, Japan).

TAT was measured by an enzyme-linked immunosorbent assay (HISCL® TAT, Sysmex, Kobe, Japan). This test was performed on a Sysmex HISCL2000i analyzer (Sysmex, Kobe, Japan). In all analyses, statistical significance was set at a *P* value less than 0.05. The standard values were as follows: D-dimer, $\leq$ 1 μg/mL; FDP, $\leq$ 5 μg/mL; SFMC, $\leq$ 7 μg/mL; and TAT, < 4 ng/mL.

Statistical analysis
Pearson's χ^2 test was used to analyze each clinical characteristic, in order to determine the factors associated with postoperative hypercoagulability. These variables were dichotomized in the analysis. Receiver operating characteristic (ROC) curves were created to determine the appropriate cutoff points. Factors with a *P* value of < 0.05 on the univariate analysis were subjected to a multivariate analysis using a logistic regression model. The results of the multivariate analysis are presented as the odds ratio (OR) and 95% CI with the corresponding *P*-value. All of the analyses were performed using the JMP software program (version 11, SAS Institute, Cary, NC, United States). The statistical methods of this study were reviewed by Minoru Hattori from Hiroshima University.

RESULTS
Patient characteristics
The final study population included 123 patients (68 males and 55 females), the median age was 67 years (range, 32 to 89 years), the median operation time was 319 min (range, 74-795), the median bleeding volume was 70 mL (range, 5-4135). The patients' characteristics and clinical data are summarized in Table 1. There were no patients with symptomatic VTE in this study population. Thirty-five patients (28%) received postoperative anticoagulant therapy. Bleeding complications occurred in 5 (14%) patients who received anticoagulant therapy (Clavien-Dindo Grade 1, *n* = 4; Grade 2, *n* = 1).

Univariate and multivariate analyses of the risk factors for D-dimer elevation on POD 7 in patients without anticoagulant therapy
We analyzed the correlation between D-dimer elevation on POD 7 and the clinical risk factors for VTE among the 88 patients who did not receive anticoagulant therapy. The median cutoff level for D-dimer on POD 7 was 6.45. In the univariate analysis the group with a higher D-dimer level ($\geq$ 6.45 μg/mL) on POD 7, included a greater number of patients of $\geq$ 75 years

Table 1 Baseline characteristics of the patients *n* (%)	
Characteristic	***n* = 123**
Age, median (range)	67 (32-89)
Sex, Female	55 (45)
BMI (kg/m²), median (range)	22.8 (15.2-33.2)
Performance status	
0-2	119 (97)
3-4	4 (3)
Surgical procedure	
Gastrectomy	32 (26)
Small bowel resection	5 (4)
Colectomy	45 (36)
Proctectomy	32 (26)
Stoma closure	2 (2)
Others	7 (6)
Surgical technique	
Laparoscopic surgery	86 (70)
Operative time (min), median (range)	319 (74-795)
Bleeding volume (ml), median (range)	70 (5-4135)
Clinical risk factors for VTE	
Malignancy	118 (96)
Metastatic disease	16 (13)
Diabetes mellitus	16 (13)
Varicose vein	1 (0.8)
Hormone therapy	4 (3)
CV catheter	4 (3)
Preoperative infection	7 (6)
Cardiovascular disease	6 (5)
Antiplatelet therapy	10 (8)
Pelvic surgery	22 (18)
Previous history of VTE	0

BMI: Body mass index; CV: Central vein; VTE: Venous thromboembolism.

of age, required a longer surgical time ($\geq$ 321 min), and had a higher levels of D-dimer, FDP, TAT, and SFMC on POD 1 than the group of patients with lower D-dimer levels (< 6.45 μg/mL) on POD 7. According to a multivariate analysis, the SFMC on POD 1 (OR = 4.31, 95%CI: 1.10-18.30, *P* = 0.03) was an independent risk factor for D-dimer elevation on POD7 (Table 2). Their cutoff points with sensitivities and specificities were determined by a ROC analysis. The cutoff point of SFMC was 3.8 μg/mL, with an area under the curve (AUC) of 0.78, a sensitivity of 63% and a specificity of 89%.

Univariate and multivariate analyses of the risk factors for SFMC elevation on POD 1 in the whole study population

We analyzed the correlation between SFMC elevation on POD 1 and the clinical risk factors and surgical factors. In the univariate analysis, there were significant differences in age, the operative time, and the administration of antiplatelet therapy. Subsequently, in the multivariate analysis, age and operative time were found to be independent risk factors for SFMC elevation on POD 1 (Table 3).

Possible indications for anticoagulant therapy based on the SFMC level on POD 1

The anticoagulant therapy group and the no anti-

coagulant therapy group (*n* = 88) were divided into two subgroups [the SFMC-high group (POD 1 SFMC $\geq$ 3.8 μg/mL) and the SFMC-low group (POD 1 SFMC < 3.8 μg/mL)], and the D-dimer levels on PODs 1, 3, and 7 were examined to confirm the patients' hypercoagulability. In the no anticoagulant therapy group, the D-dimer levels were significantly higher at every point of measurement than they were in the SFMC-low group. In the anticoagulant therapy group, however, there was no significant difference in the D-dimer levels on POD 7 (*P* = 0.14). Among the SFMC-High group, the D-dimer level on POD 7 was significantly reduced in patients who underwent anticoagulant therapy in comparison to patients who did not. This suggests the possibility that anticoagulant therapy might be indicated based on the SFMC level.

DISCUSSION

In the current study, we demonstrated the SFMC predicted the postoperative hypercoagulable state more strongly than other clinical risk factors, including the Caprini score and the levels of other fibrin related markers on POD 1.

A hypercoagulable state is a precursor condition of VTE, which is a significant complication that is associated with a poor prognosis, increased morbidity and a longer hospital stay[3]. Since it is well known that most cases of VTE are asymptomatic, perioperative patients who do not receive pharmacological prophylaxis should be carefully monitored to allow for the early detection of VTE[10]. If we could detect the hypercoagulable state and presence of VTE using a simple blood test, we could expect a dramatic reduction in unnecessary imaging examinations, which would reduce both radiation exposure and the use of contrast agents that are needed for computed tomographic pulmonary angiography (CTPA)[11,12]. The aim of a marker that identifies patients with a high risk of developing VTE will help to minimize the disadvantages of anticoagulant therapy and unnecessary radiographic examinations.

SFMC, which reflects acute intravascular fibrin formation, has been recognized as an independent marker for predicting VTE after orthopedic surgery, due to the substantial elevation of SFMC levels in patients who develop VTE[3,10,11,13]. Although SFMC is a cost-effective and safe diagnostic method, little is known about the changes in SFMC levels after gastroenterological surgery. No studies have evaluated SFMC levels or the cutoff SFMC level for the diagnosis of thrombosis after gastroenterological surgery. Since most VTE events occur during the first week after surgery, we evaluated the risk factors based on the characteristics of patients, surgical factors, and blood tests on POD 1 with the aim of detecting suspected cases of VTE during the early postoperative phase[3,7,10,11,13].

In the current study, we demonstrated that among

Table 2 The relationship between the D-dimer level on POD 7 and the clinical characteristics of patients who did not receive anticoagulant therapy

Clinical risk factors for VTE	D-dimer (POD 7)		Univariate	Multivariate		
	Low (< 6.45)	High (≥ 6.45)	P value	OR	95%CI	P value
Age						
< 75	37	26	< 0.01	2.48	0.70-9.21	0.15
≥ 75	7	18				
Sex						
Male	20	21	0.83			
Female	24	23				
Performance status						
0-2	43	43	1.00			
3-4	1	1				
Operative time (min)						
< 321	32	19	< 0.01	2.09	0.64-6.92	0.21
≥ 321	12	25				
Bleeding volume (mL)						
< 113	35	28	0.09			
≥ 113	9	16				
Laparoscopic surgery						
No	11	16	0.24			
Yes	33	28				
Malignancy						
Absence	2	3	0.64			
Presence	42	41				
Metastatic disease						
Absence	38	39	0.74			
Presence	6	5				
Diabetes mellitus						
Absence	39	39	1.00			
Presence	5	5				
Hormone therapy						
Absence	43	43	1.00			
Presence	1	1				
CV catheter						
Absence	42	43	0.55			
Presence	2	1				
Preoperative infection						
Absence	42	41	0.64			
Presence	2	3				
Antiplatelet therapy						
Absence	41	40	0.69			
Presence	3	4				
Pelvic surgery						
Absence	41	41	1.00			
Presence	3	3				
Caprini score						
< 7	24	15	0.05			
≥ 7	20	29				
Fibrin-related markers						
D-dimer (μg/mL) < 0.6	17	12	0.11			
Preoperative ≥ 0.6	13	21				
D-dimer (μg/mL) < 3.8	33	11	< 0.01	2.88	0.56-14.82	0.19
POD1 ≥ 3.8	11	33				
FDP (μg/mL) < 10.1	38	18	< 0.01	1.42	0.25-7.65	0.68
POD1 ≥ 10.1	6	26				
TAT (ng/mL) POD1						
< 8.3	34	12	< 0.01	1.83	0.44-7.27	0.39
≥ 8.3	10	32				
SFMC (μg/mL) < 3.8	39	16	< 0.01	4.31	1.10-18.30	0.03
POD1 ≥ 3.8	5	28				

VTE: Venous thromboembolism; CV: Central vein; FDP: Fibrin degradation products; TAT: Thrombin antithrombin complex; SFMC: Soluble fibrin monomer complex; POD: Postoperative day.

88 patients without anticoagulant therapy, the SFMC level on POD 1 was an independent risk factor for D-dimer elevation on POD 7. There were no significant differences in the other clinical risk factors or fibrin-related markers. With a cutoff point of 3.8 μg/mL, the diagnostic sensitivity, specificity and odds ratio of the SFMC on POD 1 were 63%, 89% and 4.31, respectively. In previous studies in which SFMC was used to predict VTE (cutoff points: 7.05-19.8 μg/mL) the sensitivity and specificity were 88% and 62 to 90%, respectively[10,13]. This difference in the cutoff points is considered to be due to the clinical endpoint (D-dimer elevation or the occurrence of VTE). An ROC analysis showed moderate accuracy in the prediction of D-dimer elevation on POD 7 using a cutoff point of 6.45 μg/mL (AUC: 0.78). Given that some reports used postoperative D-dimer cutoff values of 6.1 to 7.5 μg/mL for predicting the VTE, we consider this clinical endpoint to be reasonable[11,14].

Although, patients who are considered to have a high clinical risk for VTE based on the presence of risk factors such as pelvic surgery, obesity, and a previous history of thrombosis tend to receive appropriate perioperative anticoagulant therapy, the administration of perioperative anticoagulant therapy to patients who are deemed to have a low clinical risk of VTE is controversial[15]. Surgeons may withhold perioperative anticoagulant therapy due to the risk of bleeding complications. Major bleeding is reported to occur in 2.9% to 9.4% of patients during the period of pharmacological prophylaxis[16,17]. In the current study, post-operative bleeding complications, including subcutaneous bleeding, conjunctival bleeding, melena, and intraabdominal hemorrhage, occurred in 11.4% (5 of 35) of the patients. However, the incidence of major bleeding that necessitated a blood transfusion was 2% (1 of 35). The bleeding complications were classified as Grade 1, *n* = 4; Grade 2, *n* = 1 (Clavien-Dindo classification). We were therefore able to administer chemoprophylaxis without serious bleeding complications. Although this study did not use epidural anesthesia to avoid the risk of spinal epidural hematoma, previous studies have reported spinal

Clinical risk factors for VTE	SFMC (POD 1)		Univariate	Multivariate		
	Low (< 3.8)	High (≥ 3.8)	*P* value	OR	95%CI	*P* value
Age						
< 75	59	27	0.01	2.44	1.07-5.66	0.03
≥ 75	16	21				
Sex						
Male	45	23	0.18			
Female	30	25				
BMI (kg/m²)						
< 27	71	47	0.37			
≥ 27	4	1				
Performance status						
0-2	73	46	0.64			
3-4	2	2				
Operative time (min)						
< 321	45	18	0.01	2.33	1.08-5.12	0.02
≥ 321	30	30				
Bleeding volume (mL)						
< 113	47	28	0.63			
≥ 113	28	20				
Laparoscopic surgery						
No	25	12	0.32			
Yes	50	36				
Malignancy						
Absence	5	0	0.06			
Presence	70	48				
Metastatic disease						
Absence	65	42	0.89			
Presence	10	6				
Diabetes mellitus						
Absence	67	40	0.33			
Presence	8	8				
Varicose vein						
Absence	75	47	0.2			
Presence	0	1				
Hormone therapy						
Absence	72	47	0.55			
Presence	3	1				
CV catheter						
Absence	72	47	0.55			
Presence	3	1				
Preoperative infection						
Absence	71	45	0.83			
Presence	4	3				
Cardiovascular disease						
Absence	73	44	0.15			
Presence	2	4				
Antiplatelet therapy						
Absence	72	41	0.03	3.04	0.74-15.30	0.12
Presence	3	7				
Pelvic surgery						
Absence	62	39	0.84			
Presence	13	9				
Caprini score						
< 7	36	17	0.16			
≥ 7	39	31				
Fibrin-related markers						
D-dimer (μg/mL) < 0.6	26	11	0.12			
Preoperative ≥ 0.6	30	25				

Table 3 The relationship between the soluble fibrin monomer complex on POD 1 and the clinical characteristics of the patients

SFMC: Soluble fibrin monomer complex; POD: Postoperative day; VTE: Venous thromboembolism; BMI: Body mass index; CV: Central vein.

epidural hematoma due to anticoagulant therapy to be extremely rare[18]. Thus, the use of epidural anesthesia during anticoagulant therapy should be the subject of future studies.

In the current study, elderly patients and a longer duration of surgery had an impact on the occurrence of SFMC elevation on POD 1 (Table 3), and anticoagulant therapy inhibited D-dimer elevation on POD 7 in the SFMC-high group (Figure 2). These results suggest that the selective administration of anticoagulant therapy to the patients of the SFMC-high group, especially patients who had these two risk factors, might be effective for preventing the development of VTE.

The relationship between the preoperative SFMC and D-dimer levels and the development of VTE after surgery is important; however, the preoperative SFMC and D-dimer levels could not predict postoperative VTE in previous studies[10,13]. These studies indicate that the preoperative SFMC level was not increased in patients who developed postoperative VTE.

This study is associated with several limitations. First, because there were no cases of symptomatic VTE was found, the D-dimer level on POD 7, which is well known to have high sensitivity (79%-95%) and a negative predictive value of nearly 100%, was used for the clinical endpoint, rather than the occurrence of VTE[10,19,20]. However, D-dimer elevation can also represent infection, malignancy, heart failure, chronic renal disease and liver disease[19]. Thus, it might not reflect true VTE. It would be therefore be better to consider the inclusion of asymptomatic VTE and to confirm the cutoff points in further clinical studies. Second, the further diagnostic work-up of patients with asymptomatic VTE, such as ultrasound, CT and CTPA, was performed at the discretion of the surgical team.

In conclusion, the plasma level of SFMC on POD 1 strongly associated with D-dimer elevation on POD7 than the clinical risk factors and the other fibrin related markers, which indicated the possible role of SFMC in the prediction of hypercoagulable state and subsequent VTE. The present study also demonstrated the possibility that the plasma levels of SFMC could be used as an indication for anticoagulant therapy, and the selective administration of anticoagulant therapy to the patients of the SFMC-high group would be effective for preventing the development of VTE. We are planning to perform another prospective study to examine the protective effects against VTE that are achieved by administering anticoagulant therapy based

A

Anticoagulant therapy (*n* = 35)

B

Non-anticoagulant therapy (*n* = 88)
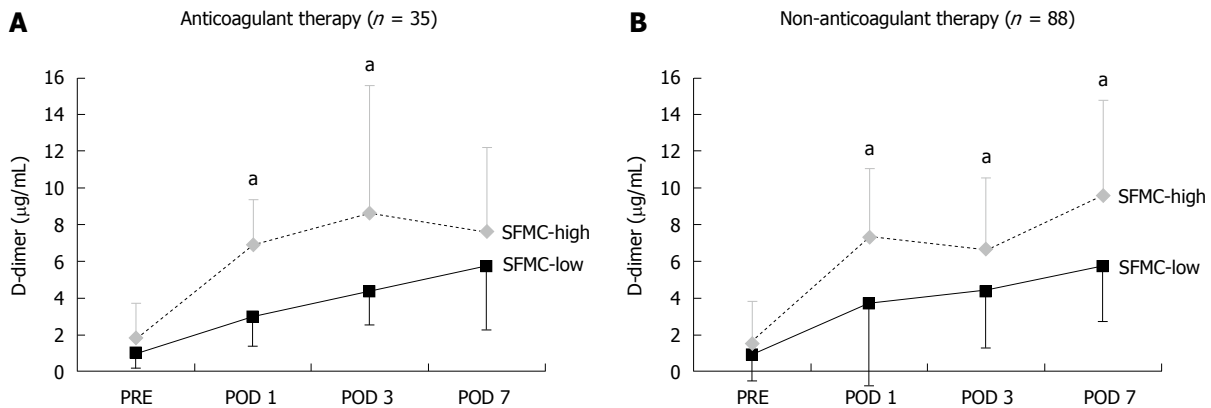
Figure 2 The postoperative kinetics of the D-dimer levels in patients who received anticoagulant therapy (A) and those who did not receive anticoagulant therapy (B). In the soluble fibrin monomer complex (SFMC)-High group, the plasma levels of D-dimer (POD 7) in patients who received anticoagulant therapy were reduced in comparison to those who did not. The mean D-dimer level ± SD in the SFMC-High group and - the SFMC-Low group. [a]$P < 0.05$.

on the plasma levels of SFMC on POD 1.

ACKNOWLEDGMENTS

COMMENTS

Background

Venous thromboembolism (VTE) remains a significant complication after gastroenterological surgery. Therefore, a diagnostic marker with high sensitivity and specificity that can be used to identify patients at high risk for a hypercoagulable state and subsequent VTE will help to minimize the disadvantages associated with anticoagulant therapy and unnecessary radiography.

Research frontiers

The risk of VTE varies according to the thrombotic risk factors of individual patients; these include age, sex, obesity, cancer, family history, infection, heart disease, respiratory disease, hormone treatment and poor functional status. However, there is no simple maker that detects the hypercoagulable state and the presence of VTE after gastroenterological surgery.

Innovations and breakthroughs

This paper reports that the soluble fibrin monomer complex (SFMC) on POD 1 was more strongly associated with D-dimer elevation on POD 7 than were the clinical risk factors or other fibrin-related markers after gastroenterological surgery.

Applications

SFMC was able to be used as a marker to predict a postoperative hypercoagulable state and subsequent VTE after gastroenterological surgery. The present study also demonstrated the possibility that the plasma levels of SFMC could be used as an indication for anticoagulant therapy for patients who have undergone gastroenterological surgery.

Terminology

SFMC appears in the bloodstream during the extremely early stage of blood coagulation. It reflects the plasmatic activation of coagulation and fibrinolysis.

Peer-review

The authors examined the role of SFMC in the prediction of hypercoagulable state after gastroenterological surgery, and they concluded that the SFMC on POD 1 strongly predicted the hypercoagulable state after gastroenterological surgery than the clinical risk factors and the other fibrin related markers. VTE

is serious problem after surgery. This article is thought to be significant for prediction of hypercoagulable state on early phase after gastroenterological surgery.

REFERENCES

1   **Yamashita Y**, Wada H, Nomura H, Mizuno T, Saito K, Yamada N, Asanuma K, Usui M, Kamimoto Y, Matsumoto T, Ohishi K, Katayama N. Elevated fibrin-related markers in patients with malignant diseases frequently associated with disseminated intravascular coagulation and venous thromboembolism. *Intern Med* 2014; **53**: 413-419 [PMID: 24583428]

2   **Hamidi S**, Riazi M. Cutoff values of plasma d-dimer level in patients with diagnosis of the venous thromboembolism after elective spinal surgery. *Asian Spine J* 2015; **9**: 232-238 [PMID: 25901235 DOI: 10.4184/asj.2015.9.2.232]

3   **Moghadamyeghaneh Z**, Hanna MH, Carmichael JC, Nguyen NT, Stamos MJ. A nationwide analysis of postoperative deep vein thrombosis and pulmonary embolism in colon and rectal surgery. *J Gastrointest Surg* 2014; **18**: 2169-2177 [PMID: 25213583 DOI: 10.1007/s11605-014-2647-5]

4   **Gould MK**, Garcia DA, Wren SM, Karanicolas PJ, Arcelus JI, Heit JA, Samama CM. Prevention of VTE in nonorthopedic surgical patients: Antithrombotic Therapy and Prevention of Thrombosis, 9th ed: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines. *Chest* 2012; **141**: e227S-e277S [PMID: 22315263 DOI: 10.1378/chest.11-2297]

5   **Guyatt GH**, Akl EA, Crowther M, Gutterman DD, Schuünemann HJ; American College of Chest Physicians Antithrombotic Therapy and Prevention of Thrombosis Panel. Executive summary: Antithrombotic Therapy and Prevention of Thrombosis, 9th ed: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines. *Chest* 2012; **141**: 7S-47S [PMID: 22315257 DOI: 10.1378/chest.1412S3]

6   **Erem HH**, Kiran RP, Remzi FH, Vogel JD. Venous thromboembolism in colorectal surgery: skip SCIP or comply? *Tech Coloproctol* 2014; **18**: 719-724 [PMID: 24562596 DOI: 10.1007/s10151-014-1129-9]

7   **Galster H**, Kolb G, Kohsytorz A, Seidlmayer C, Paal V. The pre-, peri-, and postsurgical activation of coagulation and the thromboembolic risk for different risk groups. *Thromb Res* 2000; **100**: 381-388 [PMID: 11150579]

8   **Ieko M**, Nakabayashi T, Tarumi T, Naito S, Yoshida M, Kanazawa K, Mizukami K, Koike T. Soluble fibrin monomer degradation products as a potentially useful marker for hypercoagulable states with accelerated fibrinolysis. *Clin Chim Acta* 2007; **386**: 38-45 [PMID: 17803984 DOI: 10.1016/j.cca.2007.07.023]

9   **Shimomura M**, Kochi M, Hinoi T, Egi H, Adachi T, Kobayashi T, Tashiro T, Ohdan H. Clinical significance of Pharmacological

Prophylaxis based on the Original Classification of Venous Thromboembolism after Lower Abdominal Surgery. *Hiroshima J Med Sci* 2016; **65**: 53-59

10 **Yukizawa Y**, Inaba Y, Watanabe S, Yajima S, Kobayashi N, Ishida T, Iwamoto N, Choe H, Saito T. Association between venous thromboembolism and plasma levels of both soluble fibrin and plasminogen-activator inhibitor 1 in 170 patients undergoing total hip arthroplasty. *Acta Orthop* 2012; **83**: 14-21 [PMID: 22248164 DOI: 10.3109/17453674.2011.652886]

11 **Watanabe H**, Madoiwa S, Sekiya H, Nagahama Y, Matsuura S, Kariya Y, Ohmori T, Mimuro J, Hoshino Y, Hayasaka S, Sakata Y. Predictive blood coagulation markers for early diagnosis of venous thromboembolism after total knee joint replacement. *Thromb Res* 2011; **128**: e137-e143 [PMID: 21839493 DOI: 10.1016/j.thromres.2011.07.030]

12 **Wells PS**, Anderson DR, Rodger M, Stiell I, Dreyer JF, Barnes D, Forgie M, Kovacs G, Ward J, Kovacs MJ. Excluding pulmonary embolism at the bedside without diagnostic imaging: management of patients with suspected pulmonary embolism presenting to the emergency department by using a simple clinical model and d-dimer. *Ann Intern Med* 2001; **135**: 98-107 [PMID: 11453709]

13 **Wada H**, Kobayashi T, Abe Y, Hatada T, Yamada N, Sudo A, Uchida A, Nobori T. Elevated levels of soluble fibrin or D-dimer indicate high risk of thrombosis. *J Thromb Haemost* 2006; **4**: 1253-1258 [PMID: 16706968 DOI: 10.1111/j.1538-7836.2006.01942.x]

14 **Jiang Y**, Li J, Liu Y, Li YC, Zhang WG. Risk factors for deep vein thrombosis after orthopedic surgery and the diagnostic value of D-dimer. *Ann Vasc Surg* 2015; **29**: 675-681 [PMID: 25728333 DOI: 10.1016/j.avsg.2014.12.022]

15 **Nelson DW**, Simianu VV, Bastawrous AL, Billingham RP, Fichera A, Florence MG, Johnson EK, Johnson MG, Thirlby RC, Flum DR, Steele SR. Thromboembolic Complications and Prophylaxis Patterns in Colorectal Surgery. *JAMA Surg* 2015; **150**: 712-720 [PMID: 26060977 DOI: 10.1001/jamasurg.2015.1057]

16 **Simonneau G**, Laporte S, Mismetti P, Derlon A, Samii K, Samama CM, Bergman JF. A randomized study comparing the efficacy and safety of nadroparin 2850 IU (0.3 mL) vs. enoxaparin 4000 IU (40 mg) in the prevention of venous thromboembolism after colorectal surgery for cancer. *J Thromb Haemost* 2006; **4**: 1693-1700 [PMID: 16796710 DOI: 10.1111/j.1538-7836.2006.02083.x]

17 **Agnelli G**, Bergqvist D, Cohen AT, Gallus AS, Gent M. Randomized clinical trial of postoperative fondaparinux versus perioperative dalteparin for prevention of venous thromboembolism in high-risk abdominal surgery. *Br J Surg* 2005; **92**: 1212-1220 [PMID: 16175516 DOI: 10.1002/bjs.5154]

18 **Singelyn FJ**, Verheyen CC, Piovella F, Van Aken HK, Rosencher N. The safety and efficacy of extended thromboprophylaxis with fondaparinux after major orthopedic surgery of the lower limb with or without a neuraxial or deep peripheral nerve catheter: the EXPERT Study. *Anesth Analg* 2007; **105**: 1540-157, table of contents [PMID: 18042845 DOI: 10.1213/01.ane.0000287677.95626.60]

19 **Kim YJ**, Im S, Jang YJ, Park SY, Sohn DG, Park GY. Diagnostic Value of Elevated D-Dimer Level in Venous Thromboembolism in Patients With Acute or Subacute Brain Lesions. *Ann Rehabil Med* 2015; **39**: 1002-1010 [PMID: 26798616 DOI: 10.5535/arm.2015.39.6.1002]

20 **Pabinger I**, Ay C. Biomarkers and venous thromboembolism. *Arterioscler Thromb Vasc Biol* 2009; **29**: 332-336 [PMID: 19228607 DOI: 10.1161/ATVBAHA.108.182188]

ORIGINAL ARTICLE

Observational Study

Comparing acid steatocrit and faecal elastase estimations for use in M-ANNHEIM staging for pancreatitis

M Ganesh Kamath, C Ganesh Pai, Asha Kamath, Annamma Kurien

M Ganesh Kamath, Department of Physiology, Melaka Manipal Medical College, Manipal University, Manipal 576104, India

C Ganesh Pai, Department of Gastroenterology and Hepatology, Kasturba Medical College, Manipal University, Manipal 576104, India

Asha Kamath, Department of Community Medicine, Kasturba Medical College, Manipal University, Manipal 576104, India

Annamma Kurien, Department of Pathology, Melaka Manipal Medical College, Manipal University, Manipal 576104, India

Author contributions: Pai CG proposed the study; Kamath MG and Pai CG wrote the first draft; Kamath MG and Kurien A performed the research; Kamath MG and Pai CG analysed the lab parameters; Kamath MG and Pai CG collected the data and assisted Kamath A, who performed the statistical analysis; all authors contributed to the design and interpretation of the study and to further drafts; Pai CG is the guarantor.

Institutional review board statement: The study protocol was reviewed and approved by University Ethics Committee of Manipal University.

Informed consent statement: All study participants or their legal guardians provided written informed consent prior to study enrolment.

Conflict-of-interest statement: The authors have no conflict of interest to declare.

Data sharing statement: No additional data are available.

Abstract

AIM

To compare two tests for exocrine pancreatic function (EPF) for use in M-ANNHEIM staging for pancreatitis.

METHODS

One hundred and ninety four consecutive patients with acute pancreatitis (AP; $n = 13$), recurrent acute pancreatitis (RAP; $n = 65$) and chronic pancreatitis (CP; $n = 116$) were enrolled. EPF was assessed by faecal elastase-1 (FE-1) estimation and stool fat excretion by the acid steatocrit method. Patients were classified as per M-ANNHEIM stages separately based on the results of the two tests for comparison. Independent Student's t-test, χ^2 test, Kruskal-Wallis test, Mann-Whitney U test and McNemar's test were used as appropriate.

RESULTS

Sixty-one (52.5%) patients with CP had steatorrhoea when assessed by the acid steatocrit method; 79

(68.1%) with CP had exocrine insufficiency by the FE-1 test (χ^2 test, $P < 0.001$). The results of acid steatocrit and FE-1 showed a significant negative correlation (Spearman's rho = -0.376, $P < 0.001$). A statistically significant difference was seen between the M-ANNHEIM stages as classified separately by acid steatocrit and the FE-1. Thirteen (6.7%), 87 (44.8%), 89 (45.8%) and 5 (2.5%) patients were placed in M-ANNHEIM stages 0, Ⅰ, Ⅱ, and Ⅲ respectively, with the use of acid steatocrit as against 13 (6.7%), 85 (43.8%), 75 (38.6%), and 21 (10.8%) respectively by FE-1 in stages 0, Ⅰ, Ⅱ, and Ⅲ thereby altering the stage in 28 (14.4%) patients ($P < 0.001$, McNemar's test).

CONCLUSION
FE-1 estimation performed better than the acid steatocrit test for use in the staging of pancreatitis by the M-ANNHEIM classification since it diagnosed a higher proportion of patients with exocrine insufficiency.

Key words: Chronic pancreatitis; Pancreatic function tests; Pancreatic elastase; Staging; Steatorrhoea

Core tip: Patients with acute, recurrent acute and chronic pancreatitis were classified as per M-ANNHEIM stages, separately based on the results of two exocrine function tests (acid steatocrit method and faecal elastase test) for comparison. A statistically significant difference was seen between the M-ANNHEIM stages as classified separately by the two tests. faecal elastase-1 estimation performed better than the acid steatocrit test for use in the staging of pancreatitis by the M-ANNHEIM classification since it diagnosed a higher proportion of patients with exocrine function.

INTRODUCTION
Steatorrhoea from pancreatic insufficiency increases in frequency as chronic pancreatitis (CP) advances and forms an important parameter for staging the disease in various classification systems[1-3]. The M-ANNHEIM classification, a new system for staging and assessing the severity of pancreatitis, subdivides the disease into 5 stages based on pain and pancreatic functions[1]. Different pancreatic function tests (PFT) and tests for assessing steatorrhoea have been in use for assessing exocrine pancreatic function (EPF) in patients with CP[4]. PFT have also been used for diagnosing CP

when imaging studies are inconclusive for the same as happens in early stages of the disease[4]. Direct PFT like the secretin test have a greater sensitivity and help in diagnosing CP in its moderate to late stages as compared to early stages of the disease[4]. However, the test is cumbersome, not easily available, poorly standardised across centres, poses difficulty in measuring the enzyme output and is poorly tolerated by some patients due to the need for oro-duodenal intubation[5]. The 72-h quantitative faecal fat estimation is considered the best method for assessing steatorrhoea. A major drawback of this method has been the need to collect stool specimen for 72 h and to store and process them[6].

The acid steatocrit method correlates well with the 72-h quantitative faecal fat estimation and has a sensitivity, specificity and positive predictive value of 100%, 95% and 90% respectively, and acts as an easier alternative[7,8]. The other advantages of this method are its simplicity, reliability and cost-effectiveness for evaluating steatorrhoea in CP[8-11].

Faecal elastase-1 (FE-1), is a useful indirect pancreatic function test in which a random spot stool sample can be used to identify exocrine pancreatic insufficiency (EPI) in well established CP, the situation in which steatorrhoea commonly occurs[12-14]. Studies indicate that FE-1 is useful in estimating fat malabsorption in CP and correlates well with the acid steatocrit method[15].

Not many studies have compared FE-1 and the acid steatocrit method for evaluating EPF in CP. The aim of our study was to determine the usefulness of stool fat analysis by the acid steatocrit method and FE-1 estimation in the staging of pancreatitis using the M-ANNHEIM classification system.

MATERIALS AND METHODS
Patients
Consecutive patients with pancreatitis presenting to the Department of Gastroenterology and Hepatology, Kasturba Hospital, Manipal between June 2009 and June 2013 were prospectively enrolled in this cross sectional study. Patients underwent detailed clinical evaluation and were classified to have AP, RAP and CP. AP was defined as a single episode of any two of typical upper abdominal pain, raised serum amylase and/or lipase three times above the upper limit of normal and evidence of pancreatitis on imaging[16]. Patients presenting with more than one episode of acute pancreatitis with complete resolution of symptoms in between the episodes and no evidence of CP on imaging were considered to have RAP[17,18]. CP was defined by the presence of pancreatic calcifications and/or ductal changes, visualized by ultrasonography, computed tomography (CT), endoscopic ultrasound (EUS) ("consistent with" and "suggestive of" CP by the Rosemont criteria), endoscopic retrograde cholangiopancreatography or magnetic resonance

Table 1 Demographic and clinical features of patients identified with pancreatitis based on imaging criteria

	AP (*n* = 13)	RAP (*n* = 65)	CP (*n* = 116)	*P* value
Age (yr) (mean ± SD)	29.8 ± 11.6	29.0 ± 11.5	33.3 ± 14.2	0.10
Male: female	12 : 1	57 : 8	96 : 20	0.53
Alcoholic pancreatitis	2 (15.4)	19 (29.2)	28 (24.1)	0.10
(⩾ 50 g/d)				
Idiopathic pancreatitis	11 (84.6)	46 (70.8)	88 (75.9)	0.52
Duration of symptoms (in months)	0 (0-0.2)	7.0 (3.5-24.0)	24.0 (4.0-48.0)	< 0.001
[median (interquartile range)]				
VAS (mean ± SD)	5.4 ± 2.0	6.4 ± 2.31	5.4 ± 2.5	0.02

A *P* value of < 0.05 was considered statistically significant.

cholangiopancreatography (MRCP)[19,20].

Stool samples were collected from all patients in two separate containers and one sample was stored at -80 ℃, for estimation of FE-1 by ELISA by using a monoclonal antibody based ELISA kit (ScheBo Biotech, Giessen, Germany) as per manufacturer's instructions. Values of ⩾ 200 μg per gram of stool, 100 and 200 μg per gram and < 100 μg per gram were categorised as normal, mild to moderate EPI and severe insufficiency respectively[21].

Stool fat estimation by the acid steatocrit method

Semiquantitative stool fat estimation by the acid steatocrit method was done on random spot stool samples as proposed by Tran *et al*[11]. 500 mg of stool was diluted with water and homogenized for 2 to 5 min. 500-μL aliquot of the homogenized stool were added with 100 mL of Perchloric acid and the pH was confirmed to be < 1. The mixture was aspirated into a capillary tube, sealed at one end and centrifuged at 13000 revolutions per minute for exactly 15 min[9,11]. The length of the fatty layer and the length of the solid layer were measured. Acid steatocrit (%) was obtained by the formula: fatty layer/(fatty layer + solid layer) × 100. The stool fat (in grams/day) was calculated by the equation: -0.43 + (0.45 × acid steatocrit %)[9]. Steatorrhoea was diagnosed when the stool fat excretion was 7 g/d or higher[4].

Patients were classified as per the M-ANNHEIM staging system first using the acid steatocrit method and then by using the FE-1 test also for comparison.

Statistical analysis

Independent Student's *t*-test and the χ^2 test were used as appropriate. Spearman's rho was used to analyse the correlation between the results of the two tests for exocrine function. The Kruskal-Wallis test was used to compare non normal continuous variables between the various M-ANNHEIM stages. A *P* value of < 0.05 was considered as statistically significant. The Mann-Whitney *U* test was used to compare continuous variables between any two M-ANNHEIM stages with Boneferonni adjustments for multiple pairwise comparisons considering a *P* value of < 0.008 as statistically significant for 6-pairwise

comparison. The McNemar's test was used to compare the nominal data. A *P* value of < 0.05 was considered as statistically significant. The statistical review for this study was performed by a biomedical statistician.

The study protocol was approved by the Ethics Committee of Manipal University. All study participants or their legal guardians provided written informed consent prior to study enrolment.

RESULTS

Of the 194 consecutive patients recruited, 13 (6.8%) had AP, 65 (33.5%) had RAP and 116 (59.7%) had CP. Their baseline characteristics are shown in Table 1.

Correlation between exocrine insufficiency assessed by acid steatocrit and FE-1 estimation

EPI was tested by acid steatocrit and FE-1 by ELISA in all 194 patients. Stool fat analysis by acid steatocrit method showed a significant negative correlation (Spearman's rho = -0.376, *P* < 0.001) with FE-1 indicating that both methods had a good agreement for assessing EPI. None of the patients with AP or RAP showed evidence of EPI by either test. Among a total of 116 patients with CP, 61 (52.5%) and 79 (68.1%) patients showed the presence of EPI by the acid steatocrit method and FE-1 respectively. This difference was statistically significant (χ^2 test, *P* < 0.001).

M-ANNHEIM staging using the acid steatocrit test

Since all patients in the present study consulted for abdominal pain, there were no patients with stage Ⅳ disease as per the M-ANNHEIM classification. The median (IQR) stool fat excretion levels as assessed by the acid steatocrit method were significantly different between the M-ANNHEIM stages 0, Ⅰ, Ⅱ and Ⅲ in a 6-pairwise comparison (*P* < 0.001, by Kruskal-Wallis test; Table 2). The stool fat excretion was also significantly different when compared between any two stages except between stages 0 and Ⅰ (Table 2).

M-ANNHEIM staging using FE-1 estimation

The median (IQR) FE-1 values were significantly different between the different M-ANNHEIM stages in

Table 2 Stool fat in grams/day by acid steatocrit in M-ANNHEIM stages of pancreatitis

M-ANNHEIM stage (*n* %)	Median (IQ range) of stool fat in g/d
0, 13 (6.7)	6.3 (6.0-6.6)
I , 87 (44.8)	6.3 (5.9-6.4)
II , 89 (45.8)	7.5 (6.4-10.8)
III, 5 (2.5)	15.3 (12.0-15.6)

A statistically significant difference was present between the different M-ANNHEIM stages (*P* < 0.001, Kruskal-Wallis test). Comparison between any two stages showed a statistically significant difference between stages 0 and I , and stages II and III (*P* = 0.002, Mann-Whitney *U* test) and also between stages 0 and III, I and II , I and III (*P* < 0.001; Mann-Whitney *U* test). A *P* value of < 0.008 was considered statistically significant for such comparisons between any two groups after Alpha adjustment.

Table 3 Faecal elastase-1 levels in M-ANNHEIM stages of pancreatitis

M-ANNHEIM stage (*n* %)	Median (IQ range) of stool fat in g/d
0, 13 (6.7)	289.0 (249.0-383.2)
I , 85 (43.8)	389.1 (263.2-436.1)
II , 75 (38.6)	144.3 (108.9-219.0)
III, 21 (10.8)	87.6 (41.1-119.1)

A statistically significant difference was present between the different M-ANNHEIM stages (*P* < 0.001, Kruskal-Wallis test). Comparison between stages 0 and II, 0 and III, I and II, and II and III showed a statistically significant difference (*P* < 0.001 for all comparisons, Mann-Whitney *U* test). A *P* value of < 0.008 was considered statistically significant for such comparisons between any two groups after Alpha adjustment.

a 6-pairwise comparison (*P* < 0.001, by Kruskal-Wallis test, Table 3). These values were also significantly different when compared between any two stages except between stages 0 and I (Table 3).

Tests for exocrine function - relevance to M-ANNHEIM staging

To determine the usefulness of the two methods of assessing EPI for use in the M-ANNHEIM staging, we compared the number of patients in M-ANNHEIM stages obtained separately by using acid steatocrit and FE-1 estimations. As shown in Table 4, 28 (14.4%) patients had a change in stage by using FE-1 as against the use of acid steatocrit. 7 (3.6%), 5 (2.5%), 16 (8.2%) shifted from stage I to II , II to I and II to III respectively. This difference was statistically significant (*P* < 0.001, Mc Nemar's test; Table 4).

DISCUSSION

By comparing M-ANNHEIM stages of pancreatitis as determined by using the acid steatocrit method and FE-1 levels we have shown that 14.4% of patients had a change in stage, most often a move to a higher stage, with the use of the latter. This is because FE-1 estimation confirmed EPI in a significantly higher

Table 4 Comparing the number of patients based on M-ANNHEIM staging by acid steatocrit and faecal elastase-1 estimations *n* (%)

M-ANNHEIM stages	Acid steatocrit method	FE-1 test
0	13 (06.7)	13 (06.7)
I	87 (44.8)	85 (43.8)
II	89 (45.8)	75 (38.6)
III	05 (2.5)	21 (10.8)

A *P* value < 0.05 was considered statistically significant. A statistically significant difference was present between the number of those assessed by both methods in M-ANNHEIM stages (*P* < 0.001, Mc Nemar's test). FE-1: Faecal elastase-1.

number of patients compared to the acid steatocrit method. Though the tests used in our study measure different aspects of EPI *i.e.*, enzyme secretion and fat excretion respectively, the results of the two showed a high degree of correlation as expected. The lower rate of detection of EPI by the acid steatocrit test could possibly be attributed to the disadvantages this method. These include a lack of standardisation of the test and the effect of dietary fat intake at the time of sample collection on the test results[15,22]. The number of patients in M-ANNHEIM stages 0 and III were smaller and a higher number would have enhanced the quality of this study.

Unlike with the acid steatocrit method FE-1 estimation offers many advantages. In addition to its high sensitivity for assessing moderate to severe EPI, it correlates well with the findings of imaging studies in patients with CP and unlike other pancreatic enzymes such as chymotrypsin, elastase is not degraded as it passes through the gut[6,15,23-26]. Bian *et al*[27] have shown that the secretin-enhanced MRCP (sMRCP) significantly correlates with the FE-1 test to quantify the pancreatic exocrine function in patients with CP based on the M-ANNHEIM staging. However, sMRCP has its own limitations in the detection of EPI in patients with CP, given its high cost, the semiquantitaive nature of its results and a modest sensitivity of 69%[28]. The limitations of FE-1 estimation such as its lower sensitivity for detecting mild EPI should however be kept in mind while using this test[4,6].

Estimation of 72-h stool fat excretion and the secretin test are considered the gold standard for assessing steatorrhoea and EPI respectively. It is likely that these tests would have provided different results if we had used them in the M-ANNHEIM staging of pancreatitis. A recent study showed that FE-1 is highly sensitive to diagnose EPI, but low on specificity as compared to the 72-h stool fat excretion test[29]. However, 72-h stool fat excretion and the secretin test are demanding on patients and laboratories alike and are hence uncommonly used at present[6]. It is unlikely that a simple test for steatorrhoea like the spot faecal fat test using Sudan staining would have performed

any better than FE-1 estimation but this needs to be evaluated in future studies.

Accurate staging of pancreatitis is important to study the natural history of the disease and the effect of interventions on the same. It will also help in comparing the results of different studies. It is possible that the additional use of biomarkers will improve the staging systems and this needs to be explored in future studies. An earlier report from our centre showed that serum MCP-1 levels were lower in patients with CP and EPI as compared to those diagnosed with CP but without EPI[30]. Future studies combining tests for pancreatic function and biomarkers may help in the early detection of CP.

While the assessment of EPF by acid steatocrit and FE-1 correlated well with each other the latter detected EPI in a significantly higher number, thereby placing a larger number of patients in higher stages of disease as per the M-ANNHEIM classification. We recommend that the FE-1 test should be used for staging pancreatitis by the M-ANNHEIM classification.

COMMENTS

Background
Exocrine pancreatic insufficiency (EPI) increases as chronic pancreatitis advances and this forms an important parameter for staging of chronic pancreatitis (CP) in various classification systems.

Research frontiers
Various pancreatic function tests are available to assess the exocrine pancreatic function (EPF). This study focussed on comparing faecal elastase-1 (FE-1) estimation and the results of acid steatocrit test for evaluating EPF for use in the staging of pancreatitis by the M-ANNHEIM system.

Innovations and breakthroughs
The results of this study show that stool fat analysis by acid steatocrit and FE-1 correlate well with each other. The estimation of FE-1 detected EPI, in a significantly higher number, thereby placing a larger number of patients in higher stages of disease as per the M-ANNHEIM classification.

Applications
This study shows that FE-1 is a more appropriate pancreatic function test to determine EPI and to stage pancreatitis using the M-ANNHEIM classification.

Terminology
FE-1 measures the amount of pancreatic elastase enzyme secreted into the gut by the pancreas and is estimated by the enzyme-linked immunosorbent assay technique. FE-1 is a tubeless indirect pancreatic function test which relies on the stability of pancreatic elastase as it transits through the intestine before excretion in stool. FE-1 is highly sensitive in estimating EPI during advanced stages of CP. Steatorrhoea by the acid steatocrit method is determined by diluting the stool with distilled water and homogenising it followed by mixing the stool with Perchloric acid to a pH of less than 1. The stool mixture is transferred to a capillary tube, and centrifuged to obtain a fat layer and a solid layer, which is measured by the appropriate formula to measure the stool fat content in the given stool sample.

Peer-review
The authors have produced a well designed and constructed study with useful clinical results. The design is clear, the outcomes well presented and the conclusion is also clear.

REFERENCES

1   **Schneider A**, Löhr JM, Singer MV. The M-ANNHEIM classification of chronic pancreatitis: introduction of a unifying classification system based on a review of previous classifications of the disease. *J Gastroenterol* 2007; **42**: 101-119 [PMID: 17351799]

2   **Ramesh H**. Proposal for a new grading system for chronic pancreatitis: the ABC system. *J Clin Gastroenterol* 2002; **35**: 67-70 [PMID: 12080229]

3   **Büchler MW**, Martignoni ME, Friess H, Malfertheiner P. A proposal for a new clinical classification of chronic pancreatitis. *BMC Gastroenterol* 2009; **9**: 93 [PMID: 20003450 DOI: 10.1186/1471-230X-9-93]

4   **Duggan SN**, Ní Chonchubhair HM, Lawal O, O'Connor DB, Conlon KC. Chronic pancreatitis: A diagnostic dilemma. *World J Gastroenterol* 2016; **22**: 2304-2313 [PMID: 26900292 DOI: 10.3748/wjg.v22.i7.2304]

5   **Chowdhury RS**, Forsmark CE. Review article: Pancreatic function testing. *Aliment Pharmacol Ther* 2003; **17**: 733-750 [PMID: 12641496]

6   **Lieb JG**, Draganov PV. Pancreatic function testing: here to stay for the 21st century. *World J Gastroenterol* 2008; **14**: 3149-3158 [PMID: 18506918 DOI: 10.3748/wjg.14.3149]

7   **Amann ST**, Josephson SA, Toskes PP. Acid steatocrit: a simple, rapid gravimetric method to determine steatorrhea. *Am J Gastroenterol* 1997; **92**: 2280-2284 [PMID: 9399770]

8   **Dumasy V**, Delhaye M, Cotton F, Deviere J. Fat malabsorption screening in chronic pancreatitis. *Am J Gastroenterol* 2004; **99**: 1350-1354 [PMID: 15233677 DOI: 10.1111/j.1572-0241.2004.30661.x]

9   **Bijoor AR**, Geetha S, Venkatesh T. Faecal fat content in healthy adults by the 'acid steatocrit method'. *Indian J Clin Biochem* 2004; **19**: 20-22 [PMID: 23105451 DOI: 10.1007/BF02894252]

10   **Guarino A**, Tarallo L, Greco L, Cesarano L, Guandalini S, Rubino A. Reference values of the steatocrit and its modifications in diarrheal diseases. *J Pediatr Gastroenterol Nutr* 1992; **14**: 268-274 [PMID: 1619531]

11   **Tran M**, Forget P, Van den Neucker A, Strik J, van Kreel B, Kuijten R. The acid steatocrit: a much improved method. *J Pediatr Gastroenterol Nutr* 1994; **19**: 299-303 [PMID: 7815261]

12   **Tod J**, Fine D. Fecal elastase: a useful test for pancreatic insufficiency? *Dig Dis Sci* 2010; **55**: 2709-2711 [PMID: 20838890 DOI: 10.1007/s10620-010-1409-9]

13   **Beharry S**, Ellis L, Corey M, Marcon M, Durie P. How useful is fecal pancreatic elastase 1 as a marker of exocrine pancreatic disease? *J Pediatr* 2002; **141**: 84-90 [PMID: 12091856 DOI: 10.1067/mpd.2002.124829]

14   **Carroccio A**, Verghi F, Santini B, Lucidi V, Iacono G, Cavataio F, Soresi M, Ansaldi N, Castro M, Montalto G. Diagnostic accuracy of fecal elastase 1 assay in patients with pancreatic maldigestion or intestinal malabsorption: a collaborative study of the Italian Society of Pediatric Gastroenterology and Hepatology. *Dig Dis Sci* 2001; **46**: 1335-1342 [PMID: 11414313]

15   **Girish BN**, Rajesh G, Vaidyanathan K, Balakrishnan V. Fecal elastase1 and acid steatocrit estimation in chronic pancreatitis. *Indian J Gastroenterol* 2009; **28**: 201-205 [PMID: 20177867 DOI: 10.1007/s12664-009-0079-z]

16   **Banks PA**, Bollen TL, Dervenis C, Gooszen HG, Johnson CD, Sarr MG, Tsiotos GG, Vege SS. Classification of acute pancreatitis--2012: revision of the Atlanta classification and definitions by international consensus. *Gut* 2013; **62**: 102-111 [PMID: 23100216 DOI: 10.1136/gutjnl-2012-302779]

17   **Guda NM**, Romagnuolo J, Freeman ML. Recurrent and relapsing pancreatitis. *Curr Gastroenterol Rep* 2011; **13**: 140-149 [PMID: 21286872 DOI: 10.1007/s11894-011-0176-x]

18   **Yadav D**, Hawes RH, Brand RE, Anderson MA, Money ME, Banks PA, Bishop MD, Baillie J, Sherman S, DiSario J, Burton FR, Gardner TB, Amann ST, Gelrud A, Lawrence C, Elinoff B, Greer JB, O'Connell M, Barmada MM, Slivka A, Whitcomb DC. Alcohol consumption, cigarette smoking, and the risk of recurrent acute

and chronic pancreatitis. *Arch Intern Med* 2009; **169**: 1035-1045 [PMID: 19506173 DOI: 10.1001/archinternmed.2009.125]

19 **Catalano MF**, Sahai A, Levy M, Romagnuolo J, Wiersema M, Brugge W, Freeman M, Yamao K, Canto M, Hernandez LV. EUS-based criteria for the diagnosis of chronic pancreatitis: the Rosemont classification. *Gastrointest Endosc* 2009; **69**: 1251-1261 [PMID: 19243769 DOI: 10.1016/j.gie.2008.07.043]

20 **Conwell DL**, Lee LS, Yadav D, Longnecker DS, Miller FH, Mortele KJ, Levy MJ, Kwon R, Lieb JG, Stevens T, Toskes PP, Gardner TB, Gelrud A, Wu BU, Forsmark CE, Vege SS. American Pancreatic Association Practice Guidelines in Chronic Pancreatitis: evidence-based report on diagnostic guidelines. *Pancreas* 2014; **43**: 1143-1162 [PMID: 25333398 DOI: 10.1097/MPA.0000000000000237]

21 **Ewald N**, Raspe A, Kaufmann C, Bretzel RG, Kloer HU, Hardt PD. Determinants of Exocrine Pancreatic Function as Measured by Fecal Elastase-1 Concentrations (FEC) in Patients with Diabetes mellitus. *Eur J Med Res* 2009; **14**: 118-122 [PMID: 19380282]

22 **Ramakrishna BS**. The steatocrit as a measure of fecal fat excretion: uses and pitfalls. *Indian J Gastroenterol* 2009; **28**: 195-197 [PMID: 20177864 DOI: 10.1007/s12664-009-0076-2]

23 **Dominici R**, Franzini C. Fecal elastase-1 as a test for pancreatic function: a review. *Clin Chem Lab Med* 2002; **40**: 325-332 [PMID: 12059069 DOI: 10.1515/CCLM.2002.051]

24 **Lüth S**, Teyssen S, Forssmann K, Kölbel C, Krummenauer F, Singer MV. Fecal elastase-1 determination: 'gold standard' of indirect pancreatic function tests? *Scand J Gastroenterol* 2001; **36**: 1092-1099 [PMID: 11589385]

25 **Usküdar O**, Oğuz D, Akdoğan M, Altiparmak E, Sahin B. Comparison of endoscopic retrograde cholangiopancreatography, endoscopic ultrasonography, and fecal elastase 1 in chronic pancreatitis and clinical correlation. *Pancreas* 2009; **38**: 503-506 [PMID: 19287334 DOI: 10.1097/MPA.0b013e31819f639f]

26 **Walkowiak J**, Cichy WK, Herzig KH. Comparison of fecal elastase-1 determination with the secretin-cholecystokinin test in patients with cystic fibrosis. *Scand J Gastroenterol* 1999; **34**: 202-207 [PMID: 10192202]

27 **Bian Y**, Wang L, Chen C, Lu JP, Fan JB, Chen SY, Zhao BH. Quantification of pancreatic exocrine function of chronic pancreatitis with secretin-enhanced MRCP. *World J Gastroenterol* 2013; **19**: 7177-7182 [PMID: 24222963 DOI: 10.3748/wjg.v19.i41.7177]

28 **Schneider AR**, Hammerstingl R, Heller M, Povse N, Murzynski L, Vogl TJ, Caspary WF, Stein J. Does secretin-stimulated MRCP predict exocrine pancreatic insufficiency?: A comparison with noninvasive exocrine pancreatic function tests. *J Clin Gastroenterol* 2006; **40**: 851-855 [PMID: 17016144 DOI: 10.1097/01.mcg.0000225652.00308.a2]

29 **Chowdhury SD**, Kurien RT, Ramachandran A, Joseph AJ, Simon EG, Dutta AK, David D, Kumar C B, Samuel P, Balasubramaniam KA. Pancreatic exocrine insufficiency: Comparing fecal elastase 1 with 72-h stool for fecal fat estimation. *Indian J Gastroenterol* 2016; **35**: 441-444 [PMID: 27878466 DOI: 10.1007/s12664-016-0714-4]

30 **Kamath MG**, Pai CG, Kamath A, Kurien A. Monocyte chemoattractant protein-1, transforming growth factor-beta1, nerve growth factor, resistin and hyaluronic acid as serum markers: comparison between recurrent acute and chronic pancreatitis. *Hepatobiliary Pancreat Dis Int* 2016; **15**: 209-215 [PMID: 27020638]
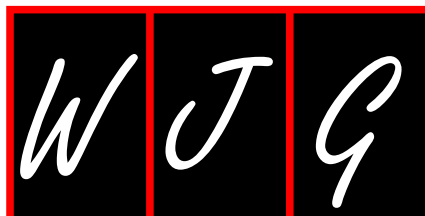
Systematic review: The placebo effect of psychological interventions in the treatment of irritable bowel syndrome

Carla E Flik, Laura Bakker, Wijnand Laan, Yanda R van Rood, André JPM Smout, Niek J de Wit

Carla E Flik, Laura Bakker, Wijnand Laan, Niek J de Wit, Department of General Practice, Julius Centre for Health Sciences and Primary Care, University Medical Centre Utrecht, 3508GA Utrecht, The Netherlands

Carla E Flik, Department of Psychiatry and Psychology, St Antonius Hospital, 3435CM Nieuwegein, The Netherlands

Yanda R van Rood, Department of Psychiatry, University Medical Centre, 2333ZA Leiden, The Netherlands

André JPM Smout, Department of Gastroenterology and Hepatology, Academic Medical Centre, 1105AZ Amsterdam, The Netherlands

Author contributions: Flik CE, Bakker L and de Wit NJ designed the study; Flik CE, Bakker L and Laan W analyzed the data and performed the calculations; Flik CE, Bakker L, Laan W and van Rood YR wrote the article in discussion with Smout AJPM and de Wit NJ, especially van Rood YR; Smout AJPM and de Wit NJ gave suggestions to improve the text and all authors contributed to the discussion of the data; all authors approved the final version of the manuscript.

Correspondence to: Dr. Carla E Flik, Department of General Practice, Julius Centre for Health Sciences and Primary Care, University Medical Centre Utrecht, Str. 6.131, P.O. Box 85500, 3508 GA Utrecht, The Netherlands. c.e.flik@umcutrecht.nl
Telephone: +31-88-7568355

Abstract

AIM

To determine the placebo response rate associated with different types of placebo interventions used in psychological intervention studies for irritable bowel syndrome.

METHODS

Randomized controlled trials comparing psychological interventions (stress management/relaxation therapy (cognitive) behavioral therapy, short-term psychodynamic therapy, and hypnotherapy) for the treatment of adult patients with irritable bowel syndrome (IBS) diagnosed with the Manning or Rome criteria with an adequate placebo control treatment and reporting data on IBS symptom severity were identified by searching PubMed, Embase, the Cochrane Library, CINAHL and PsycINFO databases. Full-text articles that were written in English and published between 1966 and February 2016 in peer-reviewed journals were selected for the present review. Placebo interventions were considered to be adequate if the number of sessions and the amount of time spent with the therapist were the same as in the active treatment. The placebo response rate (PRR) was computed for IBS symptom severity (primary outcome measure) as well as for anxiety, depression and quality of life (secondary outcome measures).

RESULTS

Six studies, with a total of 555 patients met the inclusion criteria. Four studies used an educational intervention, whereas two studies used a form of

supportive therapy as the placebo intervention. The PRR for IBS symptom severity ranged from 25% to 59%, with a pooled mean of 41.4%. The relative PRR for the secondary outcome measures ranged from 0% to 267% for anxiety, 6% to 52% for depression 20% to 125% for quality of life. The PRR associated with pharmacological treatments, treatment with dietary bran and complementary medicine ranged from 37.5% to 47%. Contrary to our expectations, the PRR in studies on psychological interventions was comparable to that in studies on pharmacological, dietary and alternative medical interventions.

CONCLUSION

The PRR is probably determined to a larger extent by patient-related factors, such as expectations and desire for the treatment to be effective, than the content of the placebo intervention.

Key words: Placebo effect; Psychological interventions; Irritable bowel syndrome; Systematic review

Core tip: This study highlights the fact that providing patients with realistic, but positive information about the expected effect of the treatment for irritable bowel syndrome is important to optimize the placebo response.

INTRODUCTION

Irritable bowel syndrome (IBS) is a chronic functional gastrointestinal disorder characterized by recurrent episodes of abdominal pain, discomfort, and altered bowel habits that are not explained by structural or biochemical abnormalities[1]. Several pathophysiological mechanisms underlying IBS have been proposed. According to the bio-psycho-social model of IBS, a disturbance in intestinal motility and enhanced visceral sensitivity interact with other factors, such as environmental influences, parent-child interactions and disturbed stress responses[2].

Because of the limited effect of pharmacotherapy[3,4], there has been increasing interest in psychological treatments for IBS. Two Cochrane reviews provided evidence for the effectiveness of cognitive behavioral therapy (CBT), interpersonal psychotherapy (IPT)[5] and hypnotherapy[6]. Another review[3]

concluded that CBT, IPT and hypnotherapy, not relaxation therapy, were more effective than typical care in relieving IBS symptoms. In 2014, a systematic review showed that relaxation therapy was effective in reducing IBS symptoms[7].

In the research on psychological treatment methods, it is possible that the treatment effect is the result of increased attention and time investment on the patient rather than the therapy itself. In randomized controlled trials, a placebo group should be used to control for this effect. The placebo group is defined as a "matched control group participating in an activity regarded therapeutically inert from the theoretical perspective of the therapy under study"[8].

Although a placebo control is different in pharmacological studies than in psychological studies, they are equally important in both cases for achieving a methodologically valid comparison. In pharmacological research the placebo response rate (PRR) is variable and may be affected by the type, dosage, size, color, frequency, and route of administration of the placebo medication[9]. In psychological interventions, the PRR may result from the consultation itself and the relationship with the physician/therapist[10]. IBS patients experienced greater benefits from augmented, positive interaction with a practitioner than from limited or no interaction at all (*i.e.*, being put on a waiting list)[10]. They also benefitted more from an increased number of office visits and a longer duration of treatment[11,12], suggesting that supportive and empathic interaction with a practitioner might influence clinical outcomes. Placebo effects can be defined as "the beneficial effects that are attributable to the responses of the patient to the context in which the treatment is delivered, rather than to specific actions of the treatment"[13]. In RCTs in which psychological interventions are studied, a control intervention with an equal number and length of sessions, using an individual or a group format and with comparably trained of therapists[8] should be used to control for these effects. Currently, researchers who examine psychological interventions debate whether and to what degree the effects of psychotherapy are based on placebo effects or therapeutic factors[8,14,15].

From a methodological perspective, the PRR is viewed as an effect that needs to be corrected for. However, from a clinical perspective, a high PRR and a good treatment response are considered to be equally positive outcomes. From this perspective, when the PRR associated with psychological interventions is larger than associated with pharmacological interventions, the psychological placebo treatment may be of greater clinical relevance. The positive relationship with the therapist can be used as an additional beneficial factor.

We presumed that the placebo response would be greater in psychological interventions than in drug trials. So far, studies on the PRR in IBS have focused

primarily on pharmacological treatments, treatment with dietary bran and complementary medicine. PRR rates in these studies ranged from 37.5% to 47%[11,16-18].

One systematic review of alternative therapies for irritable bowel syndrome included a meta-analysis of psychological therapies[19]. A separate evaluation of the results of four of the 17 included studies that used a "true placebo group" was reported. The PRR of these four studies was 30.4%.

This study searched the MEDLINE database for articles published through 2001, sample sizes were low and the IBS criteria for the inclusion of studies were not defined. Since then, results of a number of new studies have been published. The present study aims to review systematically the PRR associated with different types of placebo control interventions in studies on psychological interventions in IBS and compare them to the PRR of placebo control interventions of drug trials.

MATERIALS AND METHODS

Inclusion and exclusion criteria
Types of studies: Randomized controlled trials comparing psychological interventions for the treatment of IBS with a placebo control treatment that were written in English and published as a full text in a peer-reviewed journal, were eligible for inclusion. Cross-over studies were excluded, as were studies comparing two types of psychological therapeutic interventions without a placebo control.

Types of participants: Studies including male or female patients over the age of 18 years with IBS diagnosed according to Manning or Rome Ⅰ, Ⅱ or Ⅲ criteria were included in the analysis.

Types of interventions: In accordance with earlier Cochrane reviews[5,6], the following psychological interventions for the treatment of IBS were considered: stress management/relaxation therapy (cognitive) behavioral therapy, short-term psychodynamic therapy, and hypnotherapy.

Types of placebo treatments: Because of the potential impact of the format of the placebo intervention on the outcome[8], only studies with placebo-controlled interventions using the same number of sessions and therapeutic time as the active treatment were considered to be eligible for inclusion (For Baskin's other criteria, see Table 1). Studies using a waiting list, usual care, symptom monitoring and therapeutic contact by phone or internet, were excluded.

Types of outcome measures: Studies were eligible for inclusion if they reported improvement in IBS symptoms and/or abdominal pain (measured with a validated IBS questionnaire) and/or adequate relief of abdominal pain and discomfort or satisfactory relief of IBS symptoms as recommended by the Rome Ⅲ classification system for the design of IBS treatment trials[20].

Studies were excluded if no information on the effectiveness of the psychological interventions was available or if the proportion of patients in each group with overall symptom improvement after therapy was not reported.

Search methods to identify studies
Electronic searches: We performed a systematic search of RCTs published from 1966 to February 2016 that were available in PubMed, Embase, the Cochrane Library, CINAHL and PsychINFO databases. The following search terms were used: "irritable bowel syndrome" [MeSH] OR "colonic diseases, functional" [MeSH: NoExp] OR "irritable bowel syndrome" [tiab] OR "irritable bowel syndromes" [tiab] OR "irritable colon" [tiab] OR "mucous colitis" [tiab] OR "ibs" [tiab] OR "functional colonic disease" [tiab] OR "functional colonic diseases" [tiab] OR "spastic colon" [tiab];

Combined with: ((cognitive[tiab] OR psychological[tiab] OR psychologic[tiab] OR psychodynamic[tiab] OR psychoanalytic[tiab] OR "psycho analytic"[tiab] OR stress[tiab] OR relaxation[tiab] OR conditioning[tiab] OR "problem solving"[tiab] OR interpersonal[tiab] OR "hypno analytic"[tiab] OR behavioral[tiab] OR behavioural[tiab] OR behavior[tiab] OR behaviour[tiab]) AND (therapy[tiab] OR therapies[tiab] OR treatment[tiab] OR treatments[tiab] OR intervention[tiab] OR interventions[tiab] OR management[tiab])) OR (psychotherapy[tiab] OR psychotherapies[tiab] OR psychoeducation[tiab] OR "psycho education"[tiab] OR psychoeducational[tiab] OR psychotherapy[tiab] OR hypnotherapy[tiab] OR hypnosis[tiab] OR hypnoses[tiab] OR hypnotism[tiab] OR hypnoanalysis[tiab] OR mesmerism[tiab] OR "hypno analysis"[tiab] OR autohypnosis[tiab] OR "auto hypnosis"[tiab] OR psychoanalyses[tiab] OR psychoanalysis[tiab] OR "psycho analysis"[tiab] OR biofeedback[tiab]) OR ("Behavior Therapy"[MeSH] OR "Psychoanalysis"[MeSH] OR "Psychoanalytic Therapy"[MeSH]). No filters or limits were used.

Data collection and analysis
Study selection: Two authors (CF and LB) reviewed the title and abstract of each identified article to determine the extent to which it met eligibility criteria, such as type of study, participants, interventions, placebo treatments and outcome measures, as described previously. A manual search of the references listed in the articles retrieved from the online search was performed to identify additional studies. The full texts of the selected articles were then reviewed by the same authors to assess eligibility

based on the same criteria. Discrepancies between the selections made by CF and LB were resolved by a third author (NdW).

Data extraction: From the resulting selection of papers, information on the number of patients, patient characteristics (gender, mean age, and mean duration of illness), criteria for diagnosis (Rome I, Rome II, Rome III or Manning), treatment setting, intervention (type, group or individual delivery format, number of sessions, training of therapists and use of treatment/ placebo manual), placebo control (type, group or individual delivery format, number of sessions, training of therapists and use of treatment/placebo manual), duration of treatment, duration of the follow-up period, and results relating to the primary and secondary outcome measures were extracted.

Assessment of risk of bias: The risk of bias assessment tool developed by the Cochrane Collaboration for RCTs[21] was used. The following sources of bias can be assessed with high, low or unclear bias ratings: adequate generation of the allocation sequence; concealment of allocation to conditions; blinding of participants and personnel; handling of incomplete outcome data; and selective outcome reporting. The percentage of patients who dropped out of the intervention and placebo control group as well as the results of the intention-to treat (ITT) analysis (when provided) were added.

Outcome measures
In this review, the post-treatment IBS symptom severity scores was the primary outcome measure. Most studies presented the results of the ITT analysis, although only one study included the results of the per protocol (PP) analyses. Secondary outcome measures were improvement of symptoms of anxiety and depression as well as quality of life. Quality of life was recommended as an outcome measure by the Rome III committee, whereas anxiety and depression were chosen as secondary outcome measures due to their high rates of co-morbidity[22].

Statistical analysis
The response rate of the primary outcome measures was calculated by dividing the percentage of patients who responded according to the study criteria by the number of patients in the ITT analysis or who completed treatment. Relative placebo responses (Rel-PR) with 95% confidence intervals (95%CI) were calculated as the ratio of placebo response to active treatment response. Additionally, the mean Rel-PR across all studies was calculated.

The weighted average PRR was calculated by adding up the PRR per study multiplied by the number of patients in the placebo control group of that study and dividing the product by the total number of control

patients in all of the studies.

Criteria for response evaluation were not available for the secondary measures; therefore, PRRs for the secondary outcome measures of anxiety, depression and quality of life were calculated by setting the response rate for these measures in the active arm at 100% and computing the response rate in the placebo arm as a relative percentage of the active arm. A relative response rate > 100% indicated that the placebo intervention was more effective than the treatment intervention. To allow for comparison of the PRR between the primary and secondary outcome measures, we recalculated the rates for the primary outcome measures in this way.

For the secondary outcome measures, the PRR for the different types of placebo interventions were calculated by adding up the PRR per study multiplied with the number of patients in the placebo control group of that study and dividing the product by the total number of control patients in all of the studies.

RESULTS

Description of studies
The literature search resulted in the identification of 5169 studies. After screening the titles and abstracts, 112 studies were potentially eligible (see the flowchart in Figure 1). The manual search yielded no additional studies (Figure 1).

After reviewing the full manuscripts of these studies, 106 studies were excluded for various reasons (see the flowchart in Figure 1), leaving six eligible trials[23-28] that were included in the analysis. The characteristics of the included studies are shown in Table 1. Sample sizes ranged from 21[21] to 215[24]. Patients were recruited from primary, secondary and tertiary care institutions, although they were also partially recruited through advertisements in three studies[23-25]. The treatment setting was unclear in two of the selected studies[25,27] (Table 1).

The mean age of the study populations ranged from 31.6 to 45.5 years. The proportion of female participants ranged from 52.4% to 100%. Only one study reported the duration of IBS[26]: a median of 4 years for the intervention group and 10.5 years for the placebo group. The duration of treatment and the placebo intervention ranged from 8 wk to 3 mo. The duration of the follow-up period ranged from 3 mo to 12 mo.

Quality assessment
Four of the six studies fulfilled almost all quality criteria (Table 2).

Type of placebo interventions
Four studies used an educational program as the placebo intervention[23,24,27,28]. In these studies, educational materials were provided and discussed with
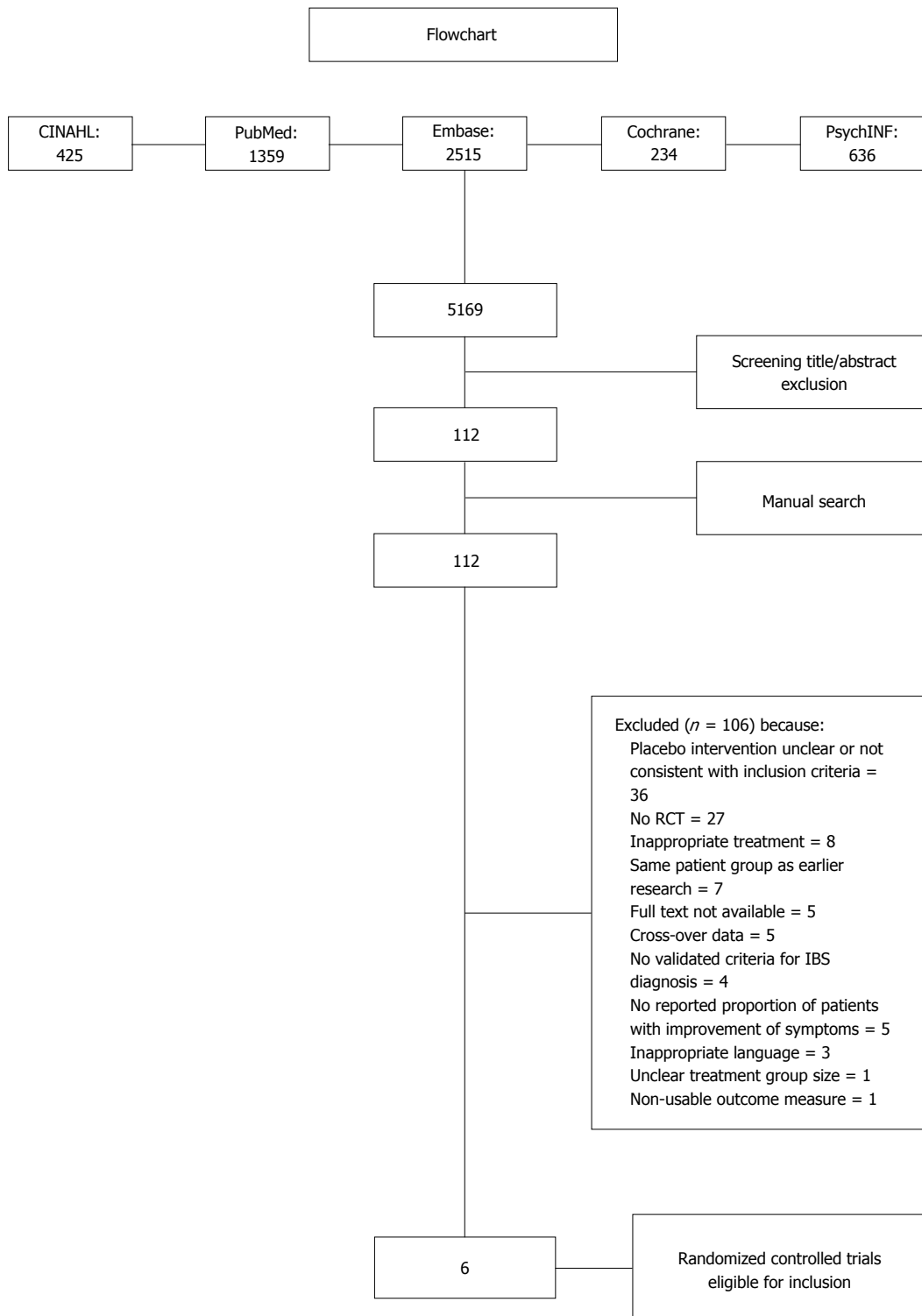
Figure 1 Flowchart of studies selected. RCT: Randomized controlled trial.

a therapist. In the study by Payne and Blanchard[27], individual cognitive therapy was compared to an educational placebo intervention delivered in a group format. The other studies compared individual CBT (with interoceptive exposure to visceral sensations) or stress management[23], individually delivered CBT[24] and autogenic training[28] to an individual educational placebo intervention.

Two studies on mindfulness and hypnotherapy delivered in a group format used support therapy as the placebo intervention[25,26]. In the study by Gaylord *et al*[25] the placebo intervention sessions were facilitated by social workers who served as group leaders, focussing on specific predesigned topics and promoting open group discussions. The placebo intervention in the study by Moser *et al*[26] consisted of

Table 1 Descriptive statistics for characteristics of included studies

Ref.	Year	Country	n	Mean age (yr)	Female sex	Recruitment	Years of illness	Criteria	Therapy	Control	Format	Ses-sions	Trained therapist intervention/ placebo	Protocol inter-vention/placebo
Craske *et al*[25] 2011	2011	United States	110	39.4	74	Community advertisement; university clinic		RII	Cognitive-behavior-al therapy	Psycho-educatio-nal support	Individual	10	Unclear/unclear	Yes/yes
Drossman *et al*[24] 2003	2003	United States and Canada	215	37.3	100	Community and hospital advertisement; physician referral in community or university-based practices		R1	Cognitive-behavior-al therapy	Psycho-educational support	Unclear	12	Yes/yes	Yes/yes
Gaylord *et al*[25] 2011	2011	United States	75	42.7	100	Local advertisement; physician care		RII	Mindful-ness	Support	Group	9	Yes/yes	Yes/yes
Moser *et al*[26] 2013	2013	Austria	100	45.5	79	Primary, secondary care and university clinic	4/10.5	RIII	Hypno-therapy	Support	Group	10	Yes/yes	Yes/no
Payne *et al*[27] 1995	1995	United States	34	40.1	85	Personal physician	16	RI	Cognitive therapy	Psycho-educatio-nal support	Therapy: individual; Control: group	10	Yes/unclear	Yes/yes
Shinozaki[28] 2010	2010	Japan	21	31.6	52	University clinic			Relax	Psycho-educatio-nal support	Individual	8	Yes/unclear	Yes/yes

doctor's visits of the same duration as the treatment.

Placebo response
Primary outcome measure: One of the six six studies investigated the effects of two separate psychological interventions and compared them with the effect of one placebo intervention[23], which brings the total number of outcomes to seven (see Table 3). All studies reported a significant reduction in IBS symptoms for at least one of the treatment interventions. For the response rate for the primary outcome measure of the placebo and active intervention arms, see Table 3. We performed the calculations using post-treatment figures. However, for the study by Craske *et al*[23], we used the figures at three-month follow-up because only they were reported.

Rel-PRs ranged from 0.33 (95%CI: 0.12-0.94) in the study by Payne and Blanchard[27] to 1.1 (95%CI: 0.7-1.73) in the study by Craske[23]. For details on the Rel-PRs, see Figure 2.

After adjusting for study sample size, the weighted average PRR for all studies was 41.4%. In subgroup analysis, after adjusting for study sample size, the pooled PRR was 39.5% for the educational programs and 42.9% for the supportive interventions, including doctor's visits[26].

Secondary outcome measures: Data on anxiety were presented in five studies[23,25-28], whereas data on depression were provided in three studies[25,27,28]. Five studies assessed quality of life using the IBS-QOL or SF-36 as the outcome measure[23-26,28]. The relative PRR for anxiety ranged from 0%[23,27] to 267%[28]. The relative PRR for depression ranged from 6%[27,28] to 52%[25]. For quality of life, it ranged from 20%[26] to 125%[23]. The relative placebo responses are presented in Table 4.

With regard to the different types of placebo interventions, after adjusting for sample size, the weighted average sizes for the educational placebo interventions were 27.8% for state anxiety, 65.1% for trait anxiety, 6% for depression and 72.7% for quality of life. For the supportive interventions, they were 27.2% for anxiety, 52% for depression and 20.8% for quality of life.

Table 2 Risk of bias ratings for included studies

Ref.	Year	Random sequence generation	Allocation concealment	Blinding of participants and personnel	Blinding of outcome assessment	Incomplete outcome data	Selective reporting	Dropout treatment/placebo control (%)	ITT or PP
Craske *et al*[23] 2011	2011	Low	Low	Low	Low	Low	Low	Interoceptive Exposure:34; Stress Management: 36/16	ITT
Drossman *et al*[24] 2003	2003	Low	Low	Low	Low	Low	Low	13/24	ITT
Gaylord *et al*[25] 2011	2011	Low	Low	Low	Low	Low	Low	6/18	ITT
Moser *et al*[26] 2013	2013	Low	Low	Low	Low	Low	Low	0/2	PP
Payne *et al*[27] 1995	1995	Unclear	Unclear	Low	Unclear	Low	Low	0/0	ITT
Shinozaki[28] 2010	2010	Unclear	Unclear	High	High	Low	Low	0/0	ITT

Possible ratings were low, high or unclear risk of bias. Studies with 2 control groups were rated twice for risk of bias because of lack of blinding (rated or active control groups appear in parentheses). ITT indicates that the analysis was intent-to-treat (analyzed as randomized). PP: Per protocol.

Table 3 Placebo treatment and placebo response rate

Ref.	Placebo treatment	Primary outcome measure	Duration of treatment[1]	Follow-up	Placebo response	Treatment response
Craske *et al*[23] 2011	Psycho-educational support	BSS index	10 wk	3 mo	59% (13/22)	62% (29/47)[1] 54% (22/41)[2]
Drossman *et al*[24] 2003	Psycho-educational support	Composite score[3]	12 wk		37.3% (19/51)	70% (77/110)
Gaylord *et al*[25] 2011	Support group	IBS-SSS	8 wk	3 mo	45.2% (17.6/39) 53.1% (20.7/39)	68.8% (27.4/36) 75% (27/36)
Moser *et al*[26] 2013	Supportive talks	IBS-IS	12 wk	12 mo	40.9% (18/44) 25% (11/44)	60.8%(28/46) 54.3% (25/46)
Payne *et al*[27] 1995	Psycho-educational support	CPSR	8 wk	3 mo	25% (3/12) 18% (2/12)	75% (9/12) 83% (10/12)
Shinozaki[28] 2010	Psycho-educational support	AR	8 wk		30% (3/10)	81.8% (9/11)

[1]Cognitive behavioral treatment; [2]Stress management; [3]Composite score: Mc-Gill Pain Questionnaire; IBS-QOL; satisfaction with treatment; global well-being. IBS: Irritable bowel syndrome; BSS: Bowel syndrome severity index; IBS-SSS: IBS-Symptom Severity Score; IBS-IS: IBS-Impact Scale; AR: Adequate relief; CPSR: Composite primary symptom reduction.
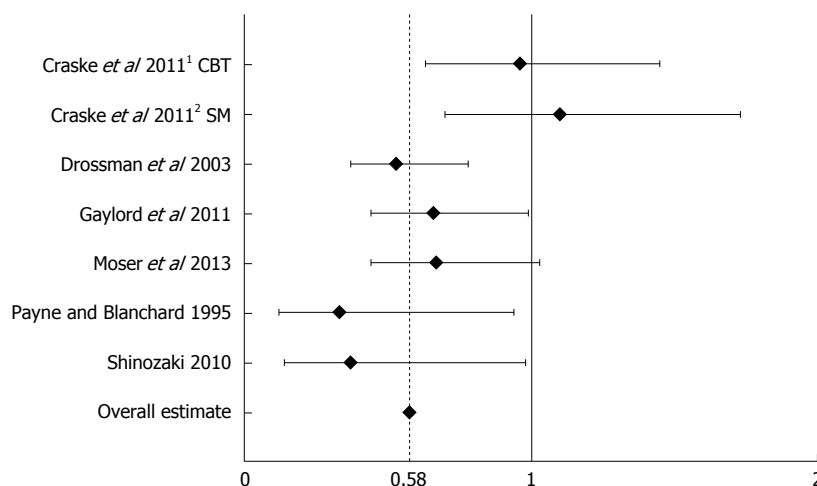
Figure 2 Relative placebo responses defined as the ratio of placebo response to active treatment response in the individual studies. The mean relative placebo responses (Rel-PR) and 95% confidence intervals are shown. [1]Cognitive behavioral treatment; [2]Stress management. CBT: Cognitive behaviour therapy; CM: Contingency management; SM: Stress management.

DISCUSSION

Summary of findings
Our results showed that the PRR in six studies investigating the effect of psychological treatment on IBS for the primary outcome varied from 25.0% to 59.0%. The pooled adjusted mean PRR was 41.4%, which is comparable to the PRR reported in studies

Table 4 Comparison of outcome measurements

Author	Symptoms	Anxiety	Depression	Quality of life
Craske CBT-IE	Bowel Symptom Severity (BSS)	VSI	-	IBS-QOL (FA and IF)
	56%/89% = 63%	0%/44% = 0%	-	FA: 31/25 = 125%
				IF: 9/10 = 84%
Craske SM	BSS	VSI	-	IBS-QOL (FA and IF)
	56%/82% = 68%	0%/23% = 0%	-	FA: 31/17 = 184%
				IF: 9/14 = 64%
Drossmann	Mc Gill pain Questionnaire	-	-	IBS-QOL
	2.77/4.58 = 60%	-	-	4.8/9.35 = 51%
Gaylord	IBS-Symptom Severity Score (IBS-SSS)	VSI Brief State Inventory-anxiety	Brief state inventory-depression	IBS-QOL
	42.2/68.8 = 61%	1.16/5.78 = 20%	0.78/1.49 = 52%	3.7/0.19 = 36%
		1.64/3.86 = 42%		
Moser	IBS-Impact Scale (IBS-IS)	HADS Hospital Anxiety and Depression Scale	-	SF-36
	40.9/60.8 = 67%	0.5/3.7 = 14%	-	24/117.9 = 20%
Payne and Blanchard	Composite Primary Symptom Reduction (CPSR)	STAI (state) STAI (trait)	BDI	-
	25%/75% = 33%	FALSE FALSE	0.4/6.3 = 6%	-
Shinozaki	Adequate Relief	State Trait Anxiety Inventory (state)	Self rating depression scale	SF-36
	Self Reported IBS Questionnaire (SIBSQ)	STAI (trait)	(SDS)	
	30/81.8 = 37%	3.2/2.8 = 114%	0.1/1.8 = 6%	15.5/58.2 = 27%
	19.6/3.2 = 612%	4/1.5 = 267%		

The percentages were calculated by dividing the treatment effect in the placebo group by the treatment effect in the intervention group and multiplying the quotient by 100. FA: Food avoidance; IF: Interference; VSI: Visceral sensitivity index.

on pharmacological therapy (37.5%)[16]; medication and dietary fibre (47%)[18], medication and alternative medicine (40.7%)[17] and complementary medicine (42.6%)[11]. Our presumption that the response to placebo interventions in studies on psychological treatment for IBS would be greater than that to pharmacological interventions, was not confirmed by our results.

Explanation of findings

Compared to the placebo medication used in the pharmacological studies, the placebo interventions used in the psychological studies involved extensive patient-professional contact. It has been proposed[10,29] that the personality of and empathy exhibited by the therapist during the placebo intervention are responsible for the placebo effect. Furthermore, the more time that the therapist spends with a patient, the greater the placebo response. Hence, one would expect that the PRR in psychological studies would be higher. The fact that we found comparable PRR to those reported in pharmacological studies is obviously inconsistent with this hypothesis. Other factors may need to be considered. Vase *et al*[30] showed that the combination of expected pain relief and desire for pain relief accounted for up to 81% of the variance in the effect of active treatment. They concluded that "adding a verbal suggestion for pain relief in drug treatment

can increase the magnitude of placebo analgesia to that of an active agent." Kirsch[14] also argued that the placebo effect is generally dependent on the activation of response expectancy in the patient. From this perspective, the PRR is determined by the expectation of and desire for symptom relief of the patient, which is influenced by the way that the therapy is introduced and executed by the nurse, doctor or therapist. A positive interpersonal encounter with affective communication and adequate information from the health professional can positively influence the patient's expectations and result in an improvement in health status[31]. Therefore, the words that a general practitioner uses to create expectations within the patient are important, in both pharmacotherapy and psychological interventions[32]. The fact that we did not find a difference in placebo response in our study supports the idea that contextual factors and cognitive and emotional changes, such as expectancy, desire and memory play a role in the development of the placebo response[33].

Strengths and limitations

An important strength of the present study is the use of strict inclusion criteria to define IBS, psychological treatment[5,6] and placebo control conditions. Although this approach also resulted in a small number of studies and a relatively low number of patients,

we consider the comparability of the format of psychological and placebo intervention to be essential for a valid assessment of the "true" placebo effect.

Comparison to the literature

After adjusting for sample size, the pooled PR in the previous systematic review by Spanier *et al*[19] was 30.4%. Three of the four studies included in that analysis were excluded in this study, which involved different inclusion and exclusion criteria. Specifically, Blanchard *et al*[34] had no strict diagnostic criteria for IBS and Shaw *et al*[35] used usual care as the control intervention, which was not an appropriate control group according to our definition.

In a recent meta-analysis by Ford *et al*[36], 31 studies were included. Five of them were also included in our review, but we excluded the remaining 26 studies for the following reasons: the IBS criteria were not clear (2 studies) or Latimers criteria were used (1 study); it was not a randomized controlled trial (1 study); the intervention used was inappropriate according to our criteria [self/management by a nurse (1 study), not by a therapist (2 studies), by e-mail (1 study)]; or the control group did not fulfill the Baskin criteria [symptom monitoring (7 studies), care as usual (6 studies), waiting list (1 study), medication (1 study) or not having the same number of therapeutic sessions (3 studies)].

It would be interesting to compare the PRR of the psychological interventions for irritable bowel syndrome to that in studies on psychological interventions for other diseases. In the systematic review entitled "Psychological Interventions for treatment of inflammatory bowel disease" located in the Cochrane database and published in 2011[37], none of the control groups in the included studies met our criteria for control groups. In a study by Keefer *et al*[38] on gut-directed hypnotherapy for ulcerative colitis published in 2013, a control group that met our criteria was used. The placebo rate was 40%, which was comparable to the placebo rate found in our research. In a systematic review published in 2005, Enck and Klosterhalfen[12] compared the PRRs for functional bowel disorders with those of non-intestinal diseases and other organic gastrointestinal diseases. Most of the studies focused on drug treatment. The authors stated that the placebo effects in functional bowel disorders were similar to those in non-intestinal diseases (depression, pain and Parkinson's disease) and not too dissimilar to those in other gastrointestinal diseases (duodenal ulcer, inflammatory bowel disease).

Secondary outcome measures

The placebo effect on the secondary outcome measures differed considerably across studies. However, the overall trends showed the greatest effects on symptom scores and the smallest effects on quality of life, anxiety and depression, which is aligned with the findings reported by Vase *et al*[30]. Pain is the main complaint of IBS patients, and almost invariably these patients possess the hope and desire that treatment will bring relief of their IBS-related pain. The combination of expected pain relief and desire for pain relief generates the largest placebo effect, and consequently, the effect on symptom scores is likely to be the greatest.

The relatively high PRR for anxiety in the study of Shinozaki *et al*[28] (267%) may have been caused by the content of the educational program, which was completely focused on dietary education. Most IBS patients have considerable anxiety surrounding the potential for dietary substances to act as complaint-inducing agents. A program with this content is apparently helpful in reducing this anxiety. In the study by Craske *et al*[23], the educational program had a positive impact on the patients' food avoidance. Additionally, the effect on the Food Avoidance scale of the IBS-QOL scale was greater than the effects in the two treatment arms (125% and 184%). The results of these studies suggest that it may be worthwhile to include an educational module in IBS treatments.

In the study by Shinozaki *et al*[28], the PRR > 100% of the Self-Reported IBS Questionnaire (SIBSQ) indicated that the placebo intervention was more effective than the treatment intervention. It is not clear why this study found a significant positive treatment effect of autogenic training on the primary outcome measure of "adequate relief" and a significant positive effect of the placebo intervention on the primary symptom measure SIBSQ.

Conclusions and clinical implications

In conclusion, despite the more extensive patient-professional contact, the PRR in the placebo arm of RCTs with psychological treatment interventions is comparable to that of RCTs on drug interventions. This finding does not support the hypothesis that the personality and empathy of the professional are the main determinants of the placebo effect. Most likely, the PR is determined to a greater extent by patient- than doctor-related factors. Particularly important is the combination of expectations about and desire for symptom relief, both of which are influenced by the way that the therapy is introduced and executed. Thus, for optimal control group comparison in studies investigating psychological treatment for IBS, patients in the control group should have similar expectations from the control intervention as patients in the active intervention arm. Therefore, future RCT's should map the expectations of patients in both RCT arms before starting the intervention.

In clinical practice, the placebo response can be used optimally by enhancing the expectations of the patient through the provision of realistic but positive information about the expected effect of the treatment. The preference of patients for a certain

treatment might be related to the expected benefit, although it could also be the result of other contextual factors, such as the way in which the treatment is delivered (group versus individually). Future research should investigate the effect of patients' preference for a certain treatment arm on the treatment outcome.

ACKNOWLEDGMENTS

COMMENTS

Background

Irritable bowel syndrome (IBS) is a common chronic functional gastrointestinal disorder characterized by recurrent episodes of abdominal pain, discomfort, and altered bowel habits that cannot be explained by structural or biochemical abnormalities. Because of the limited effect of pharmacotherapy, there has been increasing interest in psychological treatments for IBS. As in pharmacological treatments, placebo effects play a role in psychological therapies. In psychological interventions, the placebo response may result from the consultation itself and the relationship with the physician/therapist. The authors presumed that the placebo response would be greater in psychological interventions than in drug trials. Therefore, the authors compared the placebo response rate in studies on psychological interventions for IBS with the placebo response rates in pharmacological studies.

Research frontiers

Awareness that proper assessment of the effect of psychological interventions for IBS requires comparison with a placebo treatment is growing. A number of randomized placebo-controlled trials on the effect of psychological interventions on IBS have been published.

Innovations and breakthroughs

In previous meta-analyses on the placebo effect associated with psychological interventions for IBS, the criteria used for inclusion in the analyses have been liberal. For instance, some studies included patients who did not fulfill Rome criteria for IBS, whereas others used usual care as the control intervention. For our systematic review, the authors chose to include only randomized controlled trials (RCTs) that included a placebo intervention that met the strict prerequisites formulated by Baskin *et al* (2003).

Applications

Contrary to our expectations, in our study we found that, despite the more extensive patient-professional contact, the response rate in the placebo arm of RCTs with psychological treatment interventions was comparable to that of RCTs with drug interventions. Thus, it seems that the personality and empathy of the professional are not the main determinants of the placebo effect. Instead, it appears that the placebo response is more determined by patient- than doctor-related factors. For optimal control group comparison in studies investigating psychological treatment for IBS, patients in the control group should have similar expectations from the control intervention as patients in the active intervention arm. Therefore, future RCTs should map the expectations of patients in both RCT arms before starting the intervention. Future research should also explore the effect of patients' preference for a certain treatment arm on the treatment outcome.

Terminology

The diagnosis of irritable bowel syndrome is made using consensus-based criteria, the most recent of which are the Rome criteria, recurrent abdominal pain associated with two or more of the following: (1) related to defecation; (2) associated with a change in the frequency of stool; and (3) associated with a change in the form (appearance) of stool. These criteria must have been fulfilled for the last 3 mo, with symptom onset occurring at least 6 mo prior to the diagnosis. A placebo is defined as an activity regarded therapeutically inert from the theoretical perspective of the therapy under examination.

Peer-review

Good, well-conducted study. Suggested to add that a supportive doctor-patient relationship with empathy and listening is likely to maximize the placebo response to pharmacological treatment, not only in IBS but also in other disease states.

REFERENCES

1   **Guthrie E**, Thompson D. Abdominal pain and functional gastrointestinal disorders. *BMJ* 2002; **325**: 701-703 [PMID: 12351366]

2   **Spiller R**, Aziz Q, Creed F, Emmanuel A, Houghton L, Hungin P, Jones R, Kumar D, Rubin G, Trudgill N, Whorwell P. Guidelines on the irritable bowel syndrome: mechanisms and practical management. *Gut* 2007; **56**: 1770-1798 [PMID: 17488783]

3   **Brandt LJ**, Chey WD, Foxx-Orenstein AE, Schiller LR, Schoenfeld PS, Spiegel BM, Talley NJ, Quigley EM. An evidence-based position statement on the management of irritable bowel syndrome. *Am J Gastroenterol* 2009; **104** Suppl 1: S1-35 [PMID: 19521341 DOI: 10.1038/ajg.2008.122]

4   **Quartero AO**, Meineche-Schmidt V, Muris J, Rubin G, de Wit N. Bulking agents, antispasmodic and antidepressant medication for the treatment of irritable bowel syndrome. *Cochrane Database Syst Rev* 2005; **(2)**: CD003460 [PMID: 15846668 DOI: 10.1002/14651858.CD003460.pub2]

5   **Zijdenbos IL**, de Wit NJ, van der Heijden GJ, Rubin G, Quartero AO. Psychological treatments for the management of irritable bowel syndrome. *Cochrane Database Syst Rev* 2009; **(1)**: CD006442 [PMID: 19160286 DOI: 10.1002/14651858.CD006442.pub2]

6   **Webb AN**, Kukuruzovic RH, Catto-Smith AG, Sawyer SM. Hypnotherapy for treatment of irritable bowel syndrome. *Cochrane Database Syst Rev* 2007; **(4)**: CD005110 [PMID: 17943840 DOI: 10.1002/14651858.CD005110.pub2]

7   **Park SH**, Han KS, Kang CB. Relaxation Therapy for Irritable Bowel Syndrome: A Systematic Review. *Asian Nursing Research* 2014; **8**: 182-192 [DOI: 10.1016/j.am.2014.07.001]

8   **Baskin TW**, Tierney SC, Minami T, Wampold BE. Establishing specificity in psychotherapy: a meta-analysis of structural equivalence of placebo controls. *J Consult Clin Psychol* 2003; **71**: 973-979 [PMID: 14622072 DOI: 10.1037/0022-006X.71.6.973]

9   **Thompson WG**. Placebos: a review of the placebo response. *Am J Gastroenterol* 2000; **95**: 1637-1643 [PMID: 10925962 DOI: 10.1111/j.1572-0241.2000.02179.x]

10 **Kaptchuk TJ**, Kelley JM, Conboy LA, Davis RB, Kerr CE, Jacobson EE, Kirsch I, Schyner RN, Nam BH, Nguyen LT, Park M, Rivers AL, McManus C, Kokkotou E, Drossman DA, Goldman P, Lembo AJ. Components of placebo effect: randomised controlled trial in patients with irritable bowel syndrome. *BMJ* 2008; **336**: 999-1003 [PMID: 18390493 DOI: 10.1136/bmj.39524.439618.25]

11 **Dorn SD**, Kaptchuk TJ, Park JB, Nguyen LT, Canenguez K, Nam BH, Woods KB, Conboy LA, Stason WB, Lembo AJ. A meta-analysis of the placebo response in complementary and alternative medicine trials of irritable bowel syndrome. *Neurogastroenterol Motil* 2007; **19**: 630-637 [PMID: 17640177 DOI: 10.1111/j.1365-2982.2007.00937]

12 **Enck P**, Klosterhalfen S. The placebo response in functional bowel disorders: perspectives and putative mechanisms. *Neurogastroenterol Motil* 2005; **17**: 325-331 [PMID: 15916619 DOI: 10.1111/j.1365-2982.2005.00676.x]

13 **Wager TD**, Atlas LY. The neuroscience of placebo effects: connecting context, learning and health. *Nat Rev Neurosci* 2015; **16**: 403-418 [PMID: 26087681 DOI: 10.1038/nrn3976]

14 **Kirsch I**. Placebo psychotherapy: synonym or oxymoron? *J Clin Psychol* 2005; **61**: 791-803 [PMID: 15827992 DOI: 10.1002/jclp.20126]

15   **Wampold BE**, Budge SL, Laska KM, Del Re AC, Baardseth TP, Fluckiger C, Minami T, Kivlighan DM, Gunn W. Evidence-based treatments for depression and anxiety versus treatment-as-usual: a meta-analysis of direct comparisons. *Clin Psychol Rev* 2011; **31**: 1304-1312 [PMID: 21996291 DOI: 10.1016/j.cpr.2011.07.012]

16   **Ford AC**, Moayyedi P. Meta-analysis: factors affecting placebo response rate in the irritable bowel syndrome. *Aliment Pharmacol Ther* 2010; **32**: 144-158 [PMID: 20412064 DOI: 10.1111/j.1365-2036.2010.04328.x]

17   **Patel SM**, Stason WB, Legedza A, Ock SM, Kaptchuk TJ, Conboy L, Canenguez K, Park JK, Kelly E, Jacobson E, Kerr CE, Lembo AJ. The placebo effect in irritable bowel syndrome trials: a meta-analysis. *Neurogastroenterol Motil* 2005; **17**: 332-340 [PMID: 15916620 DOI: 10.1111/j.1365-2982.2015.00650.x]

18   **Spiller RC**. Impact of dietary fiber on absorption from the small intestine. *Curr Opin Gastroenterol* 1999; **15**: 100-102 [PMID: 17023927]

19   **Spanier JA**, Howden CW, Jones MP. A systematic review of alternative therapies in the irritable bowel syndrome. *Arch Intern Med* 2003; **163**: 265-274 [PMID: 12578506]

20   **Irvine EJ**, Whitehead WE, Chey WD, Matsueda K, Shaw M, Talley NJ, Veldhuyzen van Zanten SJ. Design of treatment trials for functional gastrointestinal disorders. *Gastroenterology* 2006; **130**: 1538-1551 [PMID: 16678567 DOI: 10.1053/j.gastro.2005.11.058]

21   **Higgins JPT**, Altman DG. Assessing risk of bias in included studies. In: Higgins J, Green S, editors. The Cochrane Collaboration: Cochrane handbook for systematic reviews of interventions, 2008

22   **Whitehead WE**, Palsson O, Jones KR. Systematic review of the comorbidity of irritable bowel syndrome with other disorders: what are the causes and implications? *Gastroenterology* 2002; **122**: 1140-1156 [PMID: 11910364]

23   **Craske MG**, Wolitzky-Taylor KB, Labus J, Wu S, Frese M, Mayer EA, Naliboff BD. A cognitive-behavioral treatment for irritable bowel syndrome using interoceptive exposure to visceral sensations. *Behav Res Ther* 2011; **49**: 413-421 [PMID: 21565328 DOI: 10.1016/j.brat.2011.04.001]

24   **Drossman DA**, Toner BB, Whitehead WE, Diamant NE, Dalton CB, Duncan S, Emmott S, Proffitt V, Akman D, Frusciante K, Le T, Meyer K, Bradshaw B, Mikula K, Morris CB, Blackman CJ, Hu Y, Jia H, Li JZ, Koch GG, Bangdiwala SI. Cognitive-behavioral therapy versus education and desipramine versus placebo for moderate to severe functional bowel disorders. *Gastroenterology* 2003; **125**: 19-31 [PMID: 12851867 DOI: 10.1016/S0016-5085(03)00669-3]

25   **Gaylord SA**, Palsson OS, Garland EL, Faurot KR, Coble RS, Mann JD, Frey W, Leniek K, Whitehead WE. Mindfulness training reduces the severity of irritable bowel syndrome in women: results of a randomized controlled trial. *Am J Gastroenterol* 2011; **106**: 1678-1688 [PMID: 21691341 DOI: 10.1038/ajg.2011.184]

26   **Moser G**, Trägner S, Gajowniczek EE, Mikulits A, Michalski M, Kazemi-Shirazi L, Kulnigg-Dabsch S, Führer M, Ponocny-Seliger E, Dejaco C, Miehsler W. Long-term success of GUT-directed group hypnosis for patients with refractory irritable bowel syndrome: a randomized controlled trial. *Am J Gastroenterol* 2013; **108**: 602-609 [PMID: 23419384 DOI: 10.1038/ajg.2013.19]

27   **Payne A**, Blanchard EB. A controlled comparison of cognitive therapy and self-help support groups in the treatment of irritable bowel syndrome. *J Consult Clin Psychol* 1995; **63**: 779-786 [PMID: 7593870]

28   **Shinozaki M**, Kanazawa M, Kano M, Endo Y, Nakaya N, Hongo M, Fukudo S. Effect of autogenic training on general improvement in patients with irritable bowel syndrome: a randomized controlled trial. *Appl Psychophysiol Biofeedback* 2010; **35**: 189-198 [PMID: 19997775 DOI: 10.1007/s10484-009-9125-y]

29   **Kelley JM**, Lembo AJ, Ablon JS, Villanueva JJ, Conboy LA, Levy R, Marci CD, Kerr CE, Kirsch I, Jacobson EE, Riess H, Kaptchuk TJ. Patient and practitioner influences on the placebo effect in irritable bowel syndrome. *Psychosom Med* 2009; **71**: 789-797 [PMID: 19661195 DOI: 10.1097/PSY.0b013e318acee12]

30   **Vase L**, Robinson ME, Verne GN, Price DD. The contributions of suggestion, desire, and expectation to placebo effects in irritable bowel syndrome patients. An empirical investigation. *Pain* 2003; **105**: 17-25 [PMID: 14499416]

31   **Van Dulmen AM**, Bensing JM. Health promoting effects of the physician- patient encounter. *Psychology, Health & Medicine* 2002; **7**: 3 [DOI: 10.1080/13548500220139421]

32   **Wampold BE**, Imel ZE, Minami T. The story of placebo effects in medicine: evidence in context. *J Clin Psychol* 2007; **63**: 379-390; discussion 405-408 [PMID: 17279527 DOI: 10.1002/jclp.20354]

33   **Price DD**, Finniss DG, Benedetti F. A comprehensive review of the placebo effect: recent advances and current thought. *Annu Rev Psychol* 2008; **59**: 565-590 [PMID: 17550344 DOI: 10.1146/annurev.psych.59.113006.095941]

34   **Blanchard EB**, Schwarz SP, Suls JM, Gerardi MA, Scharff L, Greene B, Taylor AE, Berreman C, Malamood HS. Two controlled evaluations of multicomponent psychological treatment of irritable bowel syndrome. *Behav Res Ther* 1992; **30**: 175-189 [PMID: 1567347]

35   **Shaw G**, Srivastava ED, Sadlier M, Swann P, James JY, Rhodes J. Stress management for irritable bowel syndrome: a controlled trial. *Digestion* 1991; **50**: 36-42 [PMID: 1804731]

36   **Ford AC**, Quigley EM, Lacy BE, Lembo AJ, Saito YA, Schiller LR, Soffer EE, Spiegel BM, Moayyedi P. Effect of antidepressants and psychological therapies, including hypnotherapy, in irritable bowel syndrome: systematic review and meta-analysis. *Am J Gastroenterol* 2014; **109**: 1350-1365; quiz 1366 [PMID: 24935275 DOI: 10.1038/ajg.2014.148]

37   **Timmer A**, Preiss JC, Motschall E, Rücker G, Jantschek G, Moser G. Psychological interventions for treatment of inflammatory bowel disease. *Cochrane Database Syst Rev* 2011; **(2)**: CD006913 [PMID: 21328288 DOI: 10.1002/14651858.CD006913.pub2]

38   **Keefer L**, Taft TH, Kiebles JL, Martinovich Z, Barrett TA, Palsson OS. Gut-directed hypnotherapy significantly augments clinical remission in quiescent ulcerative colitis. *Aliment Pharmacol Ther* 2013; **38**: 761-771 [PMID: 23957526 DOI: 10.1111/apt.12449]

Association between COX-2 -1195G>A polymorphism and gastrointestinal cancer risk: A meta-analysis

Xiao-Wei Zhang, Jun Li, Yu-Xing Jiang, Yu-Xiang Chen

Xiao-Wei Zhang, Jun Li, Yu-Xiang Chen, Department of Gastrointestinal and Vascular Surgery, Deyang People's Hospital, Deyang 618099, Sichuan Province, China

Yu-Xing Jiang, Department of General Surgery and Center for Minimal Invasive Gastrointestinal Surgery, Southwest Hospital, Third Military Medical University, Chongqing 400038, China

Abstract

AIM
To perform a meta-analysis to investigate the association between cyclooxygenase-2 (COX-2) -1195G>A gene polymorphism and gastrointestinal cancers.

METHODS
Publications related to the COX-2 -1195G>A gene polymorphism and gastrointestinal cancers published before July 2016 were retrieved from PubMed, EMBASE, Web of Science, China Biological Medicine Database, China National Knowledge Infrastructure, and CQVIP Database. Meta-analysis was performed using Stata11.0 software. The strength of the association was evaluated by calculating the combined odds ratios (ORs) and the corresponding 95%CIs. The retrieved publications were excluded or included one by one for sensitivity analysis. In addition, the funnel plot, Begg's rank correlation test, and Egger's linear regression method were applied to analyse whether the included publications had publication bias.

RESULTS
A total of 24 publications related to the COX-2 -1195G>A gene polymorphism were included, including 28 studies involving 11043 cases and 18008 controls. The meta-analysis results showed that the COX-2 -1195G>A gene polymorphism significantly correlated with an increased risk of gastrointestinal cancers, particularly gastric cancer (A *vs* G: OR = 1.35; AA/AG *vs* GG: OR = 1.54; AA *vs* GG/AG: OR = 1.43; AA *vs* GG: OR = 1.80; AG *vs* GG: OR = 1.35). Compared to the Caucasian population in America and Europe, the COX-2 -1195G>A gene polymorphism in the Asian population (A *vs* G: OR = 1.30; AA/AG *vs* GG: OR

= 1.50; AA *vs* GG/AG: OR = 1.35; AA *vs* GG: OR = 1.71; AG *vs* GG: OR = 1.37) significantly increased gastrointestinal cancer risk. The sensitivity analysis (P < 0.05) and the false positive report probability (P < 0.2) confirmed the reliability of the results.

CONCLUSION
The results showed that the COX-2 -1195G>A gene polymorphism might be a potential risk factor for gastrointestinal cancers. Further validation by a large homogeneous study is warranted.

Key words: COX-2; -1195G>A; Polymorphism; Meta-analysis; Gastrointestinal cancer

Core tip: To explore the association of the cyclo-oxygenase-2 (COX-2) (-1195G>A) polymorphism with gastrointestinal cancers, we conducted this retrospective study. According to this meta-analysis, we discovered that the COX-2 (-1195G>A) polymorphism may be a risk factor for gastrointestinal cancers and may increase the risk of gastrointestinal cancers in the Asian population. Furthermore, we applied a false-positive report probability to make the results more credible. Our findings indicated that focusing on the COX-2 (-1195G>A) polymorphism to prevent gastrointestinal cancers may be viable.

INTRODUCTION

Gastrointestinal cancers have high morbidity and mortality worldwide, with most cases being gastric cancer and colorectal cancer[1,2]. Because currently there is still no effective early diagnosis method, patients are often diagnosed at a middle or late stage; even after treatment, their quality of life and survival time are still significantly affected[3]. Improving the early diagnosis and treatment of gastric cancer and colorectal cancer has important significance in the prognosis of patients[4,5]. Therefore, studying pathogenic mechanisms of tumours, clarifying the molecular mechanism, discovering "key" molecular markers of tumours, and predicting cancer risk in a timely fashion are key to the prevention, diagnosis, and molecular targeted therapy of gastric cancer and colorectal cancer.

Previous studies have shown that cyclooxygenase-2 (COX-2) is a rate-limiting enzyme of prostaglandin synthesis[6] and is closely associated with the development of malignant tumours[7]. COX-2 is localized in the nuclear membrane under physiological conditions and can be expressed in the cytoplasm and nucleus of corresponding tissues after inflammatory stimulation to participate in inflammatory reactions and promote the formation of a tumour inflammatory microenvironment[8]. A larger amount of literature confirmed that a high COX-2 expression level was present in many malignant tumours, including breast cancer, lung cancer, liver cancer, and nasopharyngeal carcinoma. The high COX-2 expression level was not only an early event of the development of malignant tumours but was also directly correlated with the infiltration degree, lymph node metastasis, TNM stage, and patient prognosis[9-11]. Further studies indicated that the intracellular localizations of COX-2 in tumour cells of different tissues types were different[12]. COX-2 was highly expressed in gastric cancer and colorectal cancer cells; in addition, COX-2 was expressed in macrophages and fibroblasts in tumour tissues[13]. These results indicated that COX-2 expression gradually increases during the process of malignant transformation of precancerous lesions into malignant tumours, suggesting that COX-2 is involved in the developmental process of gastric cancer and colorectal cancer; however, the specific mechanism is still not clear.

The COX-2 gene is localized at q25.2-25.3 of chromosome 1 and contains 10 exons and 9 introns with a total length of approximately 8.3 kb. COX-2 is a rapid-response gene to various factors, such as inflammatory factors, tumourigenic factors, injury, and growth factors, all of which can induce its rapid expression[14,15]. There have been already many published studies on the association between COX-2 gene polymorphisms and susceptibility to gastrointestinal cancers. It is generally considered that COX-2 -765G>C and COX-2 -8473T>C gene mutations are closely associated with the development of gastrointestinal cancers[16,17]. However, the association between COX-2 -1195G>A and gastric and colorectal cancers is still unclear. Because the COX-2 gene has larger distribution differences in populations of different ethnicities and different regions and the sample size in a single study is limited, this association cannot be entirely explained. Given the current controversial study results, we aimed to perform a meta-analysis to confirm the association between the COX-2 -1195G>A polymorphism and susceptibility to gastric and colorectal cancers.

MATERIALS AND METHODS

Retrieval strategy
We performed retrieval using the MeSH terms of (COX-2 -1195G>A or COX-2 -1195G>A) and (gastrointestinal or colorectal or colon or rectal or stomach or gastric) and (cancer or tumour or carcinoma) and (polymorphism or

Table 1 Quality evaluation scale of the included literature

Criterion	Score
Representativeness of cases	
Selected from population or cancer registry	3
Selected from hospital	2
Selected from pathology archives, but without description	1
Not described	0
Source of controls	
Population-based	3
Blood donors or volunteers	2
Hospital-based (cancer-free patients)	1
Not described	0
Case-control match	
Matched by age and gender	3
Not matched by age and gender	0
Specimens used for determining genotypes	
White blood cells or normal tissues	3
Tumor tissues or exfoliated cells of tissue	0
Hardy-Weinberg equilibrium (HWE)	
Hardy-Weinberg equilibrium in control subjects	3
Hardy-Weinberg disequilibrium in control subjects	0
Total sample size	
> 1000	3
> 500 and < 1000	2
> 200 and < 500	1
< 200	0

SNP or variant or mutation) in the following databases: PubMed, EMBASE, Web of Science, China Biological Medicine Database, China National Knowledge Infrastructure, and CQVIP Database. The relevant studies in China and other countries were retrieved. The retrieval period was between the establishment of the databases and July 2016. Relevant conference papers were manually retrieved from the journal database of the Third Military Medical University library.

Inclusion criteria
The included literature in this study met the following criteria: (1) studies about the COX-2 -1195G>A gene polymorphism and susceptibility to gastrointestinal cancers; (2) case-controlled or cohort studies; (3) gastrointestinal cancer patients as the case group; and (4) enough genotype data to calculate odds ratios (ORs) and corresponding 95% confidence internals (CIs).

Exclusion criteria
The exclusion criteria were as follows: (1) the study topic of the article was not about the COX-2 -1195G>A gene polymorphism and susceptibility to gastrointestinal cancers; (2) the studies were not case-controlled or cohort studies; (3) abstracts, reviews, case reports, or repetitively published articles; and (4) the study data were not complete or the raw data could not be obtained.

Data extraction and quality evaluation
The data were independently extracted by two researchers (Xiao-Wei Zhang, Jun Li) using the unified data table. The major extracted data included the

following information: first author, publication year, country, tumour type, sources of the control group, matching criteria, genotyping method, genotype distribution in the case group and the control group, and the Hardy-Weinberg equilibrium (HWE) examination result of the control group. If the data extraction results were inconsistent, a third party was consulted to reach a consensus.

The included publications were scored using the predetermined criteria[18,19]. These criteria were extracted and modified from previous studies (Table 1). The quality evaluation scale was used to evaluate the included studies from six aspects: representativeness of cases, source of controls, case-control match, specimens used for determining genotypes, HWE, and total sample size. The scores ranged from the lowest, 0 points, to the highest, 18 points. Publications with a score < 12 were classified as "low quality" and publications with a score $\geqslant$ 12 were classified as "high quality."

Statistical analysis
The OR and 95%CI were used as the effective index of the study. $P < 0.05$ indicated that the difference was statistically significant. Five genetic models, including allele model (A *vs* G), dominant model (AA/AG *vs* GG), recessive model (AA *vs* GG/AG), homozygous model (AA *vs* GG), and heterozygous model (AG *vs* GG), were compared. The statistical significance of combined OR values were examined using the Z test, and the significance level was set at 0.05 (bilateral). The χ^2 test was used to evaluate whether the genotypes in the control group conformed to HWE. The Cochrane Q test was performed to analyse the heterogeneity among studies[20]. $P < 0.10$ was considered significantly different. In addition, the I^2 value was combined to quantitatively evaluate the level of heterogeneity. The I^2 values were between 0% and 100%; when the value was larger, the heterogeneity was higher. When the heterogeneity examination result showed $P < 0.10$ or $I^2 > 50\%$, the random effects model (DerSimonian-Laird method)[21] was used to perform the analysis; otherwise, the fixed effects model (Mantel-Haenszel method)[22] was used. The included studies were deleted one by one to perform sensitivity analysis to examine the effect of a single study on the total combined effect size. Whether the included literature had publication bias was analysed through the funnel plot[23], Egger's linear regression method[24], and Begg's rank correlation test[25]. The meta-analysis was performed using Stata11.0 software.

The method reported by Wacholder *et al*[26] was used to analyse the false positive report probability (FPRP) of each significant correlation. A prior probability of 0.001 was set to detect an OR of 1.5. When the FPRP value was lower than 0.2, the correlation was noteworthy. The statistical power and FPRP value were calculated using
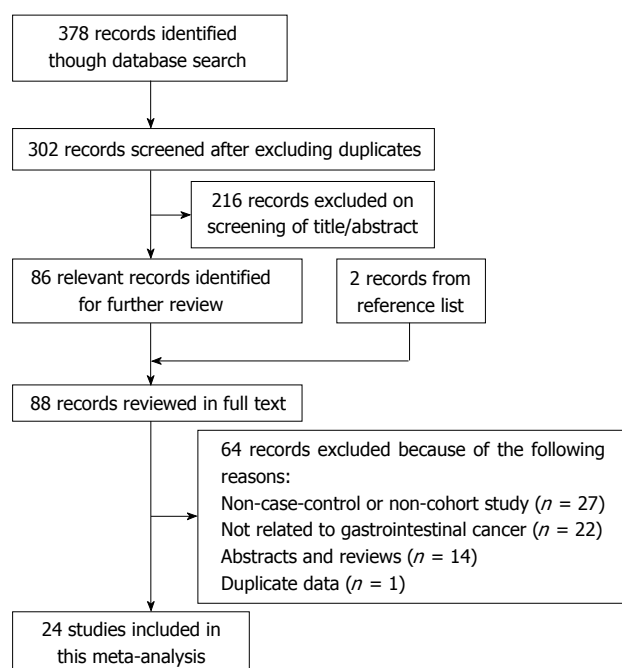
Figure 1 Flow chart of literature inclusion and exclusion.

the Excel spreadsheet provided by Wacholder *et al*[26].

RESULTS

Literature retrieval results

A total of 378 relevant publications were retrieved. After repetitive publications were excluded, there were 302 publications. Literature screening was performed according to the inclusion and exclusion criteria. Based on titles and abstracts, 216 publications that were irrelevant to the study topic were excluded. After abstracts and the full texts were further carefully read, 64 publications were excluded (27 publications of non-case-controlled and cohort studies, 22 publications irrelevant to gastrointestinal cancers, 14 publications of abstracts and reviews, and 1 repeatedly published article). Based on the references of the included literature, 2 more publications were obtained. A total of 24 publications were finally included, involving 11,043 cases and 18,008 controls (Figure 1).

Characteristics of the included studies

Among the 24 included publications (Table 2[27-49]), 11 were reports on gastric cancer and 13 on colorectal cancer; 14 were studies in Asian populations, 8 in Caucasian populations, and 2 in mixed populations. The HWE examination results of the distribution of genotypes in the control group are shown in Table 2. Among the 24 publications, the distribution of genotypes in the control groups of 19 publications conformed to HWE. The quality score of a single study ranged from 7 to 18. There were 19 publications of high quality studies ($\geqslant$ 12).

Meta-analysis results

The ORs of different comparisons and the heterogeneity examination results are shown in Table 3. The results showed that COX-2 -1195G>A gene polymorphism in all of the genetic models (A *vs* G: OR = 1.54; AA/AG *vs* GG: OR = 1.24; AA *vs* GG/AG: OR = 1.16; AA *vs* GG: OR = 1.31; AG *vs* GG: OR = 1.18) had a significant correlation with susceptibility to gastrointestinal cancers. However, when the predetermined prior probability was below 0.001, all of the FPRP values were higher than 0.2. This result indicated that the association was not noteworthy.

The subgroup analysis was performed based on tumour types (Figure 2). In the gastric cancer group (A *vs* G: OR = 1.35; AA/AG *vs* GG: OR = 1.54; AA *vs* GG/AG: OR = 1.43; AA *vs* GG: OR = 1.80; AG *vs* GG: OR = 1.35), the results showed that the COX-2 -1195G>A gene polymorphism was significantly correlated with cancer susceptibility. Analysis of FPRP in the gastric group showed that the value in the AA *vs* GG/AG model (FPRP = 0.174) was lower than 0.2, indicating that the result was noteworthy. However, the COX-2 -1195G>A gene polymorphism was not significantly correlated with susceptibility to colorectal cancer.

When subgrouping based on ethnicity (Figure 3), in the Asian population (A *vs* G: OR = 1.30; AA/AG *vs* GG: OR = 1.50; AA *vs* GG/AG: OR = 1.35; AA *vs* GG: OR = 1.71; AG *vs* GG: OR = 1.37), COX-2 -1195G>A could significantly increase the risk of developing gastrointestinal cancers. In addition, in the A *vs* G model (FPRP = 0.069), AA/AG *vs* GG model (FPRP = 0.167) and AA *vs* GG model (FPRP = 0.093), the FPRP values were lower than 0.2, indicating that the analytic results were stable and reliable. The results did not show a significant correlation between the COX-2 -1195G>A gene polymorphism and gastrointestinal cancer susceptibility in the Caucasian and mixed populations.

The subgroup analysis based on the sources of the control group showed that, in the studies based on populations from communities (A *vs* G: OR = 1.16; AA/AG *vs* GG: OR = 1.26; AA *vs* GG/AG: OR = 1.19; AA *vs* GG: OR = 1.35; AG *vs* GG: OR = 1.19), the COX-2 -1195G>A gene polymorphism significantly correlated with gastrointestinal susceptibility. The FPRP value in the A *vs* G model was lower than 0.2, indicating that the correlation was noteworthy. For studies based on populations from hospitals, none of the genetic models showed a correlation with intestinal cancers.

The subgroup analysis using the quality evaluation scores showed that, in the high quality studies (A *vs* G: OR = 1.15; AA/AG *vs* GG: OR = 1.25; AA *vs* GG/AG: OR = 1.19; AA *vs* GG: OR = 1.34; AG *vs* GG: OR = 1.19), the COX-2 -1195G>A gene polymorphism correlated with susceptibility to the development of

Table 2 Baseline information of the included studies

Ref.	Year	Country	Type of cancer	Source of controls	Matching criteria	Genotyping method	Cases			Controls			HWE	Quality score
							AA	AG	GG	AA	AG	GG		
Liu *et al*[27]	2006	China	Gastric cancer	PB	NA	DHPLC	88	116	44	375	771	377	0.626	14
Siezen *et al*[28]	2006	Netherland	Colorectal cancer	PB	Age, sex, center	PCR-RFLP	127	59	10	243	128	20	0.558	17
Siezen *et al*[28]	2006	Netherland	Colorectal cancer	PB	Age, sex, center	PCR-RFLP	283	132	19	422	226	41	0.149	18
Jiang *et al*[29]	2007	China	Gastric cancer	PB	Age, sex	PCR-RFLP	74	132	48	79	163	62	0.187	16
Tan *et al*[30]	2007	China	Colorectal cancer	PB	Age, sex	PCR-RFLP	320	502	178	308	692	300	0.020	14
Andersen *et al*[31]	2009	Denmark	Colorectal cancer	PB	Sex	Taqman	230	116	13	482	258	25	0.177	15
Hoff *et al*[32]	2009	Netherland	Colorectal cancer	HB	Age, sex	PCR-RFLP	213	101	12	232	124	13	0.471	14
Thompson *et al*[33]	2009	United States	Colorectal cancer	PB	NA	Taqman	275	138	9	297	168	15	0.131	14
Pereira *et al*[34]	2010	Portugal	Colorectal cancer	HB	NA	PCR-RFLP	70	43	4	177	73	6	0.634	10
Zhang *et al*[35]	2011	China	Gastric cancer	PB	Age, sex	PCR-RFLP	107	184	32	256	513	175	0.004	14
Zhang *et al*[36]	2011	China	Gastric cancer	PB	Age, sex	PCR-RFLP	113	175	69	241	527	217	0.027	14
Jing *et al*[37]	2012	China	Gastric cancer	PB	Age, sex	PCR-RFLP	49	87	19	51	133	53	0.059	15
Li *et al*[38]	2012	China	Gastric cancer	PB	NA	PCR-RFLP	98	145	53	73	166	80	0.461	14
Shin *et al*[39]	2012	Korea	Gastric cancer	PB	NA	PCR-RFLP	32	54	14	37	41	22	0.107	12
Zhang *et al*[40]	2012	China	Colorectal cancer	PB	NA	PCR-RFLP	77	216	50	62	184	94	0.09	12
Andersen *et al*[41]	2013	Denmark	Colorectal cancer	PB	NA	KASP™ genotyping	587	313	47	1126	560	61	0.397	15
Li *et al*[42]	2013	China	Colorectal cancer	HB	NA	PCR-RFLP	116	248	87	179	336	114	0.045	9
Makar *et al*[43]	2013	United States	Colorectal cancer	PB	Age, location, sex	Taqman	910	455	57	1198	509	67	0.162	17
Makar *et al*[43]	2013	United States	Colorectal cancer	PB	Age, location, sex	Taqman	619	287	33	958	496	63	0.905	17
Makar *et al*[43]	2013	United States	Colorectal cancer	PB	Age, location, sex	Taqman	376	185	20	509	237	29	0.829	17
Makar *et al*[43]	2013	United States	Colorectal cancer	PB	Age, location, sex	Taqman	338	138	21	558	249	20	0.206	17
Ruan *et al*[44]	2013	China	Colorectal cancer	PB	NA	PCR-RFLP	34	67	29	39	53	28	0.232	12
Pereira *et al*[45]	2014	Portugal	Colorectal cancer	HB	NA	Taqman	143	85	15	323	133	16	0.614	11
Vogel *et al*[46]	2014	Norseland	Colorectal cancer	PB	NA	KBioscience	110	24	2	209	114	11	0.337	12
Gao *et al*[47]	2015	China	Gastric cancer	PB	Age, sex	Taqman	86	137	55	74	137	57	0.664	16
Lu *et al*[17]	2015	China	Gastric cancer	HB	NA	PCR-RFLP	69	39	25	27	35	72	0.000	7
Tao *et al*[48]	2015	China	Gastric cancer	PB	Age, sex	PCR-RFLP	39	71	26	31	65	25	0.397	15
Zamudio *et al*[49]	2016	Peru	Gastric cancer	HB	NA	Taqman	85	103	32	106	139	43	0.815	9

HWE: Hardy-Weinberg equilibrium; PB: Population-based; HB: Hospital-based; DHPLC: Denaturing high performance liquid chromatography; PCR-RFLP: Polymerase chain reaction-restriction fragment length polymorphism; NA: Not available.

gastrointestinal cancers. However, the FPRP analytic values were all higher than 0.2, indicating that the analytic results were not stable. In low quality studies, the COX-2 -1195G>A gene polymorphism did not have a significant correlation with gastrointestinal cancers.

Furthermore, the subgroup analysis based on different genotyping methods showed that, in the studies using the Restriction Fragment Length Polymorphism Analysis of PCR-Amplified Fragments (PCR-RFLP) genotyping method (A *vs* G: OR =

1.23; AA/AG *vs* GG: OR = 1.46; AA *vs* GG/AG: OR = 1.24; AA *vs* GG: OR = 1.58; AG *vs* GG: OR = 1.35), the COX-2 -1195G>A gene polymorphism significantly correlated with gastrointestinal cancer susceptibility. However, the FPRP analysis showed that the evidence of the real correlation of positive results was not sufficient. For genotyping using Taqman and other technologies, the COX-2 -1195G>A gene polymorphism in none of the genetic models was significantly correlated with intestinal cancers.

Table 3 Stratified analyses of the COX-2 -1195G>A polymorphism with risk of gastrointestinal cancers

	n	Allele model (A vs G)			Dominant model (AA/AG vs GG)			Recessive model (AA vs GG/AG)			Homozygous comparison (AA vs GG)			Heterozygous comparison (AG vs GG)		
		OR (95%CI)	Ph	FPRP	OR (95%CI)	Ph	FPRP	OR (95%CI)	Ph	FPRP	OR (95%CI)	Ph	FPRP	OR (95%CI)	Ph	FPRP
Total	28	1.15 (1.04, 1.26)[1]	0.000	0.73	1.24 (1.06, 1.45)[1]	0	0.876	1.16 (1.04, 1.30)[1]	0.000	0.914	1.31 (1.08, 1.59)[1]	0.000	0.873	1.18 (1.04, 1.34)[1]	0.007	0.915
Type of cancer																
Gastric cancer	11	1.35 (1.14, 1.59)[1]	0.000	0.266	1.54 (1.20, 1.96)[1]	0.000	0.519	1.43 (1.18, 1.72)[1]	0.002	0.174	1.80 (1.36, 2.39)[1]	0.000	0.318	1.35 (1.11, 1.65)[1]	0.038	0.799
Colorectal cancer	17	1.04 (0.94, 1.15)	0.000	0.998	1.05 (0.87, 1.28)	0.002	0.998	1.04 (0.93, 1.18)	0.000	0.998	1.05 (0.83, 1.32)	0.000	0.999	1.06 (0.90, 1.25)	0.060	0.998
Ethnicity																
Asian	14	1.30 (1.14, 1.48)[1]	0.000	0.069	1.50 (1.23, 1.84)[1]	0.000	0.167	1.35 (1.14, 1.60)[1]	0.000	0.376	1.71 (1.33, 2.18)[1]	0.000	0.093	1.37 (1.15, 1.62)[1]	0.007	0.213
Caucasian	12	1.00 (0.89, 1.11)	0.000	0.999	0.91 (0.76, 1.08)	0.360	0.996	1.01 (0.89, 1.15)	0.000	0.999	0.91 (0.74, 1.11)	0.186	0.997	0.92 (0.77, 1.09)	0.749	0.997
Mixed	2	1.10 (0.93, 1.31)	0.612	0.997	1.13 (0.74, 1.73)	0.466	0.998	1.13 (0.91, 1.40)	0.781	0.996	1.20 (0.76, 1.88)	0.482	0.998	1.09 (0.69, 1.70)	0.534	0.999
Source of controls																
PB	22	1.16 (1.06, 1.25)[1]	0.000	0.09	1.26 (1.09, 1.45)[1]	0.003	0.559	1.19 (1.07, 1.33)[1]	0.000	0.685	1.35 (1.13, 1.61)[1]	0.000	0.488	1.19 (1.04, 1.36)[1]	0.031	0.914
HB	6	1.12 (0.75, 1.67)	0.000	0.998	1.14 (0.60, 2.15)	0.000	0.999	1.08 (0.72, 1.63)	0.000	0.999	1.15 (0.54, 2.45)	0.000	0.999	1.12 (0.73, 1.71)	0.021	0.998
Study quality																
High (>9)	23	1.15 (1.06, 1.25)[1]	0.000	0.504	1.25 (1.09, 1.44)[1]	0.004	0.667	1.19 (1.07, 1.32)[1]	0.000	0.502	1.34 (1.12, 1.59)[1]	0.000	0.469	1.19 (1.04, 1.35)[1]	0.038	0.873
Low (≤9)	5	1.13 (0.68, 1.86)	0.000	0.999	1.17 (0.56, 2.45)	0.000	0.999	1.09 (0.65, 1.81)	0.000	0.999	1.17 (0.48, 2.88)	0.000	0.999	1.16 (0.71, 1.90)	0.011	0.998
Genotyping method																
PCR-RFLP	16	1.23 (1.08, 1.40)[1]	0.000	0.633	1.46 (1.19, 1.78)[1]	0.000	0.231	1.24 (1.06, 1.46)[1]	0.000	0.909	1.58 (1.23, 2.02)[1]	0.000	0.436	1.35 (1.14, 1.60)[1]	0.014	0.376
Taqman	9	0.99 (0.90, 1.08)	0.049	0.999	0.97 (0.82, 1.15)	0.428	0.999	0.99 (0.89, 1.11)	0.063	0.999	0.97 (0.79, 1.19)	0.268	0.999	0.98 (0.82, 1.17)	0.669	0.999
Other technologies	3	1.36 (0.86, 2.17)	0.000	0.997	1.16 (0.58, 2.31)	0.008	0.999	1.52 (0.84, 2.75)	0.000	0.997	1.40 (0.55, 3.53)	0.000	0.999	0.99 (0.62, 1.57)	0.118	0.999

[1]OR with statistical significance. n: Number of studies included; Ph: *P* value for heterogeneity; FPRP: False positive report probability.

Sensitivity analysis and cumulative analysis

The present study performed sensitivity analysis through gradual deletion of the included studies one by one. The OR value of the combined effect did not have a significant change, indicating that the analytic results were stable and reliable (Figure 4). A cumulative analysis based on the chronological order showed that the OR point estimate value and the corresponding CI trended to become stable and showed a good changing trend (Figure 5).

Publication bias

The funnel plot, Begg's rank correlation test, and Egger's linear correlation were used to evaluate publication bias. The funnel plots of all of the models with a correlation between the COX-2 -1195G>A gene polymorphism and gastrointestinal cancers did not have significant asymmetry. In the AA/AG vs GG model, the Begg's rank correlation test showed *P* = 0.489 and the Egger's linear correlation methods showed *P* = 0.690; they both suggested that there was no significant publication bias (Figure 6).

DISCUSSION

In addition to environmental factors, the risk of cancer is also closely associated with the genetic susceptibility of an individual. Previous genetic studies indicated that gene mutations of some inducible enzymes were closely associated with various diseases, including malignant tumours and congenital malformations. These inducible enzymes change the gene expression levels and interfere with signal transduction pathways to inhibit protein synthesis and cause mRNA instability, thus achieving the purpose of changing the encoded proteins and inducing the presence of disease events. Currently, the influences of genes and genetics on the occurrence and development of gastrointestinal cancers are similar to other important factors, such as smoking, drinking, eating habits and geographical environment. Genes and
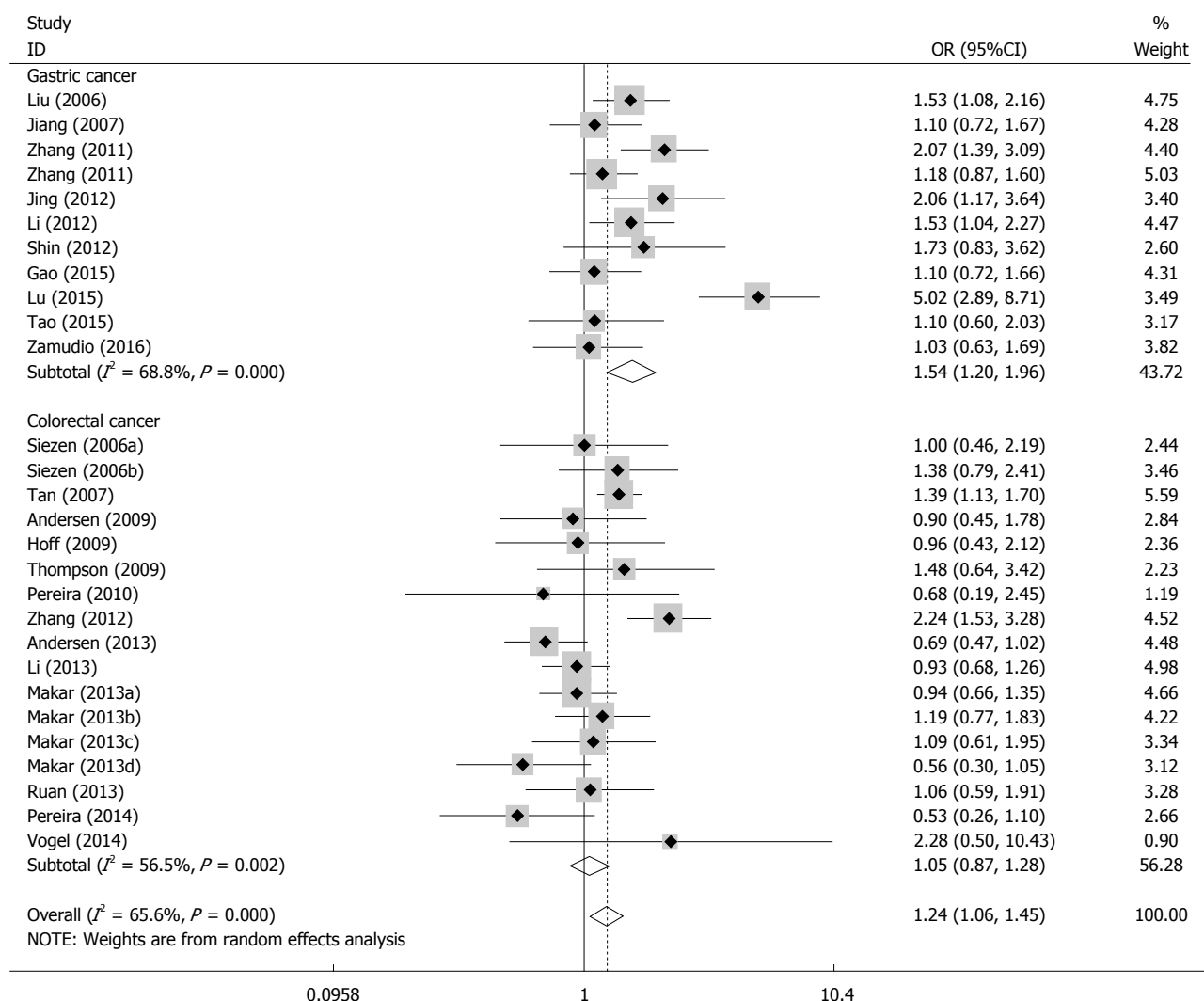
Figure 2 Forest plot of the stratified analysis of the COX-2 -1195G>A dominant model (AA/AG *vs* GG) and susceptibility to gastrointestinal cancers in different tumour types.

genetics have gradually become the hotspots of studies on the pathogenic mechanism of gastrointestinal cancers[50,51].

COX-2 overexpression can influence the tumourigenic gene features of tumour cells, including induction of anti-apoptosis, regulation of extracellular matrix adhesion, promotion of angiogenesis, increase of metastatic potential, and influence of anti-tumour effects[52-54]. Recent studies showed that the COX-2 -1195G>A gene polymorphism generated a c-MYB binding site, thus increasing the transcription activity of the COX-2 gene. c-MYB is an active transcription factor in the haematopoietic system and gastrointestinal tract. c-MYB functions on many genes to regulate the exquisite balance between cell division, differentiation and survival[55], which further confirms that the COX-2 -1195G>A polymorphism might increase susceptibility of individuals to gastrointestinal cancers. However, there were also reports showing that this polymorphism could reduce the risk of developing gastric cancer and colorectal cancer[32]. To clarify this

association, we included all case-controlled or cohort studies that met the inclusion criteria to evaluate the correlation using a meta-analysis.

Our study included 24 publications, including 11 gastric cancer publications and 13 colorectal cancer publications. A total of 11,043 cases in the case group and 18,008 cases in the control group were included. The overall meta-analysis results showed that the COX-2 -1195G>A gene in all of the genetic models (A *vs* G: OR = 1.54, 95%CI: 1.04-1.26, *P* < 0.001; AA/AG *vs* GG: OR = 1.24, 95%CI: 1.06-1.45, *P* < 0.001; AA *vs* GG/AG: OR = 1.16, 95%CI: 1.04-1.30, *P* < 0.001; AA *vs* GG: OR = 1.31, 95%CI: 1.08-1.59, *P* < 0.001; AG *vs* GG: OR = 1.18, 95%CI: 1.04-1.34, *P* = 0.007) was associated with a high risk of developing gastrointestinal cancers. The results of the publication bias and sensitivity analysis also increased the reliability of the association.

The differences in ethnicity, sources of the control population, environmental factors, and the tumour types can all change the risk of developing
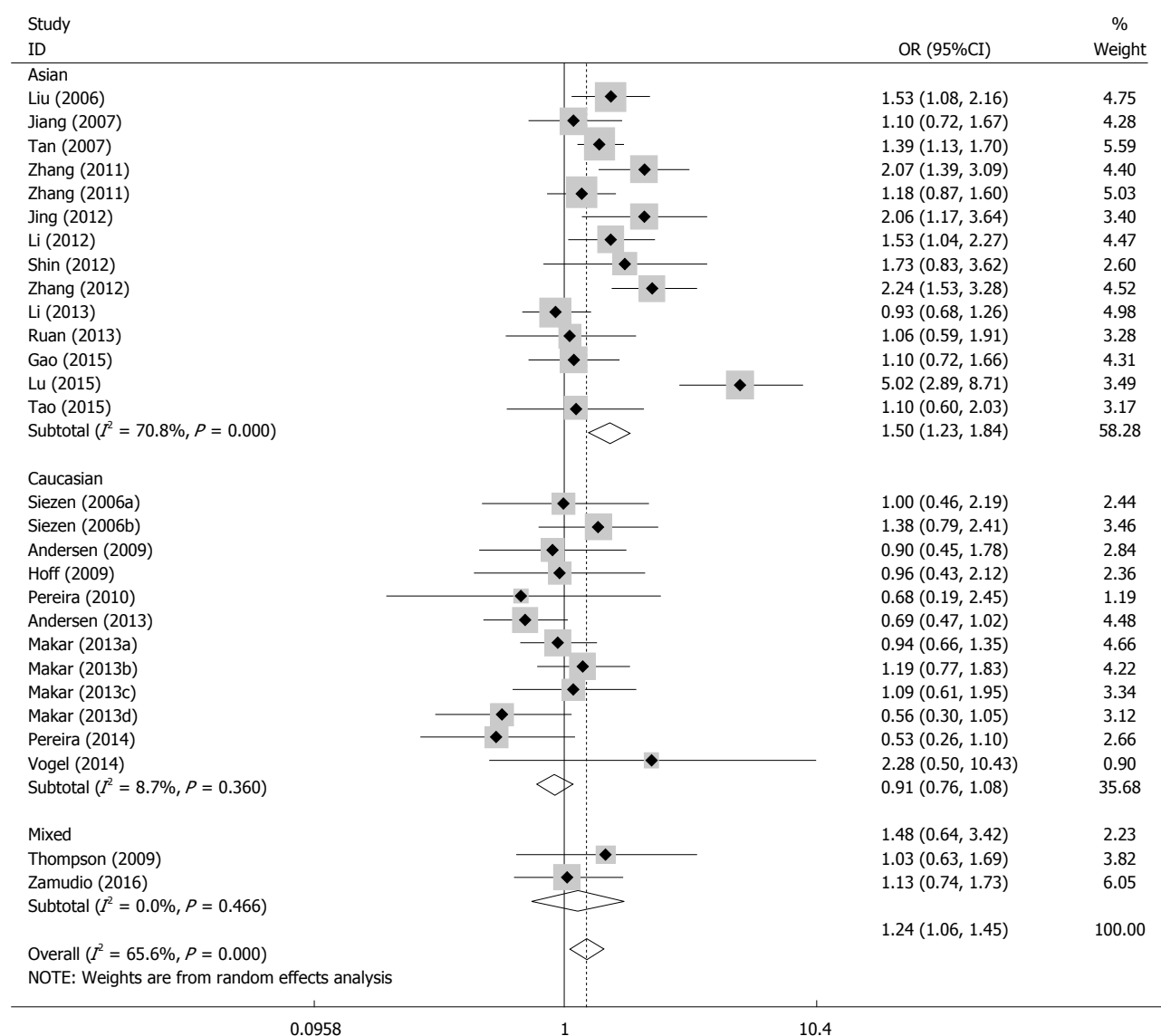
Study ID	OR (95%CI)	% Weight
Asian		
Liu (2006)	1.53 (1.08, 2.16)	4.75
Jiang (2007)	1.10 (0.72, 1.67)	4.28
Tan (2007)	1.39 (1.13, 1.70)	5.59
Zhang (2011)	2.07 (1.39, 3.09)	4.40
Zhang (2011)	1.18 (0.87, 1.60)	5.03
Jing (2012)	2.06 (1.17, 3.64)	3.40
Li (2012)	1.53 (1.04, 2.27)	4.47
Shin (2012)	1.73 (0.83, 3.62)	2.60
Zhang (2012)	2.24 (1.53, 3.28)	4.52
Li (2013)	0.93 (0.68, 1.26)	4.98
Ruan (2013)	1.06 (0.59, 1.91)	3.28
Gao (2015)	1.10 (0.72, 1.66)	4.31
Lu (2015)	5.02 (2.89, 8.71)	3.49
Tao (2015)	1.10 (0.60, 2.03)	3.17
Subtotal (I^2 = 70.8%, P = 0.000)	1.50 (1.23, 1.84)	58.28
Caucasian		
Siezen (2006a)	1.00 (0.46, 2.19)	2.44
Siezen (2006b)	1.38 (0.79, 2.41)	3.46
Andersen (2009)	0.90 (0.45, 1.78)	2.84
Hoff (2009)	0.96 (0.43, 2.12)	2.36
Pereira (2010)	0.68 (0.19, 2.45)	1.19
Andersen (2013)	0.69 (0.47, 1.02)	4.48
Makar (2013a)	0.94 (0.66, 1.35)	4.66
Makar (2013b)	1.19 (0.77, 1.83)	4.22
Makar (2013c)	1.09 (0.61, 1.95)	3.34
Makar (2013d)	0.56 (0.30, 1.05)	3.12
Pereira (2014)	0.53 (0.26, 1.10)	2.66
Vogel (2014)	2.28 (0.50, 10.43)	0.90
Subtotal (I^2 = 8.7%, P = 0.360)	0.91 (0.76, 1.08)	35.68
Mixed	1.48 (0.64, 3.42)	2.23
Thompson (2009)	1.03 (0.63, 1.69)	3.82
Zamudio (2016)	1.13 (0.74, 1.73)	6.05
Subtotal (I^2 = 0.0%, P = 0.466)		
	1.24 (1.06, 1.45)	100.00
Overall (I^2 = 65.6%, P = 0.000)		
NOTE: Weights are from random effects analysis		

0.0958   1   10.4

Figure 3 Forest plot of stratified analysis of the COX-2 -1195G>A dominant model (AA/AG *vs* GG) and gastrointestinal cancer susceptibility in different populations.

gastrointestinal diseases through the gene-environment interaction. Therefore, the present study performed subgroup analysis based on the different specific conditions of all of the studies. In the classification of tumour types, the results showed that the COX-2 -1195G>A gene in the AA/AG *vs* GG model had a clear correlation with the gastric cancer susceptibility but did not have a significant correlation with colorectal cancer, suggesting that this genotype might be a very important predisposing factor for gastric cancer. This result was also similar to the reported results in some literature. In addition, the subgroup analysis based on the ethnicity of the study population showed that the mutation frequency of this polymorphism in the Asian gastrointestinal cancer population was higher than that in the Caucasian population in America and Europe, suggesting that the presence of the COX-2 -1195G>A gene polymorphism might greatly increase susceptibility of the Asian

population, as represented by Chinese and Korean populations, to gastrointestinal cancers. For the mixed population from America, there were only two reports on its association with gastrointestinal cancers. This result was not sufficient to explain the issue, and studies with a larger sample size are needed to confirm its reliability. The subgroup analysis based on the sources of the control population showed that an increase in the risk of developing gastrointestinal cancers in the population from communities had a statistical correlation with the COX-2 -1195G>A polymorphism; however, this correlation in the population from hospitals was not statistically significant. These results suggested that, in the selection of the sources of controls, the hospital population was restricted by their diseases and medications; therefore, the genotyping results might be affected. Thus, samples from the community population were more representative than those from hospitals and relevant studies should
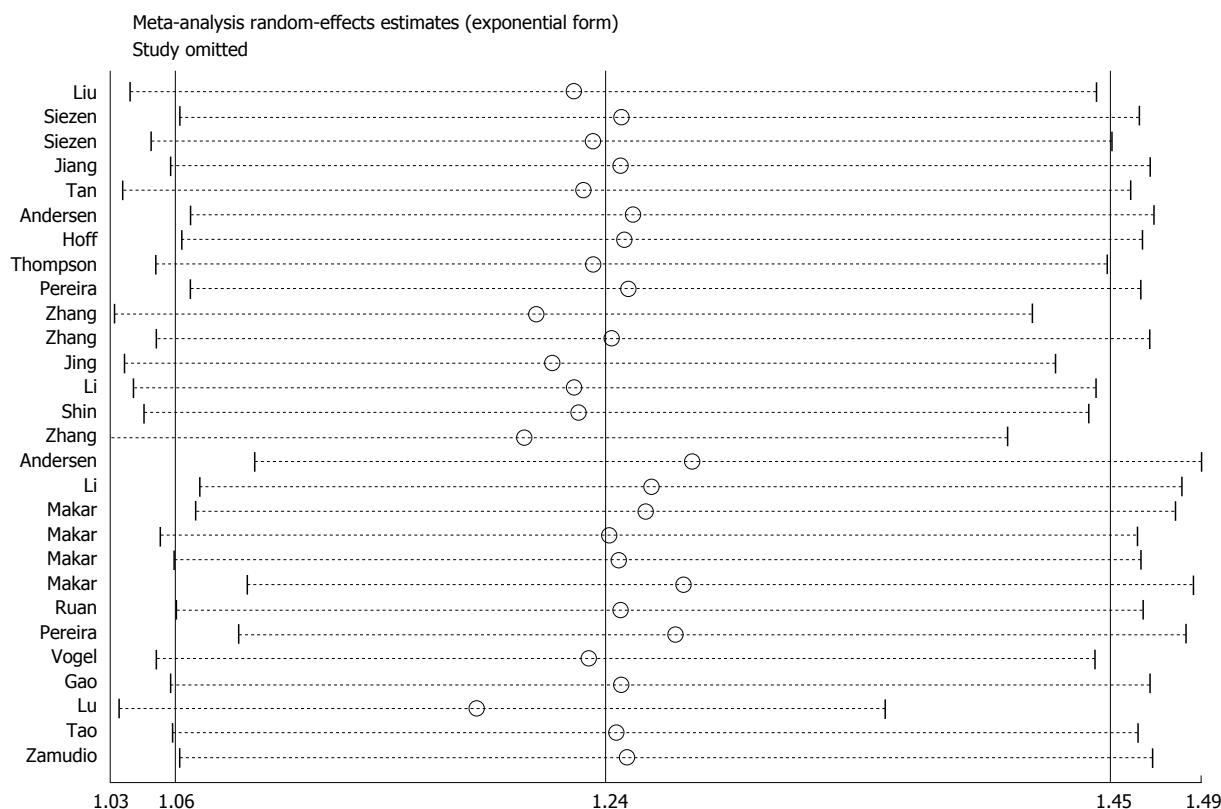
Figure 4 Analysis of the influence of a single study on the total combined OR in the dominant model (AA/AG *vs* GG).

try to select those from the community population as a control group. Furthermore, we also performed subgroup analysis based on genotyping methods and found that the statistical results among subgroups had clear differences. The differences might be because the different detection methods had different theoretical bases. To make the positive rate of our analytic results more real and reliable, we performed FPRP and found that the correlation of the COX-2 -1195G>A polymorphism in the gastric cancer recessive model (FPRP = 0.174), the allele model of the Asian population (FPRP = 0.069) and the linear model (FPRP) all passed the FPRP test. These results suggested that the correlation of these two aspects had very strong reliability and the authenticity was further confirmed.

The present study had some limitations. First, during overall and subgroup analyses, we found that there was moderate heterogeneity among samples. Although we tried to resolve this issue and used FPRP to increase the reliability of the study results, the exact source of the heterogeneity still could not be completely explained. The present study also revealed that the heterogeneity was not from a single study. The differences in the distribution of the gene polymorphism frequency among ethnic groups and other unknown factors might be the real sources of the heterogeneity. Because gastrointestinal cancers are influenced by many factors, comprehensive study and analysis should be performed in the future by combining these factors, such as diet, living habits, and

environmental exposure. Next, due to the restriction of the sample size and disease types in the included literature, we did not retrieve similar literature reports on other gastrointestinal cancers other than gastric cancer and colorectal cancer, and their association with the COX-2 -1195G>A gene polymorphism could not be clarified. Third, the present study is a meta-analysis based on the reported data of the included literature. The unreasonable data in the original studies could not be corrected and possible potential confounding factors, such as age, gender, ethnicity, specific living habits, and smoking and drinking habits, might be present. Fourth, all of the included literature was published in Chinese or English; relevant studies written in other languages may have been missed. Only including Chinese and English literature was also a reason that the sample size was not large enough, which might result in the presence of false-negative results. In addition, this meta-analysis only included published literature, and there are some relevant, important unpublished studies, which might cause a potential publication bias.

In summary, we demonstrate that the AA genotype in the COX-2 -1195G>A gene polymorphism might be an important predisposing factor for gastrointestinal cancers compared to the AG or GG phenotypes, especially for gastric cancer. In addition, compared to the included studies on American and European Caucasian populations, COX-2 -1195G>A increased susceptibility of the Asian population to gastrointestinal
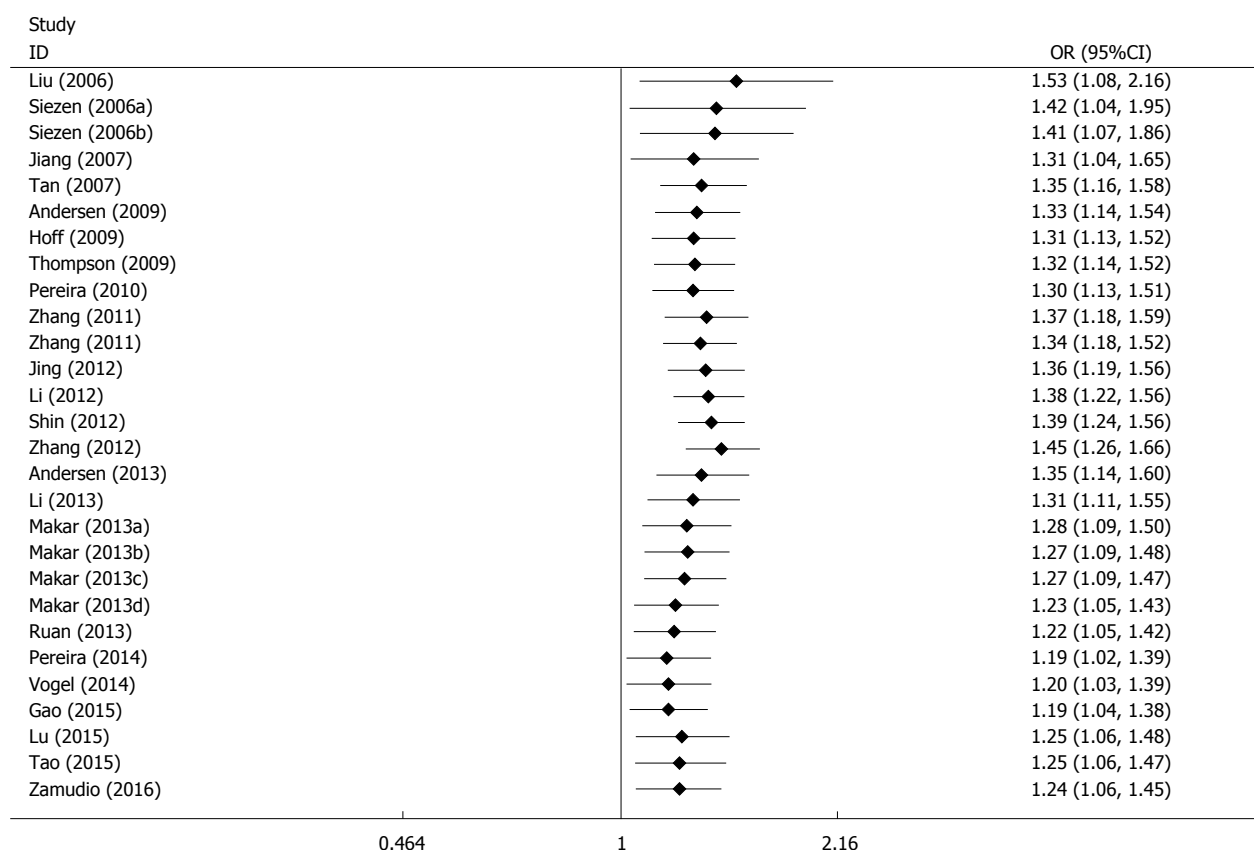
Figure 5 Cumulative meta-analysis of the COX-2 -1195G>A polymorphism and gastrointestinal cancer susceptibility in the dominant model (AA/AG *vs* GG).
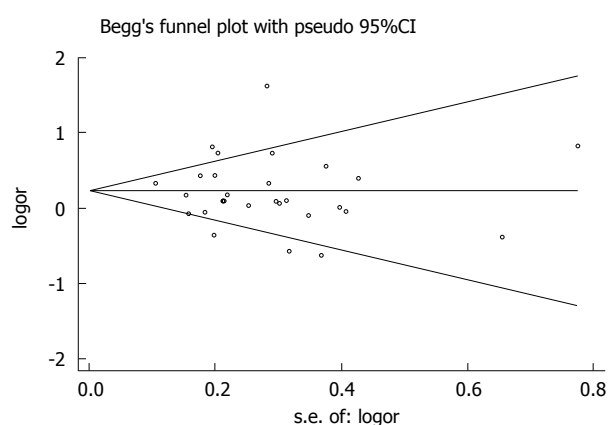

Figure 6 Begg's funnel plot of the publication bias in the COX-2 -1195G>A dominant model (AA/AG *vs* GG).

cancer. In the future, studies with larger sample sizes, more rational design, and more disease types should be performed to validate our conclusion, which can more clearly clarify the association between the COX-2 -1195G>A gene polymorphism and gastrointestinal cancers.

COMMENTS

Background
Cyclooxygenase-2 (COX-2) is closely associated with the development of

malignant tumours and is highly expressed in gastric cancer and colorectal cancer cells. Many studies have investigated the association between the COX-2 -1195G>A gene polymorphism and gastrointestinal cancers; however, the results are inconsistent.

Research frontiers
The COX-2 gene is a very important tumour-related gene with multiple SNPs. The expression level of this gene and the function of its encoded protein will be affected by some polymorphic sites, thus increasing or decreasing tumour susceptibility.

Innovations and breakthroughs
In the present study, the authors explored the COX-2 -1195G>A gene polymorphisms associated with susceptibility to gastrointestinal cancers and used an FPRP-based criterion to evaluate whether the study finding was noteworthy.

Applications
This report may present a novel site for the prevention, diagnosis, and molecular targeted therapy of gastric cancer and colorectal cancer.

Terminology
The false positive report probability (FPRP), which is the probability of no true association between a genetic variant and disease given a statistically significant finding, depends not only on the observed *P*-value but also on both the prior probability and the statistical power of the test.

Peer-review
The authors performed a meta-analysis of the association between the COX-2 -1195G>A polymorphism and gastrointestinal cancer risk, which has been extensively investigated.

REFERENCES

1   **Abdelfatah E**, Kerner Z, Nanda N, Ahuja N. Epigenetic therapy in gastrointestinal cancer: the right combination. *Therap Adv Gastroenterol* 2016; **9**: 560-579 [PMID: 27366224 DOI: 10.1177/1 756283X16644247]

2   **Torre LA**, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. *CA Cancer J Clin* 2015; **65**: 87-108 [PMID: 25651787 DOI: 10.3322/caac.21262]

3   **Rahbari M**, Rahbari N, Reissfelder C, Weitz J, Kahlert C. Exosomes: novel implications in diagnosis and treatment of gastrointestinal cancer. *Langenbecks Arch Surg* 2016; **401**: 1097-1110 [PMID: 27342853 DOI: 10.1007/s00423-016-1468-2]

4   **Karimi Kurdistani Z**, Saberi S, Tsai KW, Mohammadi M. MicroRNA-21: Mechanisms of Oncogenesis and its Application in Diagnosis and Prognosis of Gastric Cancer. *Arch Iran Med* 2015; **18**: 524-536 [PMID: 26265521]

5   **Khatkov IE**, Kagramanova AV, Zakharzhevskaya NB, Babikova EA, Generozov EV, Shcherbakov PL, Parfenov AI. [Current principles in the screening, diagnosis, and therapy of colorectal cancer]. *Ter Arkh* 2016; **88**: 90-96 [PMID: 27135106]

6   **Simmons DL**, Botting RM, Hla T. Cyclooxygenase isozymes: the biology of prostaglandin synthesis and inhibition. *Pharmacol Rev* 2004; **56**: 387-437 [PMID: 15317910 DOI: 10.1124/pr.56.3.3]

7   **Coussens LM**, Werb Z. Inflammation and cancer. *Nature* 2002; **420**: 860-867 [PMID: 12490959 DOI: 10.1038/nature01322]

8   **Sasaki Y**, Kamiyama S, Kamiyama A, Matsumoto K, Akatsu M, Nakatani Y, Kuwata H, Ishikawa Y, Ishii T, Yokoyama C, Hara S. Genetic-deletion of Cyclooxygenase-2 Downstream Prostacyclin Synthase Suppresses Inflammatory Reactions but Facilitates Carcinogenesis, unlike Deletion of Microsomal Prostaglandin E Synthase-1. *Sci Rep* 2015; **5**: 17376 [PMID: 26611322 DOI: 10.1038/srep17376]

9   **Pan J**, Kong L, Lin S, Chen G, Chen Q, Lu JJ. The clinical significance of coexpression of cyclooxygenases-2, vascular endothelial growth factors, and epidermal growth factor receptor in nasopharyngeal carcinoma. *Laryngoscope* 2008; **118**: 1970-1975 [PMID: 18758376 DOI: 10.1097/MLG.0b013e3181805134]

10 **Qin G**, Xu F, Qin T, Zheng Q, Shi D, Xia W, Tian Y, Tang Y, Wang J, Xiao X, Deng W, Wang S. Palbociclib inhibits epithelial-mesenchymal transition and metastasis in breast cancer via c-Jun/COX-2 signaling pathway. *Oncotarget* 2015; **6**: 41794-41808 [PMID: 26540629 DOI: 10.18632/oncotarget.5993]

11 **Zeng W**, van den Berg A, Huitema S, Gouw AS, Molema G, de Jong KP. Correlation of microRNA-16, microRNA-21 and microRNA-101 expression with cyclooxygenase-2 expression and angiogenic factors in cirrhotic and noncirrhotic human hepatocellular carcinoma. *PLoS One* 2014; **9**: e95826 [PMID: 24759835 DOI: 10.1371/journal.pone.0095826]

12 **Chen XL**, Su BS, Sun RQ, Zhang J, Wang YL. Relationship between expression and distribution of cyclooxygenase-2 and bcl-2 in human gastric adenocarcinoma. *World J Gastroenterol* 2005; **11**: 1228-1231 [PMID: 15754411 DOI: 10.3748/wjg.v11.i8.1228]

13 **Yashiro M**, Nakazawa K, Tendo M, Kosaka K, Shinto O, Hirakawa K. Selective cyclooxygenase-2 inhibitor downregulates the paracrine epithelial-mesenchymal interactions of growth in scirrhous gastric carcinoma. *Int J Cancer* 2007; **120**: 686-693 [PMID: 17096355 DOI: 10.1002/ijc.22329]

14 **Gu W**, Song L, Li XM, Wang D, Guo XJ, Xu WG. Mesenchymal stem cells alleviate airway inflammation and emphysema in COPD through down-regulation of cyclooxygenase-2 via p38 and ERK MAPK pathways. *Sci Rep* 2015; **5**: 8733 [PMID: 25736434 DOI: 10.1038/srep08733]

15 **Appleby SB**, Ristimäki A, Neilson K, Narko K, Hla T. Structure of the human cyclo-oxygenase-2 gene. *Biochem J* 1994; **302** (Pt 3): 723-727 [PMID: 7945196]

16 **Xu YS**, Zhao B, Long CY, Li H, Lu X, Liu G, Tang XZ, Tang WZ. Cyclooxygenase-2 promoter 765C increase of digestive tract cancer risk in the Chinese population: a meta-analysis. *Asian Pac J Cancer Prev* 2014; **15**: 4563-4566 [PMID: 24969885]

17 **Lu X**, Chen F, Liu X, Yuan D, Zi Y, He X, He R. Detection and Clinical Significance of COX-2 Gene SNPs in Gastric Cancer. *Cell Biochem Biophys* 2015; **72**: 657-660 [PMID: 27352184 DOI: 10.1007/s12013-014-0465-8]

18 **Jiang DK**, Wang WZ, Ren WH, Yao L, Peng B, Yu L. TP53 Arg72Pro polymorphism and skin cancer risk: a meta-analysis. *J Invest Dermatol* 2011; **131**: 220-228 [PMID: 20861852 DOI: 10.1038/jid.2010.270]

19 **Thakkinstian A**, McEvoy M, Minelli C, Gibson P, Hancox B, Duffy D, Thompson J, Hall I, Kaufman J, Leung TF, Helms PJ, Hakonarson H, Halpi E, Navon R, Attia J. Systematic review and meta-analysis of the association between {beta}2-adrenoceptor polymorphisms and asthma: a HuGE review. *Am J Epidemiol* 2005; **162**: 201-211 [PMID: 15987731 DOI: 10.1093/aje/kwi184]

20 **Cochran WG**. The Combination of Estimates from Different Experiments. *Biometrics* 1954; **10**: 101-129

21 **DerSimonian R**, Laird N. Meta-analysis in clinical trials. *Control Clin Trials* 1986; **7**: 177-188 [PMID: 3802833]

22 **Mantel N**, Haenszel W. Statistical aspects of the analysis of data from retrospective studies of disease. *J Natl Cancer Inst* 1959; **22**: 719-748 [PMID: 13655060]

23 **Langan D**, Higgins JP, Gregory W, Sutton AJ. Graphical augmentations to the funnel plot assess the impact of additional evidence on a meta-analysis. *J Clin Epidemiol* 2012; **65**: 511-519 [PMID: 22342263 DOI: 10.1016/j.jclinepi.2011.10.009]

24 **Egger M**, Davey Smith G, Schneider M, Minder C. Bias in meta-analysis detected by a simple, graphical test. *BMJ* 1997; **315**: 629-634 [PMID: 9310563]

25 **Begg CB**, Mazumdar M. Operating characteristics of a rank correlation test for publication bias. *Biometrics* 1994; **50**: 1088-1101 [PMID: 7786990]

26 **Wacholder S**, Chanock S, Garcia-Closas M, El Ghormli L, Rothman N. Assessing the probability that a positive report is false: an approach for molecular epidemiology studies. *J Natl Cancer Inst* 2004; **96**: 434-442 [PMID: 15026468 DOI: 10.1093/jnci/djh075]

27 **Liu F**, Pan K, Zhang X, Zhang Y, Zhang L, Ma J, Dong C, Shen L, Li J, Deng D, Lin D, You W. Genetic variants in cyclooxygenase-2: Expression and risk of gastric cancer and its precursors in a Chinese population. *Gastroenterology* 2006; **130**: 1975-1984 [PMID: 16762620 DOI: 10.1053/j.gastro.2006.03.021]

28 **Siezen CL**, Bueno-de-Mesquita HB, Peeters PH, Kram NR, van Doeselaar M, van Kranen HJ. Polymorphisms in the genes involved in the arachidonic acid-pathway, fish consumption and the risk of colorectal cancer. *Int J Cancer* 2006; **119**: 297-303 [PMID: 16482563 DOI: 10.1002/ijc.21858]

29 **Jiang GJ**, Wang HM, Zhou Y, Tan YF, Ding WL, Gao J, Ke Q, Wang Y, Shen Q, Xu YC, Shen HB. The correlation study between the nucleotide polymorphisms of cyclooxygenase-2 gene and the susceptibility to gastric cancer. *Nanjing Yike Daxue Xuebao* 2007; **27**: 890-894

30 **Tan W**, Wu J, Zhang X, Guo Y, Liu J, Sun T, Zhang B, Zhao D, Yang M, Yu D, Lin D. Associations of functional polymorphisms in cyclooxygenase-2 and platelet 12-lipoxygenase with risk of occurrence and advanced disease status of colorectal cancer. *Carcinogenesis* 2007; **28**: 1197-1201 [PMID: 17151091 DOI: 10.1093/carcin/bgl242]

31 **Andersen V**, Ostergaard M, Christensen J, Overvad K, Tjønneland A, Vogel U. Polymorphisms in the xenobiotic transporter Multidrug Resistance 1 (MDR1) and interaction with meat intake in relation to risk of colorectal cancer in a Danish prospective case-cohort study. *BMC Cancer* 2009; **9**: 407 [PMID: 19930591 DOI: 10.1186/1471-2407-9-407]

32 **Hoff JH**, te Morsche RH, Roelofs HM, van der Logt EM, Nagengast FM, Peters WH. COX-2 polymorphisms -765G--> C and -1195A--> G and colorectal cancer risk. *World J Gastroenterol* 2009; **15**: 4561-4565 [PMID: 19777615 DOI: 10.3748/wjg.15.4561]

33 **Thompson CL**, Plummer SJ, Merkulova A, Cheng I, Tucker

TC, Casey G, Li L. No association between cyclooxygenase-2 and uridine diphosphate glucuronosyltransferase 1A6 genetic polymorphisms and colon cancer risk. *World J Gastroenterol* 2009; **15**: 2240-2244 [PMID: 19437564 DOI: 10.3748/wjg.15.2240]

34   **Pereira C**, Pimentel-Nunes P, Brandão C, Moreira-Dias L, Medeiros R, Dinis-Ribeiro M. COX-2 polymorphisms and colorectal cancer risk: a strategy for chemoprevention. *Eur J Gastroenterol Hepatol* 2010; **22**: 607-613 [PMID: 20075740 DOI: 10.1097/MEG.0b013e3283352cbb]

35   **Zhang X**, Zhong R, Zhang Z, Yuan J, Liu L, Wang Y, Kadlubar S, Feng F, Miao X. Interaction of cyclooxygenase-2 promoter polymorphisms with Helicobacter pylori infection and risk of gastric cancer. *Mol Carcinog* 2011; **50**: 876-883 [PMID: 21538574 DOI: 10.1002/mc.20784]

36   **Zhang XM**, Zhong R, Liu L, Wang Y, Yuan JX, Wang P, Sun C, Zhang Z, Song WG, Miao XP. Smoking and COX-2 functional polymorphisms interact to increase the risk of gastric cardia adenocarcinoma in Chinese population. *PLoS One* 2011; **6**: e21894 [PMID: 21779349 DOI: 10.1371/journal.pone.0021894]

37   **Jing YM**, Liu J, Li SJ, Shi WJ, Cheng XL. Genetic polymorphisms in the promoter of Cyclooxygenase-2 and their association with the risk of gastric cancer. *Zhongguo Yousheng and Yichuan Zazhi* 2012; **20**: 24-25

38   **Li Y**, Dai L, Zhang J, Wang P, Chai Y, Ye H, Zhang J, Wang K. Cyclooxygenase-2 polymorphisms and the risk of gastric cancer in various degrees of relationship in the Chinese Han population. *Oncol Lett* 2012; **3**: 107-112 [PMID: 22740864 DOI: 10.3892/ol.2011.426]

39   **Shin WG**, Kim HJ, Cho SJ, Kim HS, Kim KH, Jang MK, Lee JH, Kim HY. The COX-2-1195AA Genotype Is Associated with Diffuse-Type Gastric Cancer in Korea. *Gut Liver* 2012; **6**: 321-327 [PMID: 22844559 DOI: 10.5009/gnl.2012.6.3.321]

40   **Zhang Y**, Liu CM, Peng HP, Zhang JZ, Cai XQ, Feng QL. Relationship between polymorphisms in the promoter region of the COX-2 gene and susceptibility to colorectal cancer. *Shijie Huaren Xiaohua Zazhi* 2012; **20**: 1579-1584

41   **Andersen V**, Holst R, Kopp TI, Tjønneland A, Vogel U. Interactions between diet, lifestyle and IL10, IL1B, and PTGS2/COX-2 gene polymorphisms in relation to risk of colorectal cancer in a prospective Danish case-cohort study. *PLoS One* 2013; **8**: e78366 [PMID: 24194923 DOI: 10.1371/journal.pone.0078366]

42   **Li S**, Zhao X, Wu Z, Li Y, Zhu L, Cui B, Dong X, Tian S, Hu F, Zhao Y. Polymorphisms in arachidonic acid metabolism-related genes and the risk and prognosis of colorectal cancer. *Fam Cancer* 2013; **12**: 755-765 [PMID: 23715757 DOI: 10.1007/s10689-013-9659-2]

43   **Makar KW**, Poole EM, Resler AJ, Seufert B, Curtin K, Kleinstein SE, Duggan D, Kulmacz RJ, Hsu L, Whitton J, Carlson CS, Rimorin CF, Caan BJ, Baron JA, Potter JD, Slattery ML, Ulrich CM. COX-1 (PTGS1) and COX-2 (PTGS2) polymorphisms, NSAID interactions, and risk of colon and rectal cancers in two independent populations. *Cancer Causes Control* 2013; **24**: 2059-2075 [PMID: 24022467 DOI: 10.1007/s10552-013-0282-1]

44   **Ruan YF**, Sun J, WU F, Jiang SH. Relationship between

cyclooxygenase-2 polymorphisms and colorectal cancer risk. *Int J Dig Dis* 2013; **33**: 260-263

45   **Pereira C**, Queirós S, Galaghar A, Sousa H, Pimentel-Nunes P, Brandão C, Moreira-Dias L, Medeiros R, Dinis-Ribeiro M. Genetic variability in key genes in prostaglandin E2 pathway (COX-2, HPGD, ABCC4 and SLCO2A1) and their involvement in colorectal cancer development. *PLoS One* 2014; **9**: e92000 [PMID: 24694755 DOI: 10.1371/journal.pone.0092000]

46   **Vogel LK**, Sæbø M, Høyer H, Kopp TI, Vogel U, Godiksen S, Frenzel FB, Hamfjord J, Bowitz-Lothe IM, Johnson E, Kure EH, Andersen V. Intestinal PTGS2 mRNA levels, PTGS2 gene polymorphisms, and colorectal carcinogenesis. *PLoS One* 2014; **9**: e105254 [PMID: 25166592 DOI: 10.1371/journal.pone.0105254]

47   **Gao F**, Lu L, Qin JD, Zhang B, Li JJ, Zhou CJ, Jia YB. Single Nucleotide Polymorphism in COX-2 Gene are Associated with Risk of Non-cardia Gastric Cancer. *Cancer Res Prev Treat* 2015; **42**: 470-473

48   **Tao M**, Zhang LX, Song Y, Zhuang K, Zhang NX, Zhang L. Association of COX-2 genetic polymorphisms and H.pylori infection with susceptibility of gastric cancer in Shaanxi area. *Shanxi Yike Daxue Xuebao* 2015; **46**: 17-20

49   **Zamudio R**, Pereira L, Rocha CD, Berg DE, Muniz-Queiroz T, Sant Anna HP, Cabrera L, Combe JM, Herrera P, Jahuira MH, Leão FB, Lyon F, Prado WA, Rodrigues MR, Rodrigues-Soares F, Santolalla ML, Zolini C, Silva AM, Gilman RH, Tarazona-Santos E, Kehdy FS. Population, Epidemiological, and Functional Genetics of Gastric Cancer Candidate Genes in Peruvians with Predominant Amerindian Ancestry. *Dig Dis Sci* 2016; **61**: 107-116 [PMID: 26391267 DOI: 10.1007/s10620-015-3859-6]

50   **Anand S**, Huntly BJ. Disordered signaling in myeloproliferative neoplasms. *Hematol Oncol Clin North Am* 2012; **26**: 1017-1035 [PMID: 23009935 DOI: 10.1016/j.hoc.2012.07.004]

51   **Robertson A**, Allen J, Laney R, Curnow A. The cellular and molecular carcinogenic effects of radon exposure: a review. *Int J Mol Sci* 2013; **14**: 14024-14063 [PMID: 23880854 DOI: 10.3390/ijms140714024]

52   **Chan MW**, Wong CY, Cheng AS, Chan VY, Chan KK, To KF, Chan FK, Sung JJ, Leung WK. Targeted inhibition of COX-2 expression by RNA interference suppresses tumor growth and potentiates chemosensitivity to cisplatin in human gastric cancer cells. *Oncol Rep* 2007; **18**: 1557-1562 [PMID: 17982644]

53   **Johnson GE**, Ivanov VN, Hei TK. Radiosensitization of melanoma cells through combined inhibition of protein regulators of cell survival. *Apoptosis* 2008; **13**: 790-802 [PMID: 18454317 DOI: 10.1007/s10495-008-0212-y]

54   **Palayoor ST**, Arayankalayil MJ, Shoaibi A, Coleman CN. Radiation sensitivity of human carcinoma cells transfected with small interfering RNA targeted against cyclooxygenase-2. *Clin Cancer Res* 2005; **11**: 6980-6986 [PMID: 16203791 DOI: 10.1158/1078-0432.CCR-05-0326]

55   **Ramsay RG**, Barton AL, Gonda TJ. Targeting c-Myb expression in human disease. *Expert Opin Ther Targets* 2003; **7**: 235-248 [PMID: 12667100 DOI: 10.1517/14728222.7.2.235]

CASE REPORT

Esophageal squamous papillomas with focal dermal hypoplasia and eosinophilic esophagitis

Eric A Pasman, Theresa A Heifert, Cade M Nylund

Eric A Pasman, Pediatric Residency Program, National Capital Consortium, Bethesda, MD 20889, United States

Theresa A Heifert, Cade M Nylund, Pediatric Gastroenterology, Hepatology and Nutrition Fellowship Program, National Capital Consortium, Bethesda, MD 20889, United States

Cade M Nylund, Department of Pediatrics, F. Edward Hebert School of Medicine, Uniformed Services University, Bethesda, MD 20814, United States

Conflict-of-interest statement: The authors have no disclosures. This case report discusses use of the ERBE VIO APC system (ERBE USA Inc, Marietta, Georgia) and the proprietary PRECISE setting. The authors have no affiliation with ERBE. The views expressed in this article are those of the authors and do not reflect the official policies of the Department of Army/Navy/Air Force, Department of Defense, or U.S. Government. The identification of specific products or scientific instrumentation does not constitute endorsement or implied endorsement on the part of the authors, Department of Defense, or any component agency. While we generally excise references to products, companies, manufactures, organizations, etc. in government-produced works, this report presents a special circumstance when such product inclusions become an integral part of the scientific endeavor.

Abstract

Focal dermal hypoplasia (FDH) is a rare disorder of the mesodermal and ectodermal tissues. Here we present an eight-year-old female known to have FDH who presents with poor weight gain and dysphagia. She was diagnosed with multiple esophageal papillomas and eosinophilic esophagitis. She was successfully treated with argon plasma coagulation and ingested fluticasone propionate, which has not been described previously in a child.

Key words: Focal dermal hypoplasia; Papilloma; Argon plasma coagulation; Eosinophils; Eosinophilic esophagitis; Esophageal diseases; Dysphagia

Core tip: Focal dermal hypoplasia (FDH) is a rare connective tissue disorder associated with squamous

papillomas of the esophagus in older individuals. This case discusses an 8-year-old female with FDH who presented with dysphagia. She was found to have esophageal papillomas and eosinophilic esophagitis. Treatment of eosinophilic esophagitis is highlighted. Argon plasma coagulation has been shown to be safe for use in the small diameter of the esophagus of children but not specifically for destruction of esophageal papillomas. A successful approach to debulking esophageal papillomas in a child using argon plasma coagulation is described in this case.

INTRODUCTION

Focal dermal hypoplasia (FDH) or Goltz syndrome is a rare disorder of defective ectodermal and mesodermal tissue development[1]. There are only about 300 reported cases of FDH. The disorder is inherited in an X-linked dominant manner with a female predominance of 9:1. Females are heterozygous or mosaic for mutation in the PORCN gene; affected males are typically mosaic[2,3]. The primary clinical manifestations of FDH occur due to dysplasia of the connective tissue of the skin and skeletal tissue. The dermal connective tissue is attenuated, with thin-appearing collagen fibers leading to hypoplastic and atrophic areas of skin that often follow the lines of Blaschko. Skeletal malformations include syndactyly, oligodactyly, and split-hand/foot malformation[4,5]. Cleft lip can be present leading to feeding difficulty[6]. Mucocutaneous squamous papillomas have been reported on the mouth, nose, larynx, anus, and genitals[3,7,8]. There have been reports of multiple esophageal squamous papillomas in individuals over the age of 30[9,10]. These patients were described as having chronic dysphagia. We report the presence of distal esophageal papillomas in an eight-year-old child who had no such projections seen previously on barium swallow two years prior to endoscopy.

CASE REPORT

An eight-year-old female with focal dermal hypoplasia presented to the pediatric gastroenterology clinic for poor weight gain, dysphagia, and early satiety. She previously had a gastrostomy tube placed at three weeks of age due to cleft lip and palate, which were later repaired. The gastrostomy tube remained in place and was used sporadically as the patient and the parents were motivated to transition to oral intake of formula and foods. She had a history of difficulty

with oral intake in the past; however, a tonsillectomy for tonsillar hypertrophy had led to improved feeding. Two years prior to establishing care with the pediatric gastroenterology clinic, she had a swallow study and a fluoroscopic upper gastrointestinal series that were normal. She denied odynophagia, although she endorsed the sensation of food getting stuck in her throat and chest. She reported having to clear her throat and drink water frequently during meals. She had no cough or respiratory complaints.

On exam she was very thin and emaciated with dysmorphic facial features and thin hair. She had multiple scars on her face, which appeared as an asymmetric, vascular, excoriated rash. There was a scar under her nose from the upper lip to the right of midline from previous cleft lip repair. She had a grade II/VI systolic murmur. Her lungs were clear. She had normoactive bowel sounds; her abdomen was soft, non-tender with no organomegaly. She had a low profile balloon gastrostomy tube in place.

An esophogram demonstrated multiple filling defects in the distal esophagus. The patient's history of cleft lip and facial dysmorphism precluded esophageal manometric evaluation. The medical team elected to evaluate motility with an esophageal transit study using esophageal scintigraphy, which was remarkable for delayed clearance of contrast from the esophagus, especially in the supine position. She had gastric emptying scintigraphy, which showed mild delayed emptying (39% at two hours and 85% at four hours).

An esophagogastroduodenoscopy (EGD) was performed with pancreatic stimulation; she had normal pancreatic enzyme levels. On endoscopy, however, she was noted to have multiple papillomas in her esophagus (Figure 1). Pathology of the specimen was consistent with a squamous papilloma (Figure 2). Human papilloma virus polymerase chain reaction testing was negative. She had esophageal eosinophilia on biopsies with > 80 eosinophils per high power field (hpf) in her distal esophagus and > 20 eosinophils per hpf in her proximal esophagus (Figure 3). Subsequent EGD after being on a proton pump inhibitor for over 6 weeks showed a similar number of papillomas, with biopsies revealing up to 20 eosinophils per hpf in the distal esophagus and 15 eosinophils per hpf in the proximal esophagus. At the time of the second EGD, debulking of her papillomas was completed using argon plasma coagulation. This procedure was performed using ERBE VIO APC system (ERBE USA Inc., Marietta, GA) and the PRECISE setting with an effect of 5. On re-evaluation three months after initial debulking, there was significantly less of a papilloma burden with only a small cluster of papillomas remaining. This small cluster was ablated again, using the argon plasma coagulation. Follow-up EGD demonstrated successful elimination of papillomas (Figure 4).

Options for the treatment of eosinophilic esophagitis were discussed with the patient and parents. Allergy testing directed diet was not pursued. Given her skin
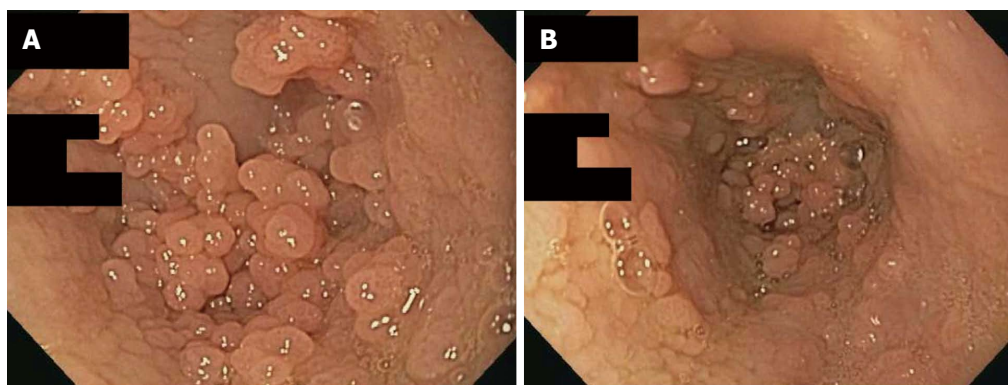
Figure 1 Esophageal endoscopic exam demonstrating multiple papillomas. A: View of esophageal papillomas in distal esophagus immediately above the level of the lower esophageal sphincter; B: View of the esophageal papillomas and thicken esophageal mucusa in the distal esophagus.
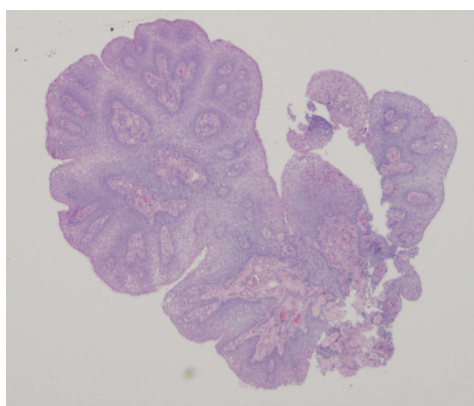

Figure 2 Low power cross section of squamous papilloma obtained from esophagus.

Figure 4 Esophageal endoscopic follow up exam post-treatment with argon plasma coagulation and debulking of the papillomas.
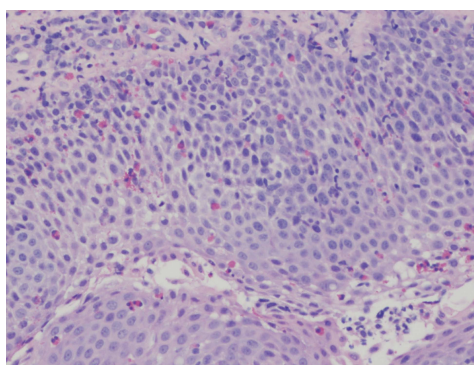

Figure 3 High power view of papilloma demonstrating eosinophilic infiltration.

disorder the implementation and interpretation of skin prick testing or skin atopy patch testing would have been technically difficult. Empiric food elimination diet or elemental diets were also presented but declined by the family as they felt any progress made in her transition from gastrostomy feeds to oral feeds would be lost with the initiation of restrictive diets or unpalatable formula. To treat the eosinophilic esophagitis, the patient was instructed to take fluticasone propionate metered dose inhaler 440 mcg
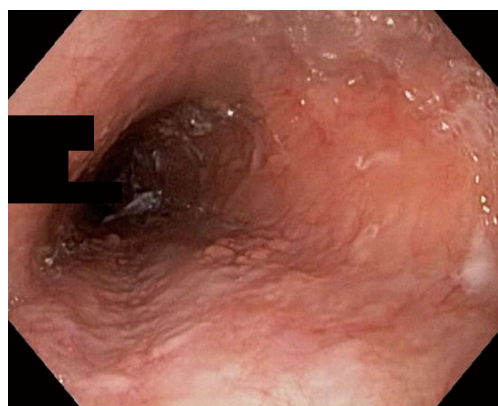
directly into the mouth, swallowed rather than inhaled, twice a day. Her biopsy at three-month follow-up EGD demonstrated 15 eosinophils per hpf. The patient reported resolution of her dysphagia but continued early satiety with solid food. A combination of treatment with the promotility stimulant erythromycin ethylsuccinate 3 mg/kg per dose prior to each meal, along with introduction of overnight formula feedings *via* gastrostomy tube, facilitated adequate weight gain.

DISCUSSION

This case presents a child with FDH, a connective tissue disorder known to be associated with the formation of squamous papillomas in the nose, mouth, pharynx, airway, rectum, vagina and esophagus. The esophageal papillomas are hypothesized to be related to the high incidence of severe gastroesophageal reflux starting in infancy in FDH[9]. In two previous case reports, esophageal papillomas were noted at 30 and 56 years old; both patients were female with oral papillomas. The older patient had complained of dysphagia since she was a teen but did not definitely have esophageal papillomas identified until age 56[9,10]. Based on radiographic studies performed two years prior to our

patient's presentation, it is likely that she developed her papillomas between ages six and eight. What is unique in this scenario is that our patient is much younger than what has previously been reported in the literature about the development of esophageal squamous papillomas. Her complaint of dysphagia was supported by poor esophageal transit on nuclear medicine study.

Further complicating this patient's presentation was eosinophilic esophagitis. There is evidence that connective tissue disorders are a risk factor for eosinophilic esophagitis. Ehler's Danlos, Marfan, and Loeys-Dietz syndromes all have significantly higher rates of eosinophilic esophagitis than expected rates in the general population[11]. Although the exact cause of this association is unknown, it is speculated that it is likely related to poor esophageal connective tissue repair as well as to increases in immune modulating molecules in the esophageal tissue. To our knowledge this is the only report of a patient with FDH and eosinophilic esophagitis.

Although the delayed esophageal clearance was most likely related to the distal esophageal papillomas, it is known that motility deficits can lead to food impaction in eosinophilic esophagitis[12]. This can lead to serious complications such as perforation. Treatment of eosinophilic esophagitis has been shown to decrease the risk of impaction[13]. The patient in this case showed a partial histological response to ingested steroid therapy with a decrease in her mucosal eosinophil count. Her dysphagia improved with simultaneous treatment of her esophageal papillomas and eosinophilic esophagitis. We can only speculate, but suspect the largest symptomatic response as far as improved dysphagia was from debulking the papillomas.

Argon plasma coagulation has been shown to be an effective treatment for esophageal pathology with mucosal overgrowth such as Barrett's esophagus[14]. Furthermore, it has been shown to be a safe treatment in children. Di Nardo *et al*[15] recently described a group of children with esophageal inlet patch who were unresponsive to proton pump inhibitor alone but responded well to argon plasma coagulation. Papillomatosis disease of the airway has been treated effectively with argon plasma coagulation; however, the technique does not appear to have been applied to esophageal papillomas[16]. We utilized the PRECISE setting, which is an ERBE proprietary setting. This setting creates superficial coagulation and tissue destruction using a low-energy output per unit time, which allows for cautery in temperature-sensitive or thin-walled structures[17]. The use of argon plasma coagulation in the small diameter esophagus of a child allowed the safe and controlled destruction of the papillomas while lowering the concern for unintended thermal damage. The desired endoscopic and symptomatic result was obtained using this technique.

There are multiple unique aspects to this single case description. We demonstrate that esophageal papillomas can be safely debulked using argon plasma coagulation. We also demonstrate a patient with focal dermal hypoplasia presenting with esophageal papillomas at an age much younger than previously shown in the literature. Finally, we identified a patient with both focal dermal hypoplasia and eosinophilic esophagitis. This is a potential association that has not yet been described, but has biologic plausibility given the association between eosinophilic esophagitis and connective tissue disorders. The young age of this patient and her comorbid eosinophilic esophagitis and esophageal papillomas present an argument for endoscopic evaluation of patients with focal dermal hypoplasia for pathological causes of feeding disorders or dysphagia.

COMMENTS

Case characteristics
An 8-year-old girl with focal dermal hypoplasia presented with dysphagia.

Clinical diagnosis
Multiple esophageal papillomas noted on esophagogastroduodenoscopy.

Differential diagnosis
Human papilloma virus, squamous papillomas associated with focal dermal hypoplasia.

Laboratory diagnosis
Human papilloma virus polymerase chain reaction negative.

Imaging diagnosis
Esophageal and gastric scintigraphy demonstrated a delayed esophageal clearance and mild dealyed gastric emptying.

Pathological diagnosis
Squamous cell papillomas with eosinophilic esophagitis.

Treatment
Endoscopic application of argon plasma coagulation for debulking of esophageal papillomas.Swallowed fluticasone proprionate metered dose inhaler 440 mcg twice a day for eosinophilic esophagitis.

Related reports
Focal dermal hypoplasia is a rare entity that is associated with esophageal squamous papillomas; however, these have only previously been identified in adults. Argon plasma coagulation has been used safely in children for the destruction of esophageal pathology but not specifically for papilloma removal.

Term explanation
Focal dermal hypoplasia (FDH) is a very rare connective tissue disorder.

Experiences and lessons
The authors found our patient to have both esophageal papillomas and eosinophilic esophagitis. Her papillomas were part of her rare underlying condition of focal dermal hypoplasia. Her eosinophilic esophagitis was treated using swallowed corticosteroids following accepted guidelines. They described a novel approach to treating esophageal papillomas in children using argon plasma coagulation.

Peer-review
The manuscript is an interesting case report of a rare disease (FDH) combined with eosinophilic esophagitis. It is a well written, referenced and illustrated manuscript.

REFERENCES

1 **Gorlin RJ**, Cohen MMJ, Hennekam RCM. In Oxford Monographs on Medical Genetics. Oxford: Oxford Univ. Press, 2001: 571-576

2 **Grzeschik KH**, Bornholdt D, Oeffner F, König A, del Carmen Boente M, Enders H, Fritz B, Hertl M, Grasshoff U, Höfling K, Oji V, Paradisi M, Schuchardt C, Szalai Z, Tadini G, Traupe H, Happle R. Deficiency of PORCN, a regulator of Wnt signaling, is associated with focal dermal hypoplasia. *Nat Genet* 2007; **39**: 833-835 [PMID: 17546031 DOI: 10.1038/ng2052]

3 **Sutton VR**, Van den Veyver IB. Focal Dermal Hypoplasia. 2008 May 15 [Updated 2016 Jul 21]. In: Pagon RA, Adam MP, Bird TD, editors. GeneReviews™ [Internet]. Seattle (WA): University of Washington, Seattle, 1993-2013. Available from: URL: http://www.ncbi.nlm.nih.gov/books/NBK1543/

4 **Goltz RW**. Focal dermal hypoplasia syndrome. An update. *Arch Dermatol* 1992; **128**: 1108-1111 [PMID: 1497368]

5 **Goltz RW**, Henderson RR, Hitch JM, Ott JE. Focal dermal hypoplasia syndrome. A review of the literature and report of two cases. *Arch Dermatol* 1970; **101**: 1-11 [PMID: 5416790]

6 **Ascherman JA**, Knowles SL, Troutman KC. Extensive facial clefting in a patient with Goltz syndrome: multidisciplinary treatment of a previously unreported association. *Cleft Palate Craniofac J* 2002; **39**: 469-473 [PMID: 12071796 DOI: 10.1597/1545-1569(2002)039<0469:EFCIAP>2.0.CO;2]

7 **Rhee KY**, Baek RM, Ahn KJ. Airway management in a patient with focal dermal hypoplasia. *Anesth Analg* 2006; **103**: 1342 [PMID: 17056997 DOI: 10.1213/01.ane.0000242323.73548.0b]

8 **Gordjani N**, Herdeg S, Ross UH, Grimme H, Kleinschmidt M, Brandis M. Focal dermal hypoplasia (Goltz-Gorlin syndrome) associated with obstructive papillomatosis of the larynx and hypopharynx. *Eur J Dermatol* 1999; **9**: 618-620 [PMID: 10586128]

9 **Brinson RR**, Schuman BM, Mills LR, Thigpen S, Freedman S. Multiple squamous papillomas of the esophagus associated with Goltz syndrome. *Am J Gastroenterol* 1987; **82**: 1177-1179 [PMID: 3673998]

10 **Kashyap P**, Sweetser S, Farrugia G. Esophageal papillomas and skin abnormalities. Focal dermal hypoplasia (Goltz syndrome) manifesting with esophageal papillomatosis. *Gastroenterology* 2011; **140**: 784, 1111 [PMID: 21272558 DOI: 10.1053/j.gastro.2010.02.062]

11 **Abonia JP**, Wen T, Stucke EM, Grotjan T, Griffith MS, Kemme KA, Collins MH, Putnam PE, Franciosi JP, von Tiehl KF, Tinkle BT, Marsolo KA, Martin LJ, Ware SM, Rothenberg ME. High prevalence of eosinophilic esophagitis in patients with inherited connective tissue disorders. *J Allergy Clin Immunol* 2013; **132**: 378-386 [PMID: 23608731 DOI: 10.1016/j.jaci.2013.02.030]

12 **Santander C**, Chavarría-Herbozo CM, Becerro-González I, Burgos-Santamaría D. Impaired esophageal motor function in eosinophilic esophagitis. *Rev Esp Enferm Dig* 2015; **107**: 622-629 [PMID: 26437981 DOI: 10.17235/reed.2015.3801/2015]

13 **Kuchen T**, Straumann A, Safroneeva E, Romero Y, Bussmann C, Vavricka S, Netzer P, Reinhard A, Portmann S, Schoepfer AM. Swallowed topical corticosteroids reduce the risk for long-lasting bolus impactions in eosinophilic esophagitis. *Allergy* 2014; **69**: 1248-1254 [PMID: 24894658 DOI: 10.1111/all.12455]

14 **Vance RB**, Dunbar KB. Endoscopic options for treatment of dysplasia in Barrett's esophagus. *World J Gastrointest Endosc* 2015; **7**: 1311-1317 [PMID: 26722612 DOI: 10.4253/wjge.v7.i19.1311]

15 **Di Nardo G**, Cremon C, Bertelli L, Oliva S, De Giorgio R, Pagano N. Esophageal Inlet Patch: An Under-Recognized Cause of Symptoms in Children. *J Pediatr* 2016; **176**: 99-104.e1 [PMID: 27318379 DOI: 10.1016/j.jpeds.2016.05.059]

16 **Miller SM**, Bellinger CR, Chatterjee A. Argon plasma coagulation and electrosurgery for benign endobronchial tumors. *J Bronchology Interv Pulmonol* 2013; **20**: 38-40 [PMID: 23328141 DOI: 10.1097/LBR.0b013e318282d3ca]

17 **Kähler GF**, Szyrach MN, Hieronymus A, Grobholz R, Enderle MD. Investigation of the thermal tissue effects of the argon plasma coagulation modes "pulsed" and "precise" on the porcine esophagus, ex vivo and in vivo. *Gastrointest Endosc* 2009; **70**: 362-368 [PMID: 19500786 DOI: 10.1016/j.gie.2008.11.050]

World Journal of Gastroenterology

CASE REPORT

Breast cancer metastasizing to the stomach mimicking primary gastric cancer: A case report

Kwangil Yim, Sang Mi Ro, Jieun Lee

Kwangil Yim, Department of Hospital Pathology, Seoul St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Seoul 06591, South Korea

Sang Mi Ro, Jieun Lee, Division of Medical Oncology, Department of Internal Medicine, Seoul St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Seoul 06591, South Korea

Jieun Lee, Cancer Research Institute, the Catholic University of Korea, Seoul 06591, South Korea

Abstract

Breast cancer with stomach metastasis rare with an incidence of 1% or less among metastatic breast cancer patients. We experienced a case of breast cancer metastasizing to the stomach in 65-year-old female patient. She experienced dyspepsia and poor oral intake before visiting the clinic. Diffuse infiltration with nodular mucosal thickening of the stomach wall was observed, suggesting advanced gastric cancer based on gross endoscopic finding. Spread of poorly cohesive tumor cells in the gastric mucosa observed upon hematoxylin and eosin stain resembled signet ring cell carcinoma, but diffuse positive staining for GATA3 in immunohistochemical stain allowed for a conclusive diagnosis of breast cancer metastasizing to the stomach. Based on the final diagnosis, systemic chemotherapy was administered instead of primary surgical resection. After 2 cycles of docetaxel administration, she showed a partial response based on abdominal computed tomography scan. This case is an unusual presentation of breast cancer metastasizing to the gastrointestinal tract.

Key words: Gastric cancer; Breast cancer; Metastasis; Immunohistochemical stain; GATA3; GCDFP-15

Core tip: This case report describes a patient who was clinically diagnosed as advanced gastric cancer,

but final pathological confirm diagnosis was to be breast cancer with gastric metastasis. Patient received systemic chemotherapy and is currently on partial response state at present.

INTRODUCTION

Breast cancer commonly metastasizes to bone, lung, liver, and brain, but metastasis to the gastrointestinal tract is rare[1,2]. In Korea, fewer than 10 cases of breast cancer metastasizing to the gastrointestinal tract have been reported[3]. Breast cancer with gastrointestinal metastasis requires systemic chemotherapy. However, if breast cancer with gastrointestinal metastasis is misdiagnosed as a primary gastrointestinal cancer, unnecessary surgical resection may take over place. Herein, the authors present a case of breast cancer metastasizing to the stomach, initially suspected to be primary gastric cancer. This patient was successfully treated with systemic chemotherapy.

CASE REPORT

A 65-year-old female patient was referred to the oncology department for evaluation of indigestion and epigastric discomfort. She had been previously diagnosed with breast cancer, treated with modified radical mastectomy (invasive lobular carcinoma, pT2N3M0), adjuvant chemotherapy (cyclophosphamide, methotrexate, 5-FU) and adjuvant radiation. Two years after surgery, she experienced cancer recurrence with bone metastasis and received an aromatase inhibitor (letrozole) as treatment for another 2 years. At the time she visited the oncology department, she was currently on aromatase inhibitor (letrozole). Other than breast cancer, she had no other medical history. Her last endoscopy was performed 2 years ago, with no specific findings.

Initial white blood cell (WBC) counts, hemoglobin level and hematocrit were 4790 cell/mm^3 (neutrophil count 82%, lymphocytes count 25.8%), 13.1 g/dL (normal range 13.0-18.0 g/dL), and 369000/mm^3 (normal range 150000-450000/mm^3). Other laboratory findings including those of blood chemistry and urine analysis were in the normal range. Serum carcinoembryonic antigen level was increased up to 23.25 ng/dL.

Endoscopy revealed diffuse infiltration with nodular mucosal thickening of the stomach wall, involving the lower two-thirds of the stomach body (Figure 1). Based on endoscopy, endoscopic ultrasound (Figure 2A) and abdominal CT scan (Figure 2B), advanced gastric cancer (cT3N1M0) was suspected. Hematoxylin and eosin (H&E) staining of the endoscopic biopsy revealed poorly cohesive tumor cells spreading into the gastric mucosa, suggesting signet ring cell carcinoma. However, no intracytoplasmic mucin was found in the tumor cells, with scant to moderate pinkish cytoplasm. Normal stomach glandular tissue was found in the biopsy specimen, with no cancer cells connected to the glandular structure (Figure 3A and B). These findings were not consistent with typical gastric signet ring cell carcinoma. Because the patient was diagnosed with invasive lobular carcinoma, archival breast tumor tissue was re-evaluated for comparison.

Breast tissue pathology showed a similar appearance to the endoscopic biopsy specimen, such as a de-cohesive pattern with cells arranged in an Indian file pattern, and a centrally located enlarged nucleus (Figure 3C). In the immunohistochemical (IHC) test, the tumor cells showed diffuse strong nuclear staining for GATA3 binding protein (GATA3) (Figure 3D). IHC results of gross cystic disease fluid protein-15 (GCDFP-15) (Figure 3E), E-cadherin (Figure 3F), estrogen receptor (ER, Figure 3G) and progesterone receptor (PR, Figure 3H) were negative. HER-2 IHC staining showed weak membranous staining consistent with equivocal (+2) positivity (Figure 3I). Silver in situ hybridization (SISH) for HER-2 gene was performed, and the dual-probe HER2/Chr17 ratio was 3.2 (161/51), consistent with HER-2 amplification (Figure 3J).

Based on the pathologic findings, breast cancer metastasizing to the stomach was diagnosed. The stomach metastasis developed 4 years after surgery and 2 years after the initiation of an aromatase inhibitor use. As systemic treatment, docetaxel combined with trastuzumab was considered but trastuzumab was not available due to insurance guidelines. Docetaxel (150 mg/m^2 intravenously [I.V.], day 1) was administered every 3 wk. After 2 cycles of systemic chemotherapy, follow up abdominal CT scans showed decreased stomach wall thickness, and perigastric lymph nodes showed a partial response (PR) based on the Response Evaluation Criteria in Solid Tumors (Figure 4). During 2 cycles of systemic chemotherapy, the patient's symptoms of indigestion and epigastric discomfort regressed. Currently, the patient is in persistent PR state and 6 cycles of docetaxel have been administered.

DISCUSSION

Cancer metastasizing to the gastrointestinal (GI) tract is reported to be rare, but breast cancer is the second most common cancer metastasizing to the GI tract after lung cancer[2,4]. However, the incidence of breast cancer with GI tract metastasis is reported to be 1% or lower[5,6]. Invasive lobular carcinoma tends to metastasize to the GI tract more frequently compared to invasive ductal carcinoma[7]. The most
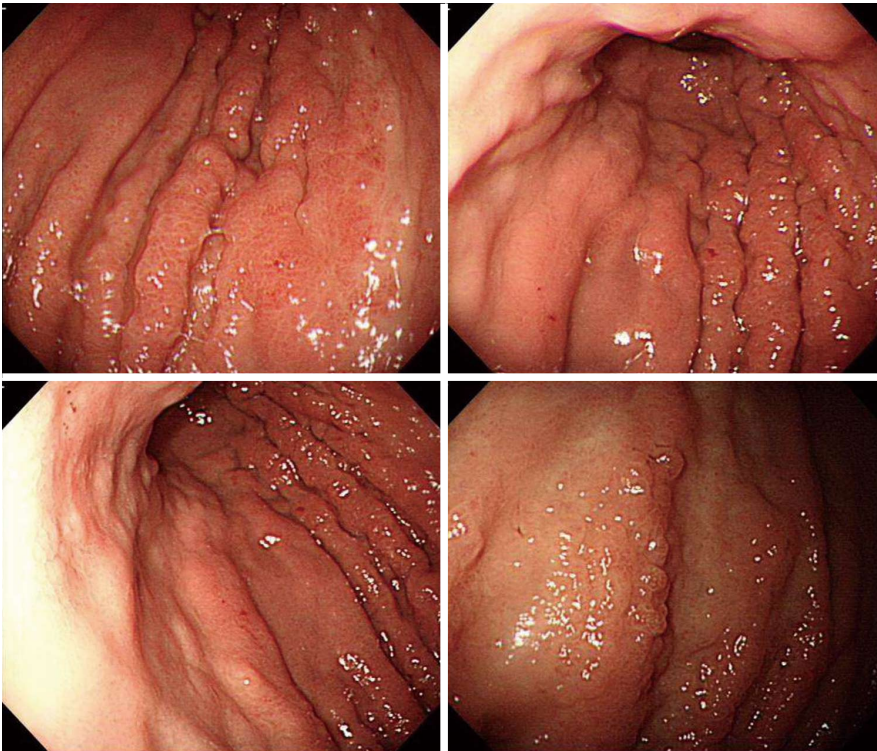
Figure 1 Upper endoscopy shows diffuse infiltrative mucosal lesion with extensive nodular thickening of the stomach wall, involving lower two-thirds of body.
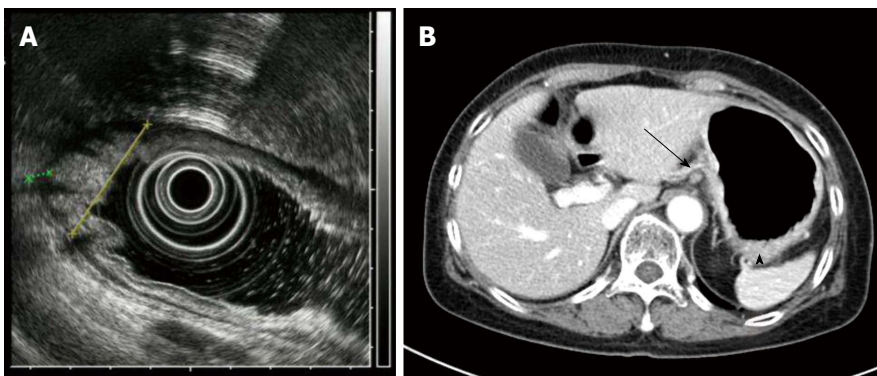

Figure 2 Endoscopic ultrasound shows subserosal invasion of the gastric lesion with lymph node involvement (A, B). Abdomen CT scan shows infiltrative gastric lesion involving cardia and angle of stomach (arrowhead) with enlarged perigastric lymph node (arrow).

common metastatic sites in the GI tract are the colon and rectum, stomach, small intestine and esophagus, in that order[4]. In Korea, 7 cases of breast cancers metastasizing to the GI tract have been reported, with 5 cases of breast cancer with gastric metastases and 2 cases of synchronous stomach and colorectal metastases[3]. The clinical characteristics of the previous cited cases are summarized in Table 1[2,3,8-19].

Most breast cancer patients with gastric metastasis present with GI symptoms[3,16], similar to primary gastric cancer. In our case, the patient complained of indigestion, early satiety, and weight loss. Endoscopy with sufficient mucosal biopsy is mandatory for the diagnosis. Diffuse infiltration of the gastric wall with linitis plastica formation may be found[2], but

approximately 50% of patients may have shallow mucosal lesion indistinguishable from benign gastric mucosal lesions[20]. Our patient showed extensive nodular mucosal thickening with a thickened gastric fold, with a primary suspicion of advanced gastric cancer.

Pathologic findings of breast cancer metastasizing to the stomach are morphologically similar to poorly cohesive gastric carcinoma, especially in invasive lobular carcinoma[21,22]. However, some morphological differences are present. In metastatic mammary carcinoma, sialomucin is present in the intracytoplasmic lumina with a central location of the nucleus. In contrast, primary gastric signet ring cell carcinoma contains clear intracytoplasmic acid mucin
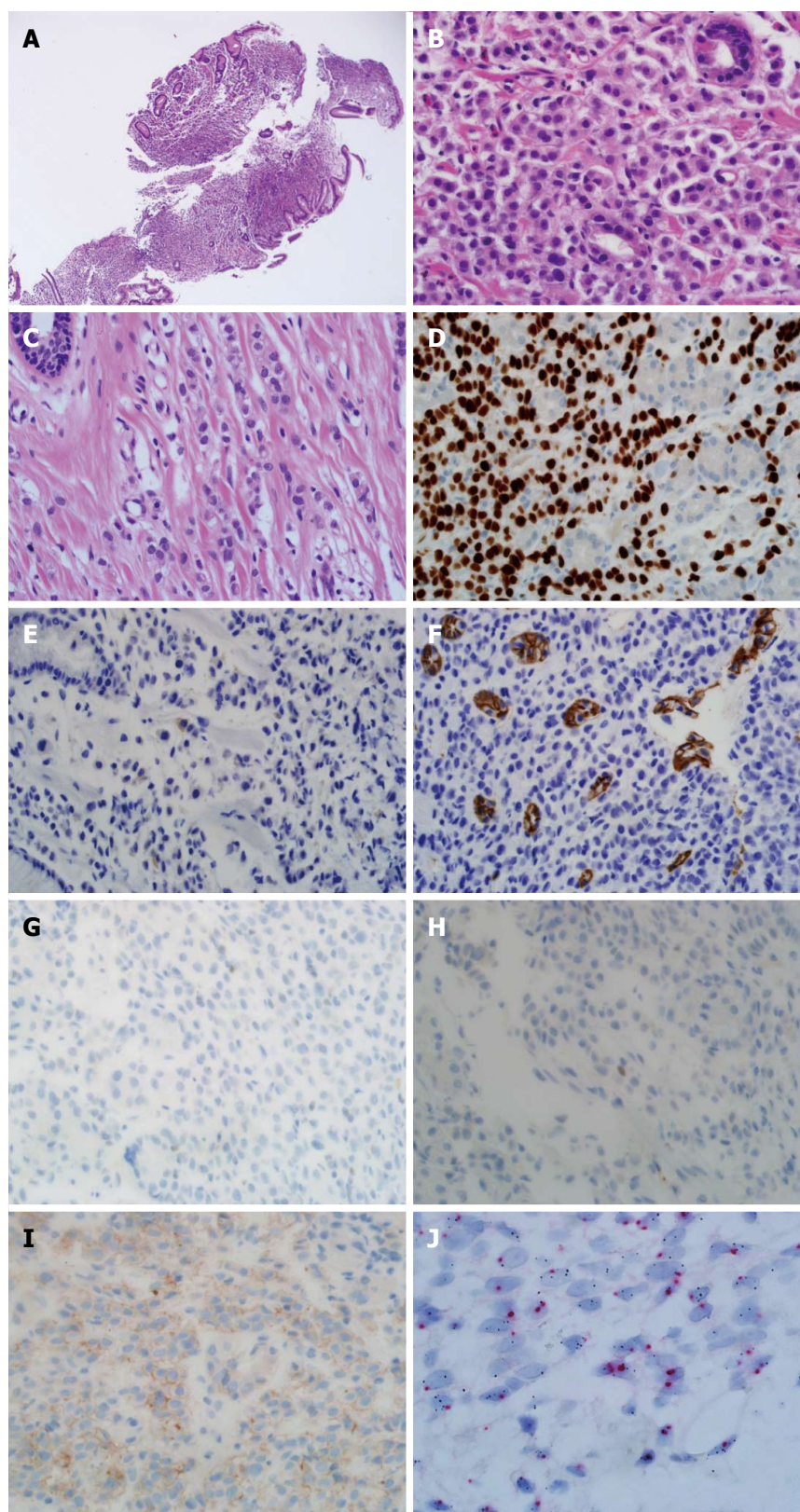
Figure 3 Pathologic features of endoscopic biopsy specimen. Discohesive tumor cells are infiltrated in the stroma of the stomach mucosal tissue (HE × 40, A). Tumor cells show enlarged centrally located nucleus without intracytoplasmic clear mucin. The tumor cells had no connection to the remained normal gastric mucosal tissue (HE × 400, B). Previous breast cancer pathology was reviewed (C). Discohesive tumor cells were arranged in indian file. The tumor cells had enlarged centrally located nucleus without intracytoplasmic mucin (HE × 400, C). Immunohistochemical stains and molecular test of tumor was done (D-J). Diffuse strong nucleus expression of GATA3 was observed (GATA3 × 400, D). Focal, less than one percentage cytoplasmic expression of GCDFP was detected (GCDFP × 400, E). Negative stain for E-cadherin (E-cadherin × 400, F). Negative stains for ER and PR (ER × 400, PR × 400, G, H). Immunohistochemical stain for HER-2 was equivocal (HER-2 × 400, I). Silver in situ hybridization (SISH) for determination of HER2 gene status. Occasional HER2 gene amplified cells were noted in the mixture with normal HE2 gene expressing cells (SISH × 1000, J).

Figure 4 Response evaluation after 2 cycles of docetaxel chemotherapy (A, B). Abdominal CT scan shows decreased perigastric lymph modes (arrows) and gastric mucosal thickening (arrowheads).
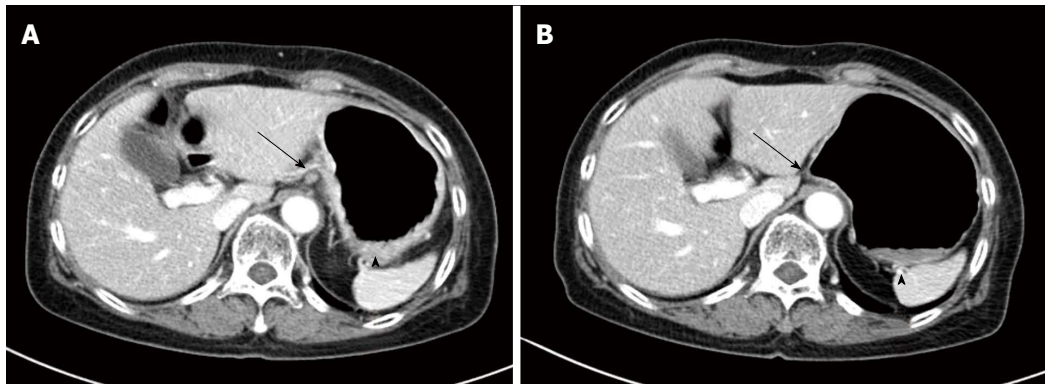
Table 1 Clinical characteristics of representative gastric metastasis of breast cancer

Ref.	Age	Duration after initial diagnosis	Clinical presentation	Endoscopy	Pathology	IHC			Surgery	Treatment	Other metastases site	Overall survival
						ER	PR	C-erbB2				
Our case	65	4	Epigastric Discomfort Indigestion	Diffuse infiltrative mucosal lesion Extensive nodular thickening	ILC	neg	neg	pos	No		Bone	-
Pera *et al*[18]	45	7	Epigastric pain heart burn	Erosion of gastric wall	ILC	pos	pos	-	Subtotal gastrectomy	H	-	-
Jones *et al*[2]	51	3	No symptom	Polyp at antrum wall	ILC	neg	neg	neg	Total gastrectomy	Palliative	Bone	-
	61	6.9	Dysphagia weight loss	Fungating mass	ILC	pos	pos	neg	No	C,R	Brain, bone, pleura	-
Eo *et al*[11]	48	9	Nausea anorexia	Elevated mucosal lesion	IDC	pos	pos	neg	No	C	Liver, bone, pleura	-
Arrangoiz *et al*[8]	70	1	Diarrhea constipation	Mucosal thickening	ILC	pos	neg	neg	No	H	Lung, rectum	-
Koike *et al*[16]	42	5	Epigastric pain	Mucosal erosion	ILC	pos	pos	neg	No	C	-	-
	54	6	Epigastric pain	Mucosal erosion	ILC	pos	pos	neg	No	C, H	Liver, bone, peritoneum	5
	54	3	Epigastric pain vomiting	Submucosal tumor	IDC	pos	pos	pos	No	C	Bone	2.3
Geredeli *et al*[12]	47	3	Increased serum CEA, CA15-3		ILC	neg	neg	neg	Subtotal gastrectomy	C	Bone	-
Buka *et al*[9]	58	1.2	Abdominal pain weight loss	Polypoid infiltration	ILC	pos	pos	neg	Total gastrectomy	C, R	Colon, pleura	7.2
Lee *et al*[17]	48	5.7	Melena	Mucosal erosion	-	-	-	-	-	C	Bone, liver	-
Yim *et al*[19]	48	Initial diagnosis	Epigastric discomfort	Mucosal erosion	ILC	neg	neg	-	No	C	Bone	-
Jeon *et al*[14]	49	5	Melena	Volcano shaped ulcers	IDC	pos	neg	-	No	C	Bone	
Kim *et al*[15]	53	10	Dyspepsia lower abdominal pain small caliper of stool	Mucosal erosion	IDC	neg	neg	-	No	C, H	Kidney, ovary, colon, bone, peritoneal LN	2.4

Hwang et al[13]	66	17	Back pain	Flat mucosal lesion	ILC	neg	pos		endoscopic mucosal resection	C	Bone	-
Cheoi et al[10]	56	4	Upper abdominal discomfort	Mucosal erosion	IDC	neg	pos	pos	-	C, H	-	1.3
Yu et al[3]	63	10	Melena small caliper of stool	Linitis plastica flat ulcer	ILC	pos	pos	pos	No	C, H	Colon, bone marrow	-

ILC: Invasive lobular carcinoma; IDC: Invasive ductal carcinoma; IHC: Immunohistochemical stain; pos: Positive; neg: Negative; C: Chemotherapy; H: Hormonal treatment.

that pushes the nucleus to the periphery[21]. In the present case, the nuclei of the tumor cells were located in the center, and there were no clear intracytoplasmic inclusions.

Also, IHC study is helpful for differential diagnosis. GCDFP-15 staining was traditionally used for differential diagnosis of mammary origin carcinoma. However, it shows relatively low sensitivity (55%-76%) for detecting a breast origin cancer[23]. Recently, GATA3 is widely known as a mammary cancer and urothelial cancer marker. GATA3 expression shows 100% positivity in involving breast lobular carcinoma and 96% positivity in breast ductal carcinoma. However, only 5% of tumors are positive for GATA3 in gastric adenocarcinoma[24]. In our case, although GCDFP-15 staining was negative, GATA3 showed diffuse strong nuclear positivity, consistent with a mammary origin of the carcinoma.

Metastatic breast cancer involving the stomach is treated with systemic agents such as cytotoxic chemotherapeutic agents or hormonal agents. Surgical resection of the stomach has a limited role in treatment, and does not affect the survival outcomes of patients presenting with gastric metastasis[4]. However, surgical treatment may have a role in palliative treatment such as relieving obstructive symptoms.

Breast cancer patients have a superior survival outcome compared to other cancers, raising the possibility of a double primary cancer during the clinical course. However, metastasis of primary breast cancer must also be considered. In a breast cancer patient who complains of gastrointestinal symptoms, prompt endoscopy and biopsy are necessary for an accurate diagnosis. Sufficient pathologic review of gastric biopsy and previous breast specimens, with immunohistochemical examination is warranted. When metastasis of breast cancer to the stomach is suspected, appropriate systemic treatment is necessary for further treatment.

COMMENTS

Case characteristics
A 65-year-old female patient who was diagnosed as metastatic breast cancer visited the hospital for evaluation of epigastric discomfort.

Clinical diagnosis
Epigastric discomfort and indigestion.

Differential diagnosis
Gastric ulcer, primary gastric cancer showed be differentiated by endoscopic biopsy.

Laboratory diagnosis
Serum carcinoembryonic antigen was increased up to 23.25 ng/dL.

Imaging diagnosis
Endoscopy showed diffuse infiltration with nodular mucosal thickening of stomach wall.

Pathological diagnosis
Metastatic invasive lobular carcinoma to stomach was diagnosed by immunohistochemical stain.

Treatment
Docetaxel 150 mg/m^2 intravenous, every 3 wk.

Related reports
Breast cancer rarely metastasize to gastrointestinal tract and should be diagnosed by careful review of the pathologic specimen. If patient have underlying breast cancer, metastatic breast cancer should be considered other than primary gastric cancer during the diagnosis.

Term explanation
GATA3 refers to GATA3 binding protein used for differential marker for diagnosis of breast cancer. Partial response (PR) means more than 30% decrease in the sum of the longest diameters of target lesions during response evaluation.

Experiences and lessons
Early differential diagnosis of metastatic breast cancer to stomach is important for appropriate systemic chemotherapy and avoidance of unnecessary surgery.

Peer-review
This is generally an interesting and useful paper.

REFERENCES

1   **Menuck LS**, Amberg JR. Metastatic disease involving the stomach. *Am J Dig Dis* 1975; **20**: 903-913 [PMID: 1190198 DOI: 10.1007/BF01070875]
2   **Jones GE**, Strauss DC, Forshaw MJ, Deere H, Mahedeva U, Mason RC. Breast cancer metastasis to the stomach may mimic primary gastric cancer: report of two cases and review of literature. *World J Surg Oncol* 2007; **5**: 75 [PMID: 17620117 DOI:

10.1186/1477-7819-5-75]

3   **Yu HA**, Kim EY, Seo MJ, Chung E, Cho MJ, Oh HJ, Jang JH, Park JC, Lee JU, Park SY. Stomach and Colon Metastasis from Breast Cancer. *Ewha Med J* 2014; **37**: 98-104 [DOI: 10.12771/emj.2014.37.2.98pISSN]

4   **McLemore EC**, Pockaj BA, Reynolds C, Gray RJ, Hernandez JL, Grant CS, Donohue JH. Breast cancer: presentation and intervention in women with gastrointestinal metastasis and carcinomatosis. *Ann Surg Oncol* 2005; **12**: 886-894 [PMID: 16177864 DOI: 10.1245/ASO.2005.03.030]

5   **Khadim MI**. The effects of Pan and its ingredients on oral mucosa. *J Pak Med Assoc* 1977; **27**: 353-356 [PMID: 413949 DOI: 10.4061/2011/413949]

6   **Matsuda I**, Matsubara N, Aoyama N, Hamanaka M, Yamagishi D, Kuno T, Tsukamoto K, Yamano T, Noda M, Ikeuchi H, Tomita N, Hirota S. Metastatic lobular carcinoma of the breast masquerading as a primary rectal cancer. *World J Surg Oncol* 2012; **10**: 231 [PMID: 23114188 DOI: 10.1186/1477-7819-10-231]

7   **Harris M**, Howell A, Chrissohou M, Swindell RI, Hudson M, Sellwood RA. A comparison of the metastatic pattern of infiltrating lobular carcinoma and infiltrating duct carcinoma of the breast. *Br J Cancer* 1984; **50**: 23-30 [PMID: 6331484]

8   **Arrangoiz R**, Papavasiliou P, Dushkin H, Farma JM. Case report and literature review: Metastatic lobular carcinoma of the breast an unusual presentation. *Int J Surg Case Rep* 2011; **2**: 301-305 [PMID: 22096760 DOI: 10.1016/j.ijscr.2011.06.010]

9   **Buka D**, Dvořák J, Richter I, Hadzi ND, Cyrany J. Gastric and Colorectal Metastases of Lobular Breast Carcinoma: A Case Report. *Acta Medica* (Hradec Kralove) 2016; **59**: 18-21 [PMID: 27131352 DOI: 10.14712/18059694.2016.50]

10   **Cheoi KS**, Eum YO, Kim HS, Lee OJ, Lee KH. A case of stomach metastasis from breast cancer. *Korean J Med* 2006; **71**: 567-572

11   **Eo WK**. Breast cancer metastasis to the stomach resembling early gastric cancer. *Cancer Res Treat* 2008; **40**: 207-210 [PMID: 19688132 DOI: 10.4143/crt.2008.40.4.207]

12   **Geredeli C**, Dogru O, Omeroglu E, Yilmaz F, Cicekci F. Gastric Metastasis of Triple Negative Invasive Lobular Carcinoma. *Rare Tumors* 2015; **7**: 5764 [PMID: 26266010 DOI: 10.4081/rt.2015.5764]

13   **Hwang SY**, Ryu DY, Park JH, Lee DW, Lee DH, Kim TO, Kim GH, Heo J, Kang DH, Song GA, Cho M, Park DY. [A case of gastric metastasis of breast carcinoma resembling early gastric cancer]. *Korean J Gastroenterol* 2005; **46**: 481-484 [PMID: 16371724]

14   **Jeon SH**, Kwon TK, Kim SH, Kwon DY, Park KS. A case of gastric metastasis from breast carcinoma manifested by upper gastrointestinal bleeding. *Korean J Gastrointest Endosc* 2002; **24**: 220-224

15   **Kim DY**, Yun T, Kim TY, Heo DS, Bang YJ. Renal, gastric, and multiple intestinal metastases of invasive ductal carcinoma of breast. *Korean J Med* 2003; **65**: S836-S840

16   **Koike K**, Kitahara K, Higaki M, Urata M, Yamazaki F, Noshiro H. Clinicopathological features of gastric metastasis from breast cancer in three cases. *Breast Cancer* 2014; **21**: 629-634 [PMID: 21779814 DOI: 10.1007/s12282-011-0284-3]

17   **Lee SI**, Moon YM, Kang JK, Park IS, Choi HJ, Kim BS, Kim TS. A case of gastric metastasis from breast cancer. *Korean J Gastroenterol* 1983; **15**: 157-162

18   **Pera M**, Riera E, Lopez R, Viñolas N, Romagosa C, Miquel R. Metastatic carcinoma of the breast resembling early gastric carcinoma. *Mayo Clin Proc* 2001; **76**: 205-207 [PMID: 11213310 DOI: 10.1016/S0025-6196(11)63129-7]

19   **Yim H**, Jin YM, Shim C, Park HB. Gastric metastasis of mammary signet ring cell carcinoma--a differential diagnosis with primary gastric signet ring cell carcinoma. *J Korean Med Sci* 1997; **12**: 256-261 [PMID: 9250925 DOI: 10.3346/jkms.1997.12.3.256]

20   **Lorimier G**, Binelli C, Burtin P, Maillart P, Bertrand G, Verriele V, Fondrinier E. Metastatic gastric cancer arising from breast carcinoma: endoscopic ultrasonographic aspects. *Endoscopy* 1998; **30**: 800-804 [DOI: 10.1055/s-2007-1001425]

21   **Chu PG**, Weiss LM. Immunohistochemical characterization of signet-ring cell carcinomas of the stomach, breast, and colon. *Am J Clin Pathol* 2004; **121**: 884-892 [PMID: 15198362 DOI: 10.1309/A09E-RYMF-R64N-ERDW]

22   **Taal BG**, Peterse H, Boot H. Clinical presentation, endoscopic features, and treatment of gastric metastases from breast carcinoma. *Cancer* 2000; **89**: 2214-2221 [PMID: 11147591]

23   **Honma N**, Horii R, Iwase T, Saji S, Younes M, Takubo K, Matsuura M, Ito Y, Akiyama F, Sakamoto G. Clinical importance of estrogen receptor-beta evaluation in breast cancer patients treated with adjuvant tamoxifen therapy. *J Clin Oncol* 2008; **26**: 3727-3734 [PMID: 18669459 DOI: 10.1200/JCO.2007.14.2968]

24   **Miettinen M**, McCue PA, Sarlomo-Rikala M, Rys J, Czapiewski P, Wazny K, Langfort R, Waloszczyk P, Biernat W, Lasota J, Wang Z. GATA3: a multispecific but potentially useful marker in surgical pathology: a systematic analysis of 2500 epithelial and nonepithelial tumors. *Am J Surg Pathol* 2014; **38**: 13-22 [PMID: 24145643 DOI: 10.1097/PAS.0b013e3182a0218f]

CASE REPORT

Multiple clear-cell sarcomas of small intestine with parotid gland metastasis: A case report

Hao Su, Wen-Sheng Liu, Wen-Hao Ren, Peng Wang, Lei Shi, Hai-Tao Zhou

Hao Su, Peng Wang, Lei Shi, Hai-Tao Zhou, Department of Colorectal Surgery, National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100021, China

Wen-Sheng Liu, Department of Head and Neck Surgery, National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100021, China

Wen-Hao Ren, Department of Pathology, National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100021, China

Abstract

Clear-cell sarcoma is a rare, malignant soft tissue tumor that displays melanocytic differentiation with a distinct molecular profile. It is rarely localized in the gastrointestinal tract. Herein we reported a case of multiple synchronous clear-cell sarcomas of the gastrointestinal tract with parotid gland metastasis. A 51-year-old male patient presented with a growing painless mass under the right ear. A preoperative positron emission tomography/computed tomography showed multiple intestinal masses and a mass in the right parotid with increased glucose uptake, and he underwent operative treatment with resection of three tumors in the jejunum and ileum and then received a right parotidectomy. Postoperative pathological examination showed that cells in the intestinal tumor were consistent with clear-cell sarcoma of the gastrointestinal tract, and the malignant cells in the parotid gland were similar to the intestinal tumor. Immunohistochemical studies revealed positive expression of HMB-45, Melan-A, and S-100. EWSR1 gene fusion transcripts were undetectable by

fluorescence *in situ* hybridization.

Key words: Clear-cell sarcomas; Clear-cell sarcomas of the gastrointestinal tract; Parotid gland metastasis; Immunohistochemistry

Core tip: Over the past 13 years, only 53 cases of clear-cell sarcomas of the gastrointestinal tract (CCS-GI) have been reported in the world. Most of the literature on CCS-GI describes a single tumor at diagnosis; our presentation is the third report of simultaneous tumors during the diagnosis to date and is the first case of CCS-GI with metastasis to the parotid gland. We also reviewed the literature on CCS-GI. Because of the high rarity, more cases need to be accumulated for further analysis.

INTRODUCTION

Clear-cell sarcoma (CCS) is a rare tumor of unknown origin that was first described by Enzinger[1] in 1965. CCS shows a predilection for the tendons or aponeuroses in the extremities in young adults aged 20-40 years[2]. Ekfors *et al*[3] described the first clear-cell sarcoma of the gastrointestinal tract (CCS-GI) in 1993, which occurred in the duodenum. Only a few cases[4] of CCS-GI have been reported. CCS-GI has specific histopathological, immunohistochemical, and genetic features. Here, we present a case of three synchronous clear-cell sarcomas in the jejunum and ileum with parotid gland metastasis.

CASE REPORT

Patient details

A 51-year-old male presented with a two-year history of a growing painless mass under the right ear, initially with a size of a soybean. The mass grew noticeably in the last six months. There was a one-year history of night sweat and frequent stool (three to four times a day). There was no history of fever, weakness, dysphagia, dyspnea, cough, hoarseness, jaundice, vomiting, melena, hematochezia, abdominal pain, abdominal distension or significant weight loss. The patient had a 5-year medical history of hypertension and he was a hepatitis-B carrier of 30 years and a smoker of 40 pack-years. There was no family history of cancer.
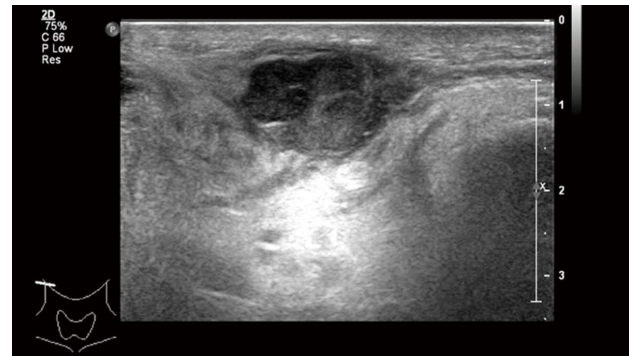
Figure 1 Ultrasonogram of the neck showed a 15 mm × 27 mm mass in the right parotid gland.

Figure 2 positron emission tomography/computed tomography showed a 36 mm × 33 mm intestinal mass with multiple peripheral lymph nodes in the right midabdomen.

On palpation, a 20 mm × 20 mm relatively well-defined and soft mass with no tenderness was observed along with multiple enlarged cervical nodules. Abdominal examination did not reveal any organomegaly or palpable lumps.

Ultrasonography of the neck two months ago revealed a relatively undefined hypoechoic mass measuring approximately 15 mm × 27 mm in its greatest dimension in the right parotid gland and submandibular gland (Figure 1) along with multiple enlarged right supraclavicular and upper cervical lymph nodes. A needle biopsy of the mass was performed and the pathologic report found malignant tumor cells. The patient was recommended for surgery for the mass in the parotid gland. The preoperative blood routine examination showed that the HGB was 106 g/L. Therefore, the patient underwent positron emission tomography/computed tomography (PET/CT). A 36 mm × 33 mm intestinal mass with increased glucose uptake, and multiple peripheral lymph nodes in the right mid-abdomen were found (Figure 2), and the maximum standard uptake value (SUV) was 6.6. An intestinal lesion with increased glucose uptake in the right hypogastrium was also seen and the SUV was 7.0. The mass in the right parotid and peripheral lymph nodes also showed increased glucose uptake, and

Figure 3 Intussusception was observed 80 cm distal to the duodenojejunal junction and the involved bowels were swollen and expanded.

Figure 4 Involved bowels with the masses and mesentery were resected with a proximal 10 cm and distal 10 cm margin.

the SUV was 10.3. Preoperative tumor makers, such as CA125, CA15-3, CA19-9,CA72-4, AFP, cyfra21-1, NSE,SCC, CEA, and ProGRP, did not show abnormal expression.

Treatment
The patient underwent an exploratory laparotomy and the excision of multiple intestinal neoplasms. Operative exploration showed no ascites, pelvic, periaortic, peritoneal, omental deposits, or liver metastasis. No tumors were palpated in the cavity of the stomach, duodenum, colon, rectum, or the mesentery root. Three masses were found at the jejunum and ileum. Intra-operatively, the first tumor was present in the jejunum, located at 80 cm distal to the duodenojejunal junction. Intussusception was observed at the point, and the involved bowels were swollen and expanded (Figure 3). The second tumor was at the end of the intussusception (approximately at the fourth loop of intestine). The third tumor was present in the ileum, located at 80 cm proximal to the ileocecal junction. These three tumors of varying sizes invaded the serosa, and the surface of the serosa had shrunk and was depressed. Multiple enlarged lymph nodes were observed in the intestinal mesentery. Following serial ligation of the mesenteric vessels, resection of the involved bowels, along with the masses and mesentery, was performed, with a proximal margin of 10 cm and a distal margin of 10 cm. The first and second tumors were removed together in one segment of the intestine (Figure 4). Then, a primary anastomosis formed. The patient recovered gradually and then underwent right parotidectomy with retention of the facial nerve, followed by right cervical lymph node dissection 17 d after abdominal surgery because the pathology of the parotid gland neoplasms was undetermined.

Postoperative pathology
Intestinal neoplasms: Upon gross examination, the specimen consisted of two segments of the small intestine: the longer one was approximately 26 cm with attached mesentery, and the other segment was

7.8 cm with attached mesentery. Two tumors were on the longer segment of intestine, one (2.5 cm × 2.2 cm × 1 cm) was at 11 cm from one margin and the other (6.5cm × 5.5cm × 4 cm) was at 19 cm from the same margin. A 2.5 cm × 1.9 cm × 1 cm tumor was on the other segment of the small intestine. The cut surface of the three tumors had hard, obscure borders that were white to tan in appearance.

Microscopically, the jejunum and ileum tissues were infiltrated with malignant cells, which was consistent with CCS-GI (a type of gastrointestinal neural ectoderm tumor, GNET) based on morphology and immunohistochemistry (Figure 5A). The tumors had invaded the mucosal and muscular layers. There was no focal necrosis, vessel invasion or nerve invasion. The mitotic index exceeded 20/10 HPFs, and the tumor was grade G3 according to the FNCLL (French Fédération Nationale des Centres de Lutte Contre le Cancer) system.

Lymph node metastases (1/29) without invasion of the outer lymph node capsule: (1) peripheral lymph nodes of the jejunum: 1/26; and (2) peripheral lymph nodes of the ileum, 0/3.

Immunohistochemistry: S100 (3+), Vim (3+), GFAP (-), HMB-45 (2+), Melan-A (2+), Melanomapan (1+), CD56 (2+), Syn (-), CgA (-), AE1/AE3 (-), CD138 (-), CD19 (-), CD20 (-), CD3 (-), CD38 (-), CD79a (-), Ki-67 (+40%), LCA (-), MUM1 (-), CD117 (lesion+), CD34 (-), DOG1 (-), CD10 (-), Calponin (-), P63 (-), EBER (-).

Gene detection: EWSR1 gene fusion transcripts were undetectable by fluorescence *in situ* hybridization (FISH).

Parotid gland neoplasms: Upon gross examination, a 1-cm diameter nodule was found in a 5.5 cm × 3 cm × 2 cm area of tissue; the cut surface of the nodule had a tough, grey-to-yellow appearance.

Microscopically, the parotid gland tissues were infiltrated with malignant cells, which was consistent with CCS morphology and immunohistochemistry and morphologically similar to the previously assessed
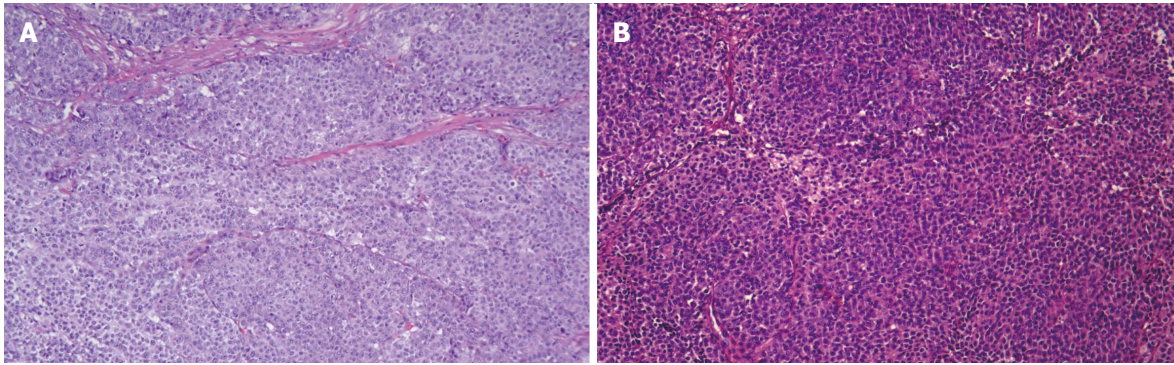
Figure 5 Microscopic observation of intestinal neoplasms and parotid gland neoplasms. A: Microphotography shows that polygonal malignant cells of intestinal neoplasms were separated by fibrous tissues, arranging in sheets and nests, with eosinophilic or clear cytoplasm and there was no exact necrosis, vessel invasion and nerve invasion. Nucleolus was obvious and the mitotic index exceeded 20/10 HPF (Hematoxylin-Eosin G × 10); B: Malignant cells of parotid gland neoplasms were similar to the intestinal tumor by microphotography (Hematoxylin-Eosin G × 10).

intestinal tumor (Figure 5B). Lymph tissues were found in the tumor and at the tumor edge, which may be metastatic lesions.

No lymph node metastases (0/30): (1) right cervical lymph nodes, level Ⅱ, 0/10; (2) right cervical lymph nodes, level Ⅲ, 0/12; (3) right cervical lymph nodes, level V, 0/5; (4) peripheral lymph nodes of the superficial lobe of the right parotid gland, 0/2; and (5) peripheral lymph nodes of the caudate lobe of the right parotid gland and tumor, 0/1.

Immunohistochemistry: S100 (3+), Melan-A (3+), Melanomapan (3+), HMB-45 (3+), AE1/AE3 (-), CK18 (-), Calponin (-), P63 (-), SMA (-).

Follow-up
Twenty days after the surgery on the parotid gland, the patient underwent CT imaging of the neck, thorax and abdominopelvic area, and no recurrence or metastasis was observed. He then started with 6 cycles of chemotherapy using an EI regimen (epirubicin 100 mg + ifosfamide 2 g D1-4+mesna 0.4 g 0 h, 4 h, and 8 h after the ifosfamide D1-4). At the time that this article was written, the patient was on the first cycle of the chemotherapy.

DISCUSSION
CCS-GI is so rare that only 53 cases (including our case) have been reported in the literature to date (Table 1)[3,5-39]. Most of the literature on CCS-GI describes the diagnosis of a single tumor; only two case reports[25,38] have described the diagnosis of two simultaneous tumors to date. CCS-GI often involves the ileum and jejunum, stomach and colon[4-7,9-12,14-35,38,39]. Because of the aggressive clinical course, regional and distant metastases are common in CCS-GI at presentation[5-7,9,10,15,17,21,25,27,29,31,37,39]. The lymph nodes, liver, and mesentery are the most common locations of the metastases at the time of presentation. The patient in our report had three synchronous masses in the jejunum and ileum, with metastasis to the parotid gland, and he attended the hospital mainly due to the

swollen parotid gland. The presence of lymph nodes both inside and outside of the parotid gland makes it a common site of metastasis for head and neck neoplasms[40], but it is a very rare metastatic site for gastrointestinal tumors. In the limited literature on CCS-GI, this is the first case of CCS-GI with metastasis to the parotid gland.

CCS-GI shows specific histopathological, immuno-histochemical, ultrastructural, and genetic features[2,4]. In 2010, Kosemehmetoglu *et al*[41] first divided CCS-GI into two subtypes according to its histomorphology: (1) CCS-like gastrointestinal tumor (CCSLGT); and (2) CCS of soft tissue (CCS-ST). However, there has been disagreement about whether these subtypes are two independent entities[31]. In 2003, Zambrano *et al*[10] reported 6 cases of CCSLGTs. They found that the CCSLGTs were at least focally positive for the S100 protein, but most did not express melanocytic markers such as HMB-45 or Melan-A. Meanwhile, Huang *et al*[36] found that certain CCS-STs were positive for the S100 protein and most could express melanocytic markers such as HMB-45 or Melan-A. Several reports found that > 90% of cases of CCS were associated with the reciprocal translocation t (12; 22) (q13; q12), resulting in fusion of the EWSR1 gene, located at 22q12, and the ATF1 gene, located at 12q13[2,41-46]. To date, these translocations have never been observed in malignant melanoma[13,22,43-46], which has a very similar histologic appearance to CCS[20]. Immunohistochemical staining of CCS reveals positivity for the S100 protein as well as melanocyte-specific markers, with this combination of staining allowing for CCS to be distinguished from malignant melanoma histologically. In our case, the tumor was consistent with CCS-GI based on morphology, was positive for the S100 protein, and expressed melanocytic markers such as HMB-45 and Melan-A, but EWSR1 gene fusion transcripts were undetectable by FISH.

Currently the most effective treatment for CCS-GI is extensive resection of the tumor and peripheral lymph nodes; chemotherapy and radiotherapy appear to have little effect[31]. The clinical behavior of CCS-GI seems to

Table 1 Clinical, pathological, immunohistochemical and genetic features of clear-cell sarcoma of the gastrointestinal tract in previously reported cases

Ref.	Age (yr)/sex	Location	Maximum diameter of tumor(cm)	S-100	HMB-45	Melan-A	Genetic findings	Outcome
Alpers *et al*[5]	26/F	Jejunum	1.5	ND	ND	ND	ND	Liver mets
Ekfors *et al*[3]	38/M	Duodenum	3.0	Positive	Positive	ND	ND	Not given
Donner *et al*[6]	37/M	Ileum	6.5	Positive	Negative	ND	t(12;22)(q13;q12-13)	Liver mets at 24 and 36 mo
Fukuda *et al*[7]	74/M	Colon	3.0	Positive	Positive	ND	EWSR1-ATF1 by RT-PCR	Liver mets at 9 mo
Hu *et al*[8]	10/M	Rectum	5.0	Positive	Positive	ND	ND	NA
Pauwels *et al*[9]	30/M	Stomach	4.0	Positive	Negative	ND	t(12;22)(q13;q12)	LN and peritoneal mets at diagnosis
Zambrano *et al*[10]	15/F	Jejunum	5.0	Positive	Negative	Negative	t(12;22)(q13;q12)	DOD 16 mo
	21/F	Jejunum	4.0	Positive	Negative	Negative	ND	DOD 12 mo
	35/F	Ileum	3.5	Positive	Negative	Negative	ND	Liver mets at 12 mo
	37/F	Ileum	4.5	Positive	Negative	Negative	ND	NA
	32/M	Ileum	5.0	Positive	Negative	Negative	ND	NA
	13/M	Stomach	6.7	Positive	Negative	Negative	ND	Local recurrence at 12 mo;2nd Local recurrence at 36 mo
Achten *et al*[11]	57/M	Jejunum	6.5	Positive	Negative	Negative	EWSR1 rearrangement by FISH	NA
Venkataraman *et al*[12]	21/F	Ileum	7.0	Positive	Negative	Negative	EWSR1 rearrangement by FISH	NA
Covinsky *et al*[13]	47/F	Pancreas	NA	Positive	Positive	Positive	EWSR1-ATF1 by RT-PCR and FISH	NED 24 mo
	85/F	Mesentery	NA	Positive	Positive	Positive	EWSR1-ATF1 by RT-PCR and FISH	DOD 1 mo
Taminelli *et al*[14]	35/M	Ileum	1.8	Positive	Negative	Positive	EWSR1-ATF1/ by RT-PCR	DOD 15 mo
Friedrichs *et al*[15]	41/M	Jejunum	8.7	Positive	Negative	Negative	EWSR1 rearrangement by FISH	Liver mets at 6 mo
Huang *et al*[16]	40/M	Stomach	3.0	Positive	Negative	Positive	ND	NED 9 mo
Antonescu *et al*[17]	81/F	Colon	7.5	Positive	Negative	Negative	EWSR1-CREB1 by RT-PCR	Mets to liver and peritoneum at 60 mo
	42/F	Ileum	5.7	Positive	Negative	Negative	EWSR1-CREB1 by RT-PCR	NA
	42/F	Ileum	3.5	Positive	Negative	Negative	EWSR1-CREB1 by RT-PCR	Peritoneal and liver mets at diagnosis
	51/F	Jejunum	NA	Positive	Negative	Negative	EWSR1 rearrangement by FISH	Peritoneal and liver mets; AWD
	18/F	Jejunum	NA	Positive	Negative	Negative	EWSR1-ATF1 by RT-PCR	Local recurrence
Granville *et al*[18]	16/M	Ileum	5.0	Positive	Negative	ND	EWSR1-ATF1 by RT-PCR; t(12;22)(q13;q12)	DOD 15 mo
Comin *et al*[19]	31/F	Ileum	2.8	Positive	Negative	Negative	EWSR1 rearrangement by FISH	NA
Lyle *et al*[20]	46/M	Jejunum	11.0	Positive	Positive	Positive	EWSR1 rearrangement by FISH; EWSR1-ATF1 by RT-PCR	NED 7 mo
	49/M	Cecum	10.5	Positive	Positive	Positive	EWSR1 rearrangement by FISH; EWSR1-ATF1 by RT-PCR	DOD 12 mo
	60/M	Jejunum	10.0	Positive	Positive	Positive	EWSR1-ATF1 by RT-PCR	DOD 28 mo
	62/M	Ileum	4.0	Positive	Positive	Positive	EWSR1 rearrangement by FISH; EWSR1-ATF1 by RT-PCR	DOD 12 mo
Abdulkader *et al*[21]	37/M	Jejunum	8.2	Positive	Negative	ND	EWSR1 rearrangement by FISH	Liver mets at 2 mo
Lagmay *et al*[22]	10/F	Stomach	7.8	Positive	Negative	Negative	EWSR1 rearrangement by FISH; EWSR1-ATF1 by RT-PCR	NED 4 mo
Joo *et al*[23]	60/M	Ileum	2.4	Positive	Negative	Negative	EWSR1 rearrangement by FISH	NA

	Age/Sex	Site	Size				Genetic	Outcome
	46/M	Jejunum	6.0	Positive	Negative	Negative	EWSR1 rearrangement by FISH	NA
Terazawa et al[24]	Early 20s/F	Ileum	3.0	Positive	ND	ND	EWSR1-ATF1 by RT-PCR	NED at 24 mo
Shenjere et al[25]	53/F	Ileum	5.0	Positive	Negative	Negative	EWSR1-ATF1 by RT-PCR	Regional LN mets at diagnosis/ NED at 7 mo
	26/F	Small and large bowel[1]	13.5/10.1	Positive	Negative	Negative	EWSR1-CREB1 by RT-PCR	NA
	66/M	Ileum	2.5	Positive	Negative	Negative	EWSR1-CREB1 by RT-PCR	Regional LN mets at diagnosis/NED
Balkaransingh et al[26]	15/M	Ileum	NA	ND	ND	ND	EWSR1 rearrangement by FISH	NA
Yang et al[27]	15/M	Ileum	4.0	Positive	ND	ND	EWSR1 rearrangement by FISH	Liver mets at 12 mo
Suárez-Vilela et al[28]	36/F	Jejunum	1.5	Positive	Negative	Negative	EWSR1 rearrangement by FISH	NA
D'Amico et al[29]	69/F	Ileum	4.0	Positive	Negative	ND	EWSR1 rearrangement by FISH	Liver mets at 2 mo
Lasithiotakis et al[30]	49/F	Jejunum	3.0	Positive	Negative	Negative	EWSR1 rearrangement by FISH	NED 20 mo
Huang et al[31]	45/F	Colon	4.0	Positive	Negative	Negative	EWSR1 rearrangement by FISH	Liver mets at 20 mo
Mallick et al[32]	45/M	Jejunum	4.4	Positive	Negative	Negative	ND	NA
Kong et al[33]	17/M	Stomach	6.0	Positive	Negative	Negative	EWSR1 rearrangement by FISH	NED 10 mo
Liu et al[34]	76/M	Jejunum	2.5	Positive	Negative	Negative	EWSR1-ATF1 by RT-PCR	NA
Thway et al[35]	36/M	Ileum	3.0	Positive	Negative	Negative	EWSR1-CREB1 by RT-PCR	DOD 7 mo
Huang et al[36]	36/M	Pancreas	4.0	Positive	Positive	Positive	EWSR1 rearrangement by FISH	Liver mets at 10 mo
Yegen et al[37]	25/F	Ileum	3.2	Positive	Negative	Negative	EWSR1 rearrangement by FISH	Liver mets at diagnosis and at 15 mo. Ovarian mets and peritoneal dissemination at 47 mo
Moslim et al[38]	57/M	Duodenum and Jejunum[2]	5.5/7.5	Positive	Negative	Positive	EWSR1 rearrangement by FISH	NED 30 mo and then DOD 4 mo later
Chen et al[39]	29/F	Jejunum	6.0	Positive	Negative	Negative	EWSR1 rearrangement by FISH	NED 17 mo
Our case	51/M	Duodenum and Jejunum[3]	6.5/2.5/2.5	Positive	Positive	Positive	EWSR1 rearrangement undetectable by FISH	NED up to date

[1]Two simultaneous tumors in small and large bowel; [2]Two simultaneous tumors in duodenum and jejunum; [3]Three simultaneous tumors in duodenum and jejunum. AWD: Alive with disease; DOD: Dead of disease; FISH: Fluorescence *in situ* hybridisation; LN: Lymph node; Mets: Metastases; NA: Not acquired; ND: Not done; NED: No evidence of disease; RT: Reverse transcription.

be highly aggressive, with high rates of local recurrence, lymph node or visceral metastases, and death, generally within < 36 mo[41,46]. In the current report, the patient underwent excision of multiple intestinal neoplasms and right parotidectomy before the first cycle of the chemotherapy and no recurrence or metastasis has been observed during the follow-up to date.

In conclusion, CCS-GI is a highly rare soft-tissue sarcoma with distinct morphological, immuno-histochemical, and genetic features. This case demonstrates that the parotid gland is a potential metastatic site for CCS-GI. Prior to developing a routine method to diagnose and treat CCS-GI, more cases need to be accumulated for further analysis.

COMMENTS

Case characteristics
A 51-year-old male presented with a two-year history of a growing painless mass lesion under the right ear that had grown noticeably over the past six months and a one-year history of night sweat and frequent stool.

Clinical diagnosis
A relatively well-defined soft mass with no tenderness was observed along with multiple enlarged cervical nodules.

Differential diagnosis
Small intestinal stromal tumors, lymphoma, head and neck neoplasm, sarcomatoid carcinoma.

Laboratory diagnosis
The patient's laboratory test had no remarkable findings.

Imaging diagnosis
Positron emission tomography/computed tomography showed an intestinal mass with involvement of multiple peripheral lymph nodes and mass in the right parotid.

Pathological diagnosis
The intestinal neoplasms and parotid gland neoplasm were consistent with

CCS based on morphology and immunohistochemistry.

Treatment
The patient underwent curative resection and postoperative chemotherapy.

Related reports
Only 53 cases of clear-cell sarcomas of the gastrointestinal tract (CCS-GI) have been reported in the literature to date, and CCS-GI shows distinct morphological, immunohistochemical, and genetic features.

Term explanation
CCS-GI is a highly rare soft tissue sarcoma.

Experiences and lessons
The present case report is the third instance of diagnosis of simultaneous multiple CCS-GIs to date and the first case of CCS-GI with metastasis to the parotid gland.

Peer-review
The authors have described a case of multiple clear-cell sarcomas of the small intestine with parotid gland metastasis. The article highlights the morphological, immunohistochemical, and genetic features of the tumors.

REFERENCES

1   **Enzinger FM**. Clear-cell sarcoma of tendons and aponeuroses. An analysis of 21 cases. *Cancer* 1965; **18**: 1163-1174 [PMID: 14332545]

2   **Hocar O**, Le Cesne A, Berissi S, Terrier P, Bonvalot S, Vanel D, Auperin A, Le Pechoux C, Bui B, Coindre JM, Robert C. Clear cell sarcoma (malignant melanoma) of soft parts: a clinicopathologic study of 52 cases. *Dermatol Res Pract* 2012; **2012**: 984096 [PMID: 22693489 DOI: 10.1155/2012/984096]

3   **Ekfors TO**, Kujari H, Isomäki M. Clear cell sarcoma of tendons and aponeuroses (malignant melanoma of soft parts) in the duodenum: the first visceral case. *Histopathology* 1993; **22**: 255-259 [PMID: 7684355]

4   **Stockman DL**, Miettinen M, Suster S, Spagnolo D, Dominguez-Malagon H, Hornick JL, Adsay V, Chou PM, Amanuel B, Vantuinen P, Zambrano EV. Malignant gastrointestinal neuroectodermal tumor: clinicopathologic, immunohistochemical, ultrastructural, and molecular analysis of 16 cases with a reappraisal of clear cell sarcoma-like tumors of the gastrointestinal tract. *Am J Surg Pathol* 2012; **36**: 857-868 [PMID: 22592145 DOI: 10.1097/PAS.0b013e31824644ac]

5   **Alpers CE**, Beckstead JH. Malignant neuroendocrine tumor of the jejunum with osteoclast-like giant cells. Enzyme histochemistry distinguishes tumor cells from giant cells. *Am J Surg Pathol* 1985; **9**: 57-64 [PMID: 2578748]

6   **Donner LR**, Trompler RA, Dobin S. Clear cell sarcoma of the ileum: the crucial role of cytogenetics for the diagnosis. *Am J Surg Pathol* 1998; **22**: 121-124 [PMID: 9422325]

7   **Fukuda T**, Kakihara T, Baba K, Yamaki T, Yamaguchi T, Suzuki T. Clear cell sarcoma arising in the transverse colon. *Pathol Int* 2000; **50**: 412-416 [PMID: 10849331]

8   **Hu XL**, Wang WX. Clear cell sarcoma of the rectum: a case report. *Zhonghua Bing Li Xue Za Zhi* 2001; **30**: 77 [DOI: 10.3760/j.issn:0529-5807.2001.01.032]

9   **Pauwels P**, Debiec-Rychter M, Sciot R, Vlasveld T, den Butter B, Hagemeijer A, Hogendoorn PC. Clear cell sarcoma of the stomach. *Histopathology* 2002; **41**: 526-530 [PMID: 12460205]

10 **Zambrano E**, Reyes-Mugica M, Franchi A, Rosai J. An osteoclast-rich tumor of the gastrointestinal tract with features resembling clear cell sarcoma of soft parts: reports of 6 cases of a GIST simulator. *Int J Surg Pathol* 2003; **11**: 75-81 [PMID: 12754623]

11 **Achten R**, Debiec-Rychter M, De Wever I, Sciot R. An unusual case of clear cell sarcoma arising in the jejunum highlights the

12 **Venkataraman G**, Quinn AM, Williams J, Hammadeh R. Clear cell sarcoma of the small bowel: a potential pitfall. Case report. *APMIS* 2005; **113**: 716-719 [PMID: 16309433 DOI: 10.1111/j.1600-0463.2005.apm_243.x]

13 **Covinsky M**, Gong S, Rajaram V, Perry A, Pfeifer J. EWS-ATF1 fusion transcripts in gastrointestinal tumors previously diagnosed as malignant melanoma. *Hum Pathol* 2005; **36**: 74-81 [PMID: 15712185 DOI: 10.1016/j.humpath.2004.10.015]

14 **Taminelli L**, Zaman K, Gengler C, Peloponissios N, Bouzourene H, Coindre JM, Hostein I, Guillou L. Primary clear cell sarcoma of the ileum: an uncommon and misleading site. *Virchows Arch* 2005; **447**: 772-777 [PMID: 16021514 DOI: 10.1007/s00428-005-0019-y]

15 **Friedrichs N**, Testi MA, Moiraghi L, Modena P, Paggen E, Plötner A, Wiechmann V, Mantovani-Löffler L, Merkelbach-Bruse S, Buettner R, Wardelmann E. Clear cell sarcoma-like tumor with osteoclast-like giant cells in the small bowel: further evidence for a new tumor entity. *Int J Surg Pathol* 2005; **13**: 313-318 [PMID: 16273186]

16 **Huang W**, Zhang X, Li D, Chen J, Meng K, Wang Y, Lu Z, Zhou X. Osteoclast-rich tumor of the gastrointestinal tract with features resembling those of clear cell sarcoma of soft parts. *Virchows Arch* 2006; **448**: 200-203 [PMID: 16220298 DOI: 10.1007/s00428-005-0051-y]

17 **Antonescu CR**, Nafa K, Segal NH, Dal Cin P, Ladanyi M. EWS-CREB1: a recurrent variant fusion in clear cell sarcoma-association with gastrointestinal location and absence of melanocytic differentiation. *Clin Cancer Res* 2006; **12**: 5356-5362 [PMID: 17000668 DOI: 10.1158/1078-0432.CCR-05-2811]

18 **Granville L**, Hicks J, Popek E, Dishop M, Tatevian N, Lopez-Terrada D. Visceral clear cell sarcoma of soft tissue with confirmation by EWS-ATF1 fusion detection. *Ultrastruct Pathol* 2006; **30**: 111-118 [PMID: 16517477 DOI: 10.1080/01913120500406400]

19 **Comin CE**, Novelli L, Tornaboni D, Messerini L. Clear cell sarcoma of the ileum: report of a case and review of literature. *Virchows Arch* 2007; **451**: 839-845 [PMID: 17636326 DOI: 10.1007/s00428-007-0454-z]

20 **Lyle PL**, Amato CM, Fitzpatrick JE, Robinson WA. Gastrointestinal melanoma or clear cell sarcoma? Molecular evaluation of 7 cases previously diagnosed as malignant melanoma. *Am J Surg Pathol* 2008; **32**: 858-866 [PMID: 18408594 DOI: 10.1097/PAS.0b013e31815b8288]

21 **Abdulkader I**, Cameselle-Teijeiro J, de Alava E, Ruiz-Ponte C, Used-Aznar MM, Forteza J. Intestinal clear cell sarcoma with melanocytic differentiation and EWS [corrected] rearrangement: report of a case. *Int J Surg Pathol* 2008; **16**: 189-193 [PMID: 18417679 DOI: 10.1177/1066896907306841]

22 **Lagmay JP**, Ranalli M, Arcila M, Baker P. Clear cell sarcoma of the stomach. *Pediatr Blood Cancer* 2009; **53**: 214-216 [PMID: 19350639 DOI: 10.1002/pbc.22014]

23 **Joo M**, Chang SH, Kim H, Gardner JM, Ro JY. Primary gastrointestinal clear cell sarcoma: report of 2 cases, one case associated with IgG4-related sclerosing disease, and review of literature. *Ann Diagn Pathol* 2009; **13**: 30-35 [PMID: 19118779 DOI: 10.1016/j.anndiagpath.2008.10.003]

24 **Terazawa K**, Otsuka H, Morita N, Yamashita K, Nishitani H. Clear-cell sarcoma of the small intestine detected by FDG-PET/CT during comprehensive examination of an inflammatory reaction. *J Med Invest* 2009; **56**: 70-75 [PMID: 19262017]

25 **Shenjere P**, Salman WD, Singh M, Mangham DC, Williams A, Eyden BP, Howard N, Knight B, Banerjee SS. Intra-abdominal clear-cell sarcoma: a report of 3 cases, including 1 case with unusual morphological features, and review of the literature. *Int J Surg Pathol* 2012; **20**: 378-385 [PMID: 22084426 DOI: 10.1177/1066896911425485]

26 **Balkaransingh P**, Saad SA, Govil SC, Thind PK, Ballance CM, Weiss AR. Clear cell sarcoma of the gastrointestinal

tract presenting as a second malignant neoplasm following neuroblastoma in infancy. *Pediatr Blood Cancer* 2012; **58**: 481-482 [PMID: 21990209 DOI: 10.1002/pbc.23330]

27 **Yang JC**, Chou AJ, Oeffinger KC, La Quaglia MP, Wolden SL. Clear cell sarcoma of the gastrointestinal tract after very low-dose therapeutic radiation therapy: a case report. *J Pediatr Surg* 2012; **47**: 1943-1945 [PMID: 23084213 DOI: 10.1016/j.jpedsurg.2012.08.014]

28 **Suárez-Vilela D**, Izquierdo FM, Tojo-Ramallo S, R Riera-Velasco J, Escobar-Stein J. Malignant gastrointestinal neuroectodermal tumor showing overlapped immunophenotype with synovial sarcoma: CD99 and SOX10 antibodies are useful in differential diagnosis. *Am J Surg Pathol* 2012; **36**: 1905-198; author reply 1908 [PMID: 23154774 DOI: 10.1097/PAS.0b013e31826f5b28]

29 **D'Amico FE**, Ruffolo C, Romeo S, Massani M, Dei Tos AP, Bassi N. Clear cell sarcoma of the ileum: report of a case and review of the literature. *Int J Surg Pathol* 2012; **20**: 401-406 [PMID: 22207412 DOI: 10.1177/1066896911428073]

30 **Lasithiotakis K**, Protonotarios A, Lazarou V, Tzardi M, Chalkiadakis G. Clear cell sarcoma of the jejunum: a case report. *World J Surg Oncol* 2013; **11**: 17 [PMID: 23351137 DOI: 10.1186/1477-7819-11-17]

31 **Huang HF**, Liu Q, Hong BU, Chen M, Chen HJ, Lin YY, Zhang HY, Pathology DO, Hospital WC and University S. Clear cell sarcoma of gastrointestinal tract: clinicopathologic analyses and review of literatures. *Linchuang Yu Shiyan Binglixue Zazhi* 2014; **30**: 383-388 [DOI: 10.13315/j.cnki.cjcep.2014.04.007]

32 **Mallick S**, Singh L, Rajan K, Sharma MC, Bansl V, Dinda AK. Malignant melanoma of soft parts with osteoclast-rich giant cells: A rare tumour of the jejunum. *Australas Med J* 2014; **7**: 181-184 [PMID: 24817912 DOI: 10.4066/AMJ.2014.1970]

33 **Kong J**, Nan LI, Shiwu WU, Guo X, Congyou GU and Feng Z. Malignant gastrointestinal neuroectodermal tumor: A case report and review of the literature. *Oncol Lett* 2014; **8**: 2687-2690 [DOI: 10.3892/ol.2014.2524]

34 **Liu C**, Ren Y, Li X, Cao Y, Chen Y, Cui X, Li L, Li F. Absence of 19 known hotspot oncogenic mutations in soft tissue clear cell sarcoma: two cases report with review of the literature. *Int J Clin Exp Pathol* 2014; **7**: 5242-5249 [PMID: 25197404]

35 **Thway K**, Judson I, Fisher C. Clear cell sarcoma-like tumor of the gastrointestinal tract, presenting as a second malignancy after childhood hepatoblastoma. *Case Rep Med* 2014; **2014**: 984369 [PMID: 24715928 DOI: 10.1155/2014/984369]

36 **Huang J**, Luo RK, Du M, Zeng HY, Chen LL, Ji Y. Clear cell sarcoma of the pancreas: a case report and review of literature. *Int J Clin Exp Pathol* 2015; **8**: 2171-2175 [PMID: 25973121]

37 **Yegen G**, Güllüoğlu M, Mete Ö, Önder S, Kapran Y. Clear cell sarcoma-like tumor of the gastrointestinal tract: a case report and review of the literature. *Int J Surg Pathol* 2015; **23**: 61-67 [PMID: 25145707 DOI: 10.1177/1066896914547046]

38 **Moslim MA**, Falk GA, Cruise M, Morris-Stiff G. Simultaneous Clear Cell Sarcomas of the Duodenum and Jejunum. *Case Rep Med* 2016; **2016**: 1534029 [PMID: 27375743 DOI: 10.1155/2016/1534029]

39 **Chen L**, Zhou AP. Small intestinal clear cell sarcoma in gestation period masquerading as an abdominal abscess. *Clin Misdiagnosis and Mistherapy* 2016; **29**: 33-34 [DOI: 10.3969/j.issn.1002-3429.2016.04.011]

40 **Park SW**, Eade T, Pang L, Wignall A, Veivers D. Role of neck dissection in metastatic squamous cell carcinoma to the parotid gland. *J Laryngol Otol* 2016; **130** Suppl 4: S54-S59 [PMID: 27488339 DOI: 10.1017/S0022215116008343]

41 **Kosemehmetoglu K**, Folpe AL. Clear cell sarcoma of tendons and aponeuroses, and osteoclast-rich tumour of the gastrointestinal tract with features resembling clear cell sarcoma of soft parts: a review and update. *J Clin Pathol* 2010; **63**: 416-423 [PMID: 20418233 DOI: 10.1136/jcp.2008.057471]

42 **Coindre JM**. New WHO classification of tumours of soft tissue and bone. *Ann Pathol* 2012; **32**: S115-S116 [PMID: 23127926 DOI: 10.1016/j.annpat.2012.07.006]

43 **Wang WL**, Mayordomo E, Zhang W, Hernandez VS, Tuvin D, Garcia L, Lev DC, Lazar AJ, López-Terrada D. Detection and characterization of EWSR1/ATF1 and EWSR1/CREB1 chimeric transcripts in clear cell sarcoma (melanoma of soft parts). *Mod Pathol* 2009; **22**: 1201-1209 [PMID: 19561568 DOI: 10.1038/modpathol.2009.85]

44 **Panagopoulos I**, Mertens F, Isaksson M, Mandahl N. Absence of mutations of the BRAF gene in malignant melanoma of soft parts (clear cell sarcoma of tendons and aponeuroses). *Cancer Genet Cytogenet* 2005; **156**: 74-76 [PMID: 15588860 DOI: 10.1016/j.cancergencyto.2004.04.008]

45 **Panagopoulos I**, Mertens F, Dêbiec-Rychter M, Isaksson M, Limon J, Kardas I, Domanski HA, Sciot R, Perek D, Crnalic S, Larsson O, Mandahl N. Molecular genetic characterization of the EWS/ATF1 fusion gene in clear cell sarcoma of tendons and aponeuroses. *Int J Cancer* 2002; **99**: 560-567 [PMID: 11992546 DOI: 10.1002/ijc.10404]

46 **Langezaal SM**, Graadt van Roggen JF, Cleton-Jansen AM, Baelde JJ, Hogendoorn PC. Malignant melanoma is genetically distinct from clear cell sarcoma of tendons and aponeurosis (malignant melanoma of soft parts). *Br J Cancer* 2001; **84**: 535-538 [PMID: 11207050 DOI: 10.1054/bjoc.2000.1628]

LETTERS TO THE EDITOR

Helicobacter is preserved in yeast vacuoles! Does Koch's postulates confirm it?

Nader Alipour, Nasrin Gaeini

Nader Alipour, Department of Medical Microbiology and Biotechnology, DEU University and Graduate of Middle East Technical university (METU), Ankara 06800, Turkey

Nasrin Gaeini, Sıfa Medical Center, Tatlikuyu, Gebze 51338, Kocaeli, Turkey

Abstract

The manuscript titled "Vacuoles of Candida yeast behave as a specialized niche for *Helicobacter pylori* (*H.*
pylori)" not only has not been prepared in a scientific manner but the methodology used was not adequate, and therefore the conclusion reached was not correct. First of all, "yeast" is a broad terminology covering a great number of genera and species of unicellular micro-organisms. The authors should have defined the organism with its binary scientific name. This measure would allow experiment reproduction by the scientific community. Moreover, the criteria established by Robert Koch to identify a specific microorganism or pathogen was not adopted in the methodology used. Regarding the methodology applied, use of the chicken egg-yolk (IgY) antibody and PCR of the apparently tainted yeast population to prove *H. pylori* existence in the yeast vacuoles might be main factors for their wrong conclusions. Bacterial tropism toward yeast extract is a known phenomenon, and yeast extract is one of the main ingredients in culture media. Their internalization through phagocytosis or similar pathways does not seem possible or practical because of the thick and cellulosic yeast wall. While the small size of yeast cells does not support their ability in harboring several *H. pylori*, other observations such as inefficiency of anti-fungal therapy as anti-*Helicobacter* therapy strongly reject the conclusion reached by the above-mentioned article.

Core tip: An article titled "Vacuoles of Candida yeast behave as a specialized niche for *Helicobacter pylori*," was published in the *World Journal of Gastroenterology* (2014; 20: 5263-5273). This "letter to the editor" is intended to demonstrate the shortcomings of that article related to the methodologies applied, the conclusion reached and the outcomes presented.

TO THE EDITOR

We read with interest the review article titled "Vacuoles of *Candida* yeast behave as a specialized niche for *Helicobacter pylori* (*H. pylori*)" by Siavoshi *et al*[1]. Based on the other research articles by the same authors, this review article concludes that: *H. pylori* are able to penetrate into the "*Candida*" yeast, multiply inside its vacuoles, and potentially transfer into the daughter cells when the yeast cells are dividing. They hypothesized that the yeast can act as the vehicle in transferring *Helicobacter* into human. They have included figures demonstrating the presence of several *Helicobacter* in *Candida* yeast cells. For the following reasons, we do not agree with their methodology and conclusion.

Yeast is a general word that describes a great amount of genera and species of unicellular micro-organisms, including beverage yeasts, baker's yeasts, fruit yeasts, food yeasts, industrial yeasts, environmental yeasts, pathogenic yeasts, *etc*. A binary scientific name must be indicated in scientific manuscripts (*e.g., Saccharomyces cerevisiae, Saccharomyces boulardii, Candida albicans, Candida tropicalis, Candida krusei etc*). They can be differentiated from each other by simple biochemical tests. Without their identifications, other scientists will not be able to reproduce these outcomes in their own laboratories.

Koch's postulates requires relying on the defined standard methods and criteria. Culturing and sub-culturing micro-organisms in the required culture media is one of these criteria. The authors have not practiced these postulates due to the fastidious nature of *H. pylori*.

Authors have used IgY "chicken egg-yolk antibodies" against *H. pylori*, to demonstrate the presence of *H. pylori* in yeast cells. However, IgY is not accurate enough for such an experiment. Human serum IgG antibodies of *H. pylori* from positive duodenal ulcer patients could be a more reliable tool than chicken IgY. *Campylobacter* which share similar antigenic cross reaction with *H. pylori* is present in the normal flora of poultry and chicken gut. So, antibodies produced in chicken eggs cannot be accurate enough.

Bacterial tropism toward yeast extract is a known phenomenon and has been reported by others[2,3]. This is a natural tropism of living bacteria toward food, and is not a novel finding. Similarly, to *Acanthamoeba* protozoa[4,5] histological smears from different body fluids demonstrate their co-existence. Such observation would not be possible if the *Helicobacter* was inside vacuoles embedded in the yeast cells! Yeast extract is one of the main ingredients in the culture media.

Internalization of food particles or bacteria into a eukaryotic cell may adopt different pathways such as phagocytosis and receptor- or transporter-mediated transportation. Bacteria mostly enter larger cells with soft and flexible membranes, such as the white blood cells (through phagocytosis). The thick and cellulosic nature of the yeast cell wall limits its phagocytic ability and the direct entrance of large particles. On the other hand, such ability can be easily acceptable in the case of protozoa and amoeba with their pseudopods and softer membranes. Cells that are specialized in bacteria internalization and ingestion are known as "Bacterivores". *Helicobacter* must have magic ability in passing through the thick cellulosic cell wall of the yeast (like the internationally-known magician "David Cooperfield" who appeared to pass through the Great Wall of China only to have the reality demonstrated to be a trick of the camera in the show). As the larger size of *Acanthamoeba* indicates, these cells can internalize several *Helicobacter*. Therefore, ingestion of *Helicobacter* by *Acanthamoeba* seems more logical than their ingestion by yeast cells.

Antibiotics are very slow in their entry into the yeast cells compared to their entrance into the *Acanthamoeba*. Therefore, it is very hard to imagine complete eradication of the *Helicobacter* by antibiotics if they are internalized into the *Candida* cells. Such eradication will not be difficult, as it happens in infected patient treatments, if the *Helicobacter* are located on the surface of the yeast cells.

Moreover, the prevalence of *Helicobacter* infection should be higher in females than males and patients with human immunodeficiency virus, due to the higher yeast infection rates in these two groups. In fact, the situation is the other way around[5-7].

Interestingly, several articles in the literature have shown similarity in prevalence of *Acanthamoeba* in drinking water sampled from different geographical locations and the prevalence of *H. pylori* in patients[8-19]. While we cannot observe such overlap between yeast and *H. pylori* incidences, it is more logical to believe that yeast cannot be a reservoir of *H. pylori* but that *Acanthamoeba* can play such a role.

Moreover, anti-*Helicobacter* therapies, including anti-fungal drug usage, have not shown statistically significant difference upon comparison with no treatment[17,20].

The above arguments reject the idea of yeast harboring *H. pylori* in its vacuole. In theoretical analysis, internalization of *H. pylori* by yeast cells can be out of two possibilities: The *Helicobacter* should cross the yeast external wall and then cross the specific vacuole membrane where they will be trapped even if they could multiply, or they should be internalized *via* phagocytosis to end up in a digestive vacuole and be digested. If we imagine that *H. pylori*

may infect the yeast cells in a way comparable to viral infection, it will be an exception for bacterial pathogenesis and the propagation mechanism. Such an idea needs an accurate and reliable study, however. The positive PCR reaction stated in Siavoshi *et al*[1]'s article, ought to have stemmed from the *Helicobacter* located on the surface of the yeast cells.

In conclusion, with all respect to the authors of the above-mentioned review article, we believe that the interpretation of their observation is totally wrong. While being aware of the symbiotic nature of *H. pylori* and *Candida* yeast and the close relationship between these two organisms, they went wrong on internalization and survival of bacterial colonies inside yeast vacuoles.

Our initial response to the Siavoshi *et al*[1] article was published as an independent manuscript[18]. We ought to admit that this was not an appropriate approach to make our thought and beliefs known. We were directed to express our comments in the form of the present "Letter to the Editor". We hope this manuscript will clarify the issue and set the record straight.

ACKNOWLEDGMENTS

REFERENCES

1   **Siavoshi F**, Saniee P. Vacuoles of Candida yeast as a specialized niche for Helicobacter pylori. *World J Gastroenterol* 2014; **20**: 5263-5273 [PMID: 24833856 DOI: 10.3748/wjg.v20.i18.5263]

2   **Ansorg R**, Schmid EN. Adhesion of Helicobacter pylori to yeast cells. *Zentralbl Bakteriol* 1998; **288**: 501-508 [PMID: 9987188]

3   **Winiecka-Krusnell J**, Wreiber K, von Euler A, Engstrand L, Linder E. Free-living amoebae promote growth and survival of Helicobacter pylori. *Scand J Infect Dis* 2002; **34**: 253-256 [PMID: 12064686]

4   **Siavoshi F**, Salmanian AH, Akbari F, Malekzadeh R, Massarrat S. Detection of Helicobacter pylori-specific genes in the oral yeast. *Helicobacter* 2005; **10**: 318-322 [PMID: 16104948 DOI: 10.1111/j.1523-5378.2005.00319.x]

5   **Dutt P**, Chaudhary S, Kumar P. Oral health and menopause: a comprehensive review on current knowledge and associated dental management. *Ann Med Health Sci Res* 2013; **3**: 320-323 [PMID: 24116306 DOI: 10.4103/2141-9248.117926]

6   **Tylenda CA**, Larsen J, Yeh CK, Lane HC, Fox PC. High levels of oral yeasts in early HIV-1 infection. *J Oral Pathol Med* 1989; **18**: 520-524 [PMID: 2575167 DOI: 10.1111/j.1600-0714.1989.tb01355.x]

7   **Hoffmann JN**, You HM, Hedberg EC, Jordan JA, McClintock MK. Prevalence of bacterial vaginosis and Candida among postmenopausal women in the United States. *J Gerontol B Psychol Sci Soc Sci* 2014; **69** Suppl 2: S205-S214 [PMID: 25360022 DOI: 10.1093/geronb/gbu105]

8   **Coşkun KA**, Ozçelik S, Tutar L, Elaldı N, Tutar Y. Isolation and identification of free-living amoebae from tap water in Sivas, Turkey. *Biomed Res Int* 2013; **2013**: 675145 [PMID: 23971043 DOI: 10.1155/2013/675145]

9   **Eftekhar M**, Athari A, Haghighi A, Mosaffa N, Shahram F, Abadi A. Seroprevalence of Acanthamoeba Antibodies in Rheumatoid Arthritis Patients by IFAT, Tehran, Iran 2007. *Iran J Parasitol* 2010; **5**: 35-40 [PMID: 22347233]

10   **Bagheri H**, Shafiei R, Shafiei F, Sajjadi S. Isolation of acanthamoeba spp. From drinking waters in several hospitals of iran. *Iran J Parasitol* 2010; **5**: 19-25 [PMID: 22347240]

11   **Mahmoudi MR**, Taghipour N, Eftekhar M, Haghighi A, Karanis P. Isolation of Acanthamoeba species in surface waters of Gilan province-north of Iran. *Parasitol Res* 2012; **110**: 473-477 [PMID: 21748347 DOI: 10.1007/s00436-011-2530-1]

12   **Nazar M**, Haghighi A, Niyyati M, Eftekhar M, Tahvildar-Biderouni F, Taghipour N, Abadi A, Nazemalhosseini Mojarad E, Athari A. Genotyping of Acanthamoeba isolated from water in recreational areas of Tehran, Iran. *J Water Health* 2011; **9**: 603-608 [PMID: 21976207 DOI: 10.2166/wh.2011.152]

13   **Moosazadeh M**, Lankarani KB, Afshari M. Meta-analysis of the Prevalence of Helicobacter Pylori Infection among Children and Adults of Iran. *Int J Prev Med* 2016; **7**: 48 [PMID: 27076886 DOI: 10.4103/2008-7802.177893]

14   **Egemen A**, Yilmaz O, Akil I, Altuğlu I. Evaluation of association between hepatitis A and Helicobacter pylori infections and routes of transmission. *Turk J Pediatr* 2006; **48**: 135-139 [PMID: 16848113]

15   **Maraki S**, Mouzas IA, Kontoyiannis DP, Chatzinikolaou I, Tselentis Y, Samonis G. Prospective evaluation of the impact of amoxicillin, clarithromycin and their combination on human gastrointestinal colonization by Candida species. *Chemotherapy* 2001; **47**: 215-218 [PMID: 11306791]

16   **Uspenskiĭ IuP**, Sheviakov MA. [Diseases associated with Helicobacter pylori and Candida spp.: clinical logic of combined studies]. *Eksp Klin Gastroenterol* 2005; **(3)** 16-19, 100 [PMID: 16255548]

17   **Sasaki K**. Candida-associated gastric ulcer relapsing in a different position with a different appearance. *World J Gastroenterol* 2012; **18**: 4450-4453 [PMID: 22969213 DOI: 10.3748/wjg.v18.i32.4450]

18   **Alipour N**, Gaeini N, Taner A, Yıldız F, Masseret S, Malfertheiner P. Retracted: Vacuoles of Acanthamoeba castellanii Behave as a Specialized Shelter (host) for Helicobacter pylori. *Helicobacter* 2015; **20**: 485-485

19   **Montalbano Di Filippo M**, Santoro M, Lovreglio P, Monno R, Capolongo C, Calia C, Fumarola L, D'Alfonso R, Berrilli F, Di Cave D. Isolation and molecular characterization of free-living amoebae from different water sources in Italy. *Int J Environ Res Public Health* 2015; **12**: 3417-3427 [PMID: 25811766 DOI: 10.3390/ijerph120403417]

20   **Baingana RK**, Kiboko Enyaru J, Davidsson L. Helicobacter pylori infection in pregnant women in four districts of Uganda: role of geographic location, education and water sources. *BMC Public Health* 2014; **14**: 915 [PMID: 25190150 DOI: 10.1186/1471-2458-14-915]

1 2>

9 771007 932045