

World Journal of *Gastrointestinal Pathophysiology*

World J Gastrointest Pathophysiol 2017 February 15; 8(1): 1-10





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2016-2019

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FIELD OF VISION

- 1 Impaired inactivation of digestive proteases: The possible key factor for the high susceptibility of germ-free and antibiotic-treated animals to gut epithelial injury
Qin X

ORIGINAL ARTICLE

Observational Study

- 3 Polyp detection rate and pathological features in patients undergoing a comprehensive colonoscopy screening
Asadzadeh Aghdaei H, Nazemalhosseini Mojarad E, Ashtari S, Pourhoseingholi MA, Chaleshi V, Anaraki F, Haghazali M, Zali MR

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World Journal of Gastrointestinal Pathophysiology
Volume 8 Number 1 February 15, 2017

ABOUT COVER

Editorial Board Member of *World Journal of Gastrointestinal Pathophysiology*, Guang-Yu Yang, MD, PhD, Professor, Department of Pathology, Northwestern University, Chicago, IL 60611, United States

AIM AND SCOPE

World Journal of Gastrointestinal Pathophysiology (*World J Gastrointest Pathophysiol*, *WJGP*, online ISSN 2150-5330, DOI: 10.4291), is a peer-reviewed open access academic journal that aims to guide clinical practice and improve diagnostic and therapeutic skills of clinicians.

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We encourage authors to submit their manuscripts to *WJGP*. We will give priority to manuscripts that are supported by major national and international foundations and those that are of great basic and clinical significance.

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NAME OF JOURNAL
World Journal of Gastrointestinal Pathophysiology

ISSN
ISSN 2150-5330 (online)

LAUNCH DATE
April 15, 2010

Frequency
Quarterly

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PUBLICATION DATE
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Impaired inactivation of digestive proteases: The possible key factor for the high susceptibility of germ-free and antibiotic-treated animals to gut epithelial injury

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Author contributions: Qin X contributes solely to this paper.

Conflict-of-interest statement: There is no conflict of interest.

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Manuscript source: Invited manuscript

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Received: October 9, 2016

Peer-review started: October 11, 2016

First decision: December 13, 2016

Revised: December 16, 2016

Accepted: January 2, 2017

Article in press: January 3, 2017

Published online: February 15, 2017

Abstract

Recent study shows that germ-free and antibiotic-treated animals are highly susceptible to gut epithelial injury. This paper addresses that impaired inactivation of digestive proteases may be the key factor for the increased susceptibility.

Key words: Digestive proteases; Germ-free; Antibiotics; Gut microbiota; Gut epithelial injury

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Core tip: This paper addresses that impaired inactivation of digestive proteases may be the possible key factor for the high susceptibility of germ-free and antibiotic-treated animals to gut epithelial injury.

Qin X. Impaired inactivation of digestive proteases: The possible key factor for the high susceptibility of germ-free and antibiotic-treated animals to gut epithelial injury. *World J Gastrointest Pathophysiol* 2017; 8(1): 1-2 Available from: URL: <http://www.wjgnet.com/2150-5330/full/v8/i1/1.htm> DOI: <http://dx.doi.org/10.4291/wjgp.v8.i1.1>

COMMENTARY ON HOT TOPICS

I read with great interest the paper by Hernández-Chirlaque *et al*^[1] regarding the high susceptibility of germ-free and antibiotic-treated mice to epithelial injury. It is found that germ-free mice treated with dextran sulfate sodium (DSS) showed only minimal inflammation (no colonic thickening, lower myeloperoxidase activity, IL-6, IL-17, TNF-alpha and IFN-gamma secretion by splenocytes and mesenteric cell cultures, *etc.*), but enhanced hemorrhage, epithelial injury and mortality along with weakened intestinal barrier. Animals treated with antibiotics also showed similar but less severe changes with intermediate effects. That paper discussed the interaction among gut bacteria, immune cells, and epithelial cells through inflammatory mediators, cytokines, bioactive molecules, receptors, ligands, tran-

scription factors, and pathways, but failed to provide a coherent mechanistic explanation. Here I suggest that impaired inactivation of digestive proteases may be the key factor for the observed increased susceptibility. Studies have well documented that large amount of digestive proteases can be found in the large intestine of germ-free or antibiotics-treated animals but not in animals raised at conventional condition^[2-7]. As we know, the gut is mainly protected by a layer of mucus secreted from goblet cells. Mucin, the structural molecule of mucus, contains a central core of peptide and side carbohydrate branches that can constitute up to 85% of the molecule. These carbohydrate side chains greatly retarded degradation of the protective mucus layer and damage of the underlying gut tissue by the digestive proteases being existed in germ-free or antibiotics-treated animals. However, the great destructive potency of these digestive proteases would be released and manifested once the gut barrier is broken as occurred either shortly after treatment with high concentrations of DSS or prolonged treatment with lower dose of DSS, resulting in the severe damage of epithelial cells at gut surface and then blood vessels in the mucosa, leading to excessive blood loss and enhanced mortality, even in the absence of remarkably inflammation. This would be similar to the so-called tryptic hemorrhagic necrosis of the gut as seen in animals under shock, which can be greatly prevented by previous inactivation of digestive proteases or ligation of pancreatic duct^[8]. In fact, evidences I collected during the last fifteen years make me to believe that impaired inactivation of digestive proteases due to reduction in gut bacteria along with the improved hygiene and inhibition by some dietary chemicals such as saccharin and sucralose may have also played critical causative role in the pathogenesis of inflammatory bowel disease (IBD) in human^[9-11]. Thus, impaired inactivation of digestive proteases in colitis and

IBD would be worthwhile for further study.

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P- Reviewer: Matowicka-Karna J, Yang CH, Zhu YL S- Editor: Qi Y
L- Editor: A E- Editor: Li D



Observational Study

Polyp detection rate and pathological features in patients undergoing a comprehensive colonoscopy screening

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Supported by Gastroenterology and Liver Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

Institutional review board statement: The study was reviewed and approved by the Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

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

Conflict-of-interest statement: There are no conflicts of interest to report.

Data sharing statement: No additional data are available.

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Manuscript source: Invited manuscript

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Received: September 13, 2016
Peer-review started: September 14, 2016
First decision: October 21, 2016
Revised: November 23, 2016
Accepted: December 7, 2016
Article in press: December 9, 2016
Published online: February 15, 2017

Abstract

AIM

To identify the prevalence, and clinical and pathologic characteristic of colonic polyps among Iranian patients undergoing a comprehensive colonoscopy, and determine the polyp detection rate (PDR) and adenoma detection rate (ADR).

METHODS

In this cross-sectional study, demographics and epidemiologic characteristics of 531 persons who underwent colonoscopies between 2014 and 2015 at Mehrad gastrointestinal clinic were determined. Demographics, indication for colonoscopy, colonoscopy findings, number of polyps, and histopathological characteristics of the polyps were examined for each person.

RESULTS

Our sample included 295 (55.6%) women and 236 (44.4%) men, with a mean age of 50.25 ± 14.89 years. Overall PDR was 23.5% (125/531). ADR and colorectal cancer detection rate in this study were 12.8% and 1.5%, respectively. Polyps were detected more significantly frequently in men than in women (52.8% *vs* 47.2%, $P < 0.05$). Polyps can be seen in most patients after the age of 50. The average age of patients with cancer was significantly higher than that of patients with polyps (61.3 years *vs* 56.4 years, $P < 0.05$). The majority of the polyps were adenomatous. More than 50% of the polyps were found in the rectosigmoid part of the colon.

CONCLUSION

The prevalence of polyps and adenomas in this study is less than that reported in the Western populations. In our patients, distal colon is more susceptible to developing polyps and cancer than proximal colon.

Key words: Adenoma detection; Polyp detection; Iran; Colonoscopy; Screening

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Core tip: One of the major reasons for colonoscopy is detection of colon polyps, such as adenomas. Early diagnosis and endoscopic removal of adenomatous polyps is one of the main objectives for screening and prevention of colorectal cancer (CRC). Given that, only few studies are available in the national literature regarding the assessment of colorectal polyps, but none has explicitly noted the rate of polyp detection. Nevertheless, our study provides comprehensive information about clinical and epidemiological features of colorectal polyps. Therefore, the results of this study can provide a good infrastructure for the next preventive program and have clinical implications for CRC screening.

Asadzadeh Aghdaei H, Nazemalhosseini Mojarad E, Ashtari S, Pourhoseingholi MA, Chaleshi V, Anaraki F, Haghazali M, Zali MR. Polyp detection rate and pathological features in patients undergoing a comprehensive colonoscopy screening. *World J Gastrointest Pathophysiol* 2017; 8(1): 3-10 Available from: URL: <http://www.wjgnet.com/2150-5330/full/v8/i1/3.htm> DOI:

INTRODUCTION

Colorectal cancer (CRC) is the third most common malignant disease in both men and women worldwide, accounting for more than 8% of mortality in the world with approximately 1.4 million new cases a year^[1-4].

CRC is also the third most common cancer in men and women in Asia^[5,6]. In the Asia-Pacific region, the incidence varies between regions, with high incidence in Australia, and Eastern Asia, and low incidence in South-central Asia^[7]. According to the International Agency for Research on Cancer, the incidence of CRC in many Asian countries is similar to that in many Western countries^[8]. CRC is also the third most common cancer in Iranians, after excluding skin cancer, and it occurs at younger ages with an increasing trend similar to that in the Asia-Pacific countries^[1,9]. These increasing rates may result from the young age structure and low rates of colon cancer in older people of these countries^[6,10,11].

Almost all CRCs develop from colorectal polyps. Over a period of ten years, most of adenomatous polyps can be converted to colon carcinoma^[12,13]. Given that the process of conversion of colorectal adenomas into adenocarcinoma is very long and slow^[14], early detection and endoscopic removal of these precancerous lesions are very effective in reducing the incidence and mortality rate of CRC^[15-17].

CRC is a suitable disease for screening^[18]. However, due to a lack of comprehensive screening strategy and public acceptance, this program is not implemented in many countries. Nevertheless, access to the CRC screening is an important key to reducing the burden of CRC. Endoscopic screening is comprised of four techniques including sigmoidoscopy, colonoscopy, barium enema, and computed tomographic colonography^[19]. Colonoscopy is a highly specific and the most effective screening tool for detecting colonic polyps and CRC^[20].

Limited data are available in the national literature regarding the assessment of colorectal polyps^[21-25]. Understanding of the prevalence of colorectal polyps especially adenomas in the general population would help clarify the efficacy of a CRC screening program. Therefore, updating the current knowledge in the scope of colorectal polyps and CRC is essential. Hence, identifying the features of colon polyps (*e.g.*, distribution, location, and histology type) has great implications for developing national screening guidelines for CRC^[26,27]. In this study, we aimed to determine the baseline polyp and adenoma prevalence in persons who underwent colonoscopies for various indications as well as opportunistic screening for CRC. We also assessed the polyp detection rate (PDR) and adenoma detection rate (ADR), and evaluated the clinical and histological

Table 1 Patients' characteristics and colonoscopy findings

Variable	All (n = 531)
Sex	
Male, n (%)	236 (44.4)
Female, n (%)	295 (55.6)
Age	
Mean years (SD)	50.3 ± 15.4
Age groups	
≤ 50 yr	250 (47.1)
> 50 yr	281 (52.9)
Family history	
Yes	150 (28.2)
No	381 (71.8)
Indication, n (%)	
Screening	120 (22.6)
Gastrointestinal bleeding	90 (17.0)
Constipation	82 (15.5)
Diarrhea	74 (14.0)
Abdominal pain	70 (13.3)
Inflammatory bowel disease	65 (12.2)
Others	29 (5.4)
Patients with at least 1 polyp, n (%)	
Neoplastic polyps	68 (54.4)
Non-neoplastic polyps	57 (45.6)
Cancer, n (%)	8 (1.5)

characteristics of colorectal polyps in Iranian patients and Iranian volunteers for CRC screening.

MATERIALS AND METHODS

Study design

In this cross-sectional study, all data were extracted from a colonoscopy database and pathology reports maintained by Mehrad gastrointestinal clinic in Iran. We included all persons aged 15 to 85 years, who underwent their first time colonoscopy during 2014-2015. Patients who had previously been identified with colon polyp or colorectal malignancies including CRC, colonic resection, active colitis, active diverticulitis and familial adenomatous polyposis were excluded from the study. We collected the data on demographic variables, indications for colonoscopy, and family history of colorectal malignancies. Family history was defined as having a first degree relative with CRC. For all colorectal lesions, data on clinical and pathological features (*i.e.*, number, size, site, and grade of dysplasia) were obtained. The study was approved by the Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences.

Polyp classification

All polyps identified during colonoscopy were biopsied or removed endoscopically and submitted for histopathology. The overall PDR was defined as the proportion of procedures in which at least one polyp was detected over the total number of colonoscopies. ADR was defined as the number of colonoscopies in which one or more adenomas was detected, divided by the total number of colonoscopies performed by the endoscopist^[28].

Pathological features of colorectal lesions were determined using the World Health Organization criteria^[28]. Hyperplastic and adenomatous polyps were classified as non-neoplastic polyps and neoplastic polyps, respectively. Microscopically, adenomas were categorized architecturally as serrated, tubular, tubular-villous, and villous.

The locations of the polyps were defined as proximal colon including the transverse colon, hepatic flexure, ascending colon, and cecum, and distal colon including the rectum, sigmoid, descending colon, and splenic flexure.

The polyp size was classified as small (< 5 mm), medium (5-9 mm), or large (> 10 mm). Estimation of polyp size was performed by the endoscopist using the diameter of the open biopsy forceps, which is about 8 mm. In the event of multiple polyps, only the size of the largest was considered for the purposes of analysis. Degrees of dysplasia observed in the adenomas were graded as low (mild and moderate) or high grade (severe). Patients with no polyps were regarded as normal.

Statistical analysis

Categorical variables are expressed as numbers and percentages. χ^2 or Fisher's exact test, where appropriate, was used for analysis of categorical variables. Continuous variables are expressed as medians, or as means and standard deviation, and 95%CI as appropriate. The Student's *t*-test was used for comparisons of means. All analyses were performed using SPSS version 21.0 (SPSS INC, Chicago, IL, United States). A two-tailed *P* < 0.05 was considered statistically significant.

RESULTS

Demographic and historical data

During the period of study (2014-2015), 531 persons met the inclusion criteria. Our sample included 55.6% of women and 44.4% of men. The age range was 15-85 years with a mean of 50.3 ± 15.4 years and a median of 52 years. The patients were divided into two age-groups (≤ 50 and > 50 years). The majority of patients was over 50 years (52.9%). One hundred and fifty (28.2%) patients had a family history of polyps or CRC in our study.

The most common reasons for colonoscopy included screening (asymptomatic adults aged 50 years and older and with a family history of CRC) in 22.6%, and lower gastrointestinal bleeding in 17.0%. Other indications for colonoscopy were constipation (15.4%), diarrhea (13.9%), abdominal pain (13.1%), inflammatory bowel disease (12.2%), and others (5.4%). Other referral indications included bloating, reflux, weight loss, anemia, fatty liver, and irritable bowel syndrome (Table 1).

Study outcomes and colonoscopy findings

Based on colonoscopy findings, the overall PDR was

Table 2 Detection rates of different histologic types of polyps and cancer by gender

Histologic type of polyps	Gender		Total
	Male (%)	Female (%)	
Neoplastic			
Tubular	18 (53.0)	16 (47.0)	34 (100)
Tubulo-villous	13 (68.4)	6 (31.6)	19 (100)
Villous	4 (40.0)	6 (60.0)	10 (100)
Serrated	3 (60.0)	2 (40.0)	5 (100)
Adenomatous polyps	38 (55.9)	30 (44.1)	68 (100)
Non-neoplastic			
Hyperplastic	28 (40.6)	29 (45.3)	57 (100)
Total PDR	66 (52.8)	59 (47.2)	125 (100)
Cancer	5 (62.5)	3 (37.5)	8 (100)
Total	69 (51.9)	64 (48.1)	133 (100)

PDR: Polyp detection rate.

Table 3 Detection rates of different histologic types of polyps and cancer by age

Histologic type of polyps	Age-groups		Total
	≤ 50 yr	> 50 yr	
Neoplastic			
Tubular	6 (17.6)	28 (82.4)	34 (100)
Tubulo-villous	7 (36.8)	12 (63.2)	19 (100)
Villous	3 (30.0)	7 (70.0)	10 (100)
Serrated	3 (60.0)	2 (40.0)	5 (100)
Adenomatous polyps	19 (28.0)	49 (72.0)	68 (100)
Non-neoplastic			
Hyperplastic	19 (33.3)	38 (66.7)	57 (100)
Total PDR	38 (30.4)	87 (69.6)	125 (100)
Cancer	1 (12.5)	7 (87.5)	8 (100)
Total	39 (29.3)	94 (70.7)	133 (100)

PDR: Polyp detection rate.

Table 4 Detection rates of different histologic types of polyps and cancer by family history

Histologic type of polyps	Family history		Total
	No (%)	Yes (%)	
Neoplastic			
Tubular	26 (76.4)	8 (23.6)	34 (100)
Tubulo-villous	13 (68.4)	6 (31.6)	19 (100)
Villous	5 (50.0)	5 (50.0)	10 (100)
Serrated	2 (40.0)	3 (60.0)	5 (100)
Adenomatous polyps	46 (67.6)	22 (32.4)	68 (100)
Non-neoplastic			
Hyperplastic	46 (80.7)	11 (19.3)	57 (100)
Total PDR	92 (73.6)	33 (26.4)	125 (100)
Cancer	0 (0)	8 (100)	8 (100)
Total	92 (69.2)	41 (30.8)	133 (100)

PDR: Polyp detection rate.

Table 5 Detection rates of different histologic types of polyps by location

Histologic type of polyps	Location		Total
	Proximal colon (%)	Distal colon (%)	
Neoplastic			
Tubular	10 (26.3)	28 (73.7)	38 (100)
Tubulo-villous	9 (40.9)	13 (59.1)	22 (100)
Villous	3 (30.0)	7 (70.0)	10 (100)
Serrated	3 (37.5)	5 (62.5)	8 (100)
Adenomatous polyps	25 (32.0)	53 (68.0)	78 (100)
Non-neoplastic			
Hyperplastic	19 (31.7)	41 (68.3)	60 (100)
Total PDR	44 (31.9)	94 (68.1)	138 (100)

PDR: Polyp detection rate.

23.5% (125/531). According to histopathology results from 125 patients with at least one polyp, 54.4% of the lesions were neoplastic polyps and 45.6% were hyperplastic polyps (non-neoplastic polyps). The overall ADR in this study was 12.8% (68/531). The percentage of male patients with polyps was significantly higher than that of female patients (52.8% vs 47.2%, $P < 0.05$). CRC was detected in 1.5% (8/531) of the total population (men, 62.5%; women, 37.5%) (Table 2). The mean age of patients with polyp was 56.4 ± 13.5 years. Polyps can be seen in most patients after the age of 50 (69.6%). CRC was more frequently observed in patients aged from 60 to 80 years. The average age of patients with cancer was significantly higher than that of those with polyps (61.3 ± 19.7 years vs 56.4 ± 13.5 years, $P < 0.05$) (Table 3). In addition, the relationship between PDR and family history of CRC or polyps was assessed and this was found not to be statistically significant ($P > 0.05$), while all patients with CRC in this study had a family history (Table 4).

Histopathological characteristics of the polyps

Totally 138 polyps were removed by colonoscopy in this

study. Among the 138 polyps, 56.5% were neoplastic and the others were non-neoplastic. All non-neoplastic polyps were hyperplastic. Among the 78 neoplastic polyps, the common histologic types were tubular adenomas (48.7%), tubule-villous adenomas (28.2%), villous adenomas (12.8%), and serrated adenomas (10.2%).

The prevalence of polyps in distal colon was higher than that in proximal colon (68.1% vs 31.9%, $P < 0.05$). Accordingly, most of cancers were located in the distal colon compared with the proximal colon (75% vs 25%, $P < 0.05$) (Table 5). Overall polyps were frequently detected in the rectum (32.0%), sigmoid (24.6%), transverse colon (16%), and ascending and descending colon (10.1%), and the others located in the cecum (5%), and splenic and hepatic flexure (2.1%) (Table 6).

Data about the size of polyps were available for only 75 polyps; 33.3% were smaller than 5 mm as small size, 40% were between 5–9 mm as medium size and 26.7% were more than 10 mm as large size (Table 7). According to the degree of dysplasia observed in the adenomas, most of patients (52.6%) had mild grade, 24.4% had moderate, and 23% had severe grade of dysplasia (Table 8).

Table 6 Detection rates of different histologic types of polyps by colonic segments

Polyps	Location								Total
	Ascending colon	Transverse colon	Descending colon	Sigmoid	Rectum	Cecum	Hepatic flexure	Splenic flexure	
Tubular	3 (7.9)	5 (13.2)	4 (10.5)	7 (18.4)	16 (42.1)	1 (2.6)	1 (2.6)	1 (2.6)	38 (100)
Tubulo-villous	4 (18.2)	3 (13.6)	2 (9.1)	6 (27.3)	5 (22.7)	2 (9.1)	0 (0)	0 (0)	22 (100)
Villous	0 (0)	1 (10)	0 (0)	2 (20)	5 (50)	2 (20)	0 (0)	0 (0)	10 (100)
Serrated	0 (0)	3 (37.5)	1 (12.5)	3 (37.5)	1 (12.5)	0 (0)	0 (0)	0 (0)	8 (100)
Hyperplastic	7 (11.7)	10 (16.7)	7 (11.7)	16 (26.7)	17 (28.3)	2 (3.3)	0 (0)	1 (1.7)	60 (100)
Total	14 (10.1)	22 (16.0)	14 (10.1)	34 (24.6)	44 (32.0)	7 (5.1)	1 (0.7)	2 (1.4)	138 (100)

Table 7 Detection rates of different histologic types of polyps by size

Size	Histologic type					Total
	Hyperplastic	Tubular	Tubul-villous	villous	Serrated	
Small	11 (44.0)	0 (0)	14 (56.0)	0 (0)	0 (0)	25 (100)
Medium	14 (46.7)	0 (0)	12 (40.0)	0 (0)	4 (13.3)	30 (100)
Large	0 (0)	8 (40.0)	3 (15.0)	9 (45.0)	0 (0)	20 (100)
Unknown	35 (55.6)	2 (3.2)	9 (14.3)	13 (20.6)	4 (6.3)	63 (100)
Total	60 (43.5)	10 (7.2)	38 (27.5)	22 (16.0)	8 (5.8)	138 (100)

Data available for only 75 polyps.

Table 8 Detection rates of different histologic types of polyps by degree of dysplasia

Grade	Histologic type				Total
	Tubular	Tubul-villous	Villous	Serrated	
Mild	21 (51.2)	11 (26.9)	6 (14.6)	3 (7.3)	41 (100)
Moderate	11 (57.9)	3 (15.8)	3 (15.8)	2 (10.5)	19 (100)
Severe	6 (33.3)	8 (44.5)	1 (5.5)	3 (16.7)	18 (100)
Total	38 (48.7)	22 (28.2)	10 (12.9)	8 (10.2)	78 (100)

DISCUSSION

The PDR and ADR rates obtained in this study are low, when compared to the figures from most Western and some Asian countries. In a large multicenter study from Italy, the median detection rate for polyps was 35%^[29]. A large colonoscopy series from Spain reported a PDR of 45.8%^[30]. Similar studies from Mayo Clinic in the United States and France reported PDR of 49% and 35.5%, and ADR of 31% and 17.7%, respectively^[31,32]. In some Asian countries like Korea, China and Thailand, PDR and ADR were similar to those in Europeans and Americans^[33-36]. However, our findings are similar to reports from Kuwait, Malaysia and Oman where PDR of 20% and ADR of 10%, 11.5% and 12.1% were reported, respectively^[37-39]. While in African countries like Nigeria these rates were reported to be lower than our results (PDR, 16.1%; ADR, 6.8%)^[40]. The mean age of the studied population was relatively young (50.3 years) and it might decrease the ADR because adenomas have been demonstrated to be more frequent in those older than 50 years of age.

The overall estimate for PDR in our study was 23.5%, while ADR was 12.8%. The percentage of male patients with polyps was significantly higher than that of female patients (52.8% vs 47.2%, $P < 0.05$). These data are consistent with the other reports that support

gender differences in the prevalence of colon polyps and cancer^[27,40,41].

Older age is the most important predictor for the prevalence of adenomas and cancer. In our study, the PDR and cancer prevalence reached a peak in the 6th decade of life. Nonetheless, the average age of patients with cancer was significantly higher than that of patients with polyps (61.3 years vs 56.4 years, $P < 0.05$). Studies from the Middle East and the Western countries also mentioned a significant increase in the risk of CRC, in particular after the age of 50 years^[24,28,42].

Based on increasing prevalence of CRC in the sixth decade of life, regular screening should begin at the age of 50. However, people at higher risk of developing CRC should begin screening at a younger age^[43,44].

The tubular type was the most common histological feature of adenomas in the present study, in accordance with the results of other reports^[21,27,45]. Polyps were detected significantly in distal (left sided) colon, comparable with results from Asian and the Western countries^[46,47]. Nonetheless, because of the significance of adenomatous polyps and hyperplastic polyps present in proximal colon, the location of polyps is not helpful for distinguishing between neoplastic and non-neoplastic polyps. So, complete colonoscopy is recommended in screening guidelines for colon cancer^[43,48]. In addition, this study showed that only 56.6% of the polyps were found in the rectum and sigmoid region. Our study did not find any association between the age and location of polyps. This is in contrast with previous studies showing that the incidence of right sided polyps increased with increasing age^[44].

With regard to the size of polyps, we observed that the hyperplastic polyps and tubular adenoma were always smaller than 10 mm, while tubulo-villous and villous adenoma were always bigger than 10 mm. So,

removing the polyps which are larger than 10 mm is recommended^[36,49].

The study faced some limitations. First, this study was not population-based, therefore, the selection bias of the study population must be kept in mind. Second, our sample included mostly symptomatic patients, in which the estimates may be different from screening studies with asymptomatic individuals. Nevertheless, the results of this study can provide a good infrastructure for the next preventive program and have clinical implications for CRC screening.

In conclusion, PDR, ADR and CRC detection rate in this study were 23.5%, 12.8% and 1.5%, respectively. Most of the polyps and CRC were identified in patients aged 50 years or older. The majority of the polyps were adenomatous. More than 50% of the polyps were found in the rectosigmoid part of the colon. Finally, our study did not find any association between the family history and PDR.

COMMENTS

Background

Colorectal cancer (CRC) is the third most common malignant disease in both men and women worldwide, accounting for more than 8% of cancer-related death in the world with approximately 1.4 million new cases a year. Almost all CRCs develop from colorectal polyps. CRC largely can be prevented by the detection and removal of adenomatous polyps, and survival is significantly better when CRC is diagnosed while still localized. When CRC is found at an early stage before it has spread, the 5-year relative survival rate is about 90%. But only about 4 out of 10 CRCs are found at this early stage. When cancer has spread outside the colon or rectum, survival rates are lower.

Research frontiers

Early diagnosis and endoscopic resection of adenomatous polyps is the main approach for screening and prevention of CRC. This study aimed at identifying the prevalence, and clinical and pathologic characteristic of colonic polyps among Iranian patients undergoing a comprehensive colonoscopy and determine the polyp detection rate (PDR) and adenoma detection rate (ADR).

Innovations and breakthroughs

Only few studies are available in the national literature regarding the assessment of colorectal polyps, but none has explicitly noted the rate of polyp detection. Nevertheless, the study provides comprehensive information about clinical and epidemiological features of colorectal polyps.

Applications

Older age is the most important predictor for the prevalence of adenomas and cancer. Based on the results of this study, the PDR and cancer prevalence reached a peak in the 6th decade of life. Given the increased prevalence of CRC in the sixth decade of life, regular screening beginning at the age of 50 is the key to preventing CRC.

Terminology

The overall PDR was defined as the proportion of procedures in which at least one polyp was detected over the total number of colonoscopies. ADR was defined as the number of colonoscopies in which one or more adenomas was detected, divided by the total number of colonoscopies performed by the endoscopist.

Peer-review

Authors report in this paper the detection rates by colonoscopy for cancer and adenoma in an Iranian population.

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P- Reviewer: Rey JF, Sali L **S- Editor:** Ji FF **L- Editor:** Wang TQ
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World Journal of Gastrointestinal Pathophysiology
Volume 8 Number 2 May 15, 2017

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World Journal of Gastrointestinal Pathophysiology (*World J Gastrointest Pathophysiol*, *WJGP*, online ISSN 2150-5330, DOI: 10.4291), is a peer-reviewed open access academic journal that aims to guide clinical practice and improve diagnostic and therapeutic skills of clinicians.

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INDEXING/ABSTRACTING

World Journal of Gastrointestinal Pathophysiology is now indexed in PubMed, PubMed Central.

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NAME OF JOURNAL
World Journal of Gastrointestinal Pathophysiology

ISSN
ISSN 2150-5330 (online)

LAUNCH DATE
April 15, 2010

Frequency
Quarterly

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PUBLICATION DATE
May 15, 2017

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INSTRUCTIONS TO AUTHORS
<http://www.wjgnet.com/bpg/gerinfo/204>

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Nonalcoholic fatty liver disease: Diagnostic biomarkers

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Author contributions: Adibi P had the original idea; Hadizadeh F and Faghihimani E performed the literature review and drafted the manuscript; Adibi P performed critical revision of the manuscript.

Conflict-of-interest statement: The authors declare no conflict of interest.

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Manuscript source: Invited manuscript

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Received: June 27, 2016

Peer-review started: June 29, 2016

First decision: August 5, 2016

Revised: January 10, 2017

Accepted: February 8, 2017

Article in press: February 13, 2017

Published online: May 15, 2017

Abstract

Nonalcoholic fatty liver disease is a common medical condition worldwide and its prevalence has increased

notably in the past few years due to the increases in prevalence of obesity and metabolic syndrome. However, diagnosis of this disease is still a matter of debate because of disease variations and pathophysiologic alterations. Specific single markers have gained considerable attention recently, among them markers related to hepatic pathophysiology, inflammation, adipocytokines and so forth. But, it seems that no single marker is sufficient for diagnosis and staging of the disease, and applying a panel including different types of tests may be more useful.

Key words: Nonalcoholic fatty liver disease; Non-alcoholic steatohepatitis; Liver fibrosis; Cirrhosis

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Core tip: Nonalcoholic fatty liver disease is a pandemic disease in both developed and developing countries. There is emerging scientific evidence in this field that needs to be classified and summarized to make conceptual maps for researchers as well as practitioners. This article aimed to cover diagnostic markers in this disease, considering limitations and applications.

Hadizadeh F, Faghihimani E, Adibi P. Nonalcoholic fatty liver disease: Diagnostic biomarkers. *World J Gastrointest Pathophysiol* 2017; 8(2): 11-26 Available from: URL: <http://www.wjgnet.com/2150-5330/full/v8/i2/11.htm> DOI: <http://dx.doi.org/10.4291/wjgp.v8.i2.11>

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is one of the most common chronic hepatic diseases in many developed countries. It is also a serious health problem all over the world^[1]. This disease includes a broad range of hepatic disorders from simple fat accumulation in hepatic cells (simple steatosis) to hepatic tissue

inflammation and fibrosis (steatohepatitis) and finally cirrhosis and even hepatocellular carcinoma^[2-6]. The prevalence of this disease has considerably increased in the past years, and different studies have reported 2-fold increase in its prevalence within 10 years in many countries^[7-9].

Diagnosis of NAFLD is of special importance because of the odds of its progressing to more critical stages. Furthermore, in cases of diagnosis at early stages, it is possible to prevent disease aggravation by applying simple approaches such as increasing physical activity and diet modification. Even in more advanced stages of the disease, such as non-alcoholic steatohepatitis (NASH), it is still important to diagnose it as early as possible due to its potential for progressing to cirrhosis^[10]. In patients suspected of having cirrhosis, it is necessary to take other diagnostic measures into consideration, such as studying complications of portal hypertension (*e.g.*, screening for gastroesophageal varicose veins) and examining the risk of hepatocellular carcinoma development^[11-14]. So, it is important to be able to differentiate between simple steatosis and steatohepatitis as the beginning step of the progression towards severe and more progressive stages of the disease, such as fibrosis and cirrhosis^[15,16].

At present, liver biopsy is the gold standard for diagnosing nonalcoholic fatty liver, but this method is not only invasive and expensive but also has the important limitations of pain, reluctance of patients, risk of severe complications and being subject to sampling error. All these challenges have increased motivation for finding/applying noninvasive methods for diagnosis of the different stages of NAFLD^[17]. In this study, we provide a brief review of the individual main indices invented for the diagnosis and prediction of different stages of NAFLD.

HEPATIC MARKERS

More than 60 transamination reactions have been recognized in liver, among which only the transaminases of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are of clinical value. Both of these markers, but especially ALT, are commonly used in clinical medicine as indicators of hepatic damage^[18]. In many studies on patients suffering from NAFLD, increased levels of aminotransferases along with diabetes have been considered as independent predictors of moderate to severe fibrosis^[19] in patients with fatty liver who are at risk of progression to advanced fibrosis and only these two variables have been shown to have a significant association with steatohepatitis^[20].

ALT

In many people, the asymptomatic increase of transaminases level, particularly ALT, has been observed, and the prevalence of this type of case (asymptomatic increase of ALT) has been reported differently in different populations (7.3% in the United States), 15.24% in

Spain, and 21.7% in Scotland^[18,21], but it generally seems that it has a prevalence of 4% to 21.7%^[18]. Of note, almost one-third of the people who have shown increased level of serum transaminases in their first test have demonstrated normal level of enzyme in the second one^[18].

High waist circumference, high body mass index (BMI), male sex, alcohol consumption and young age are considered as strong predictors for rising ALT level^[21]. Moreover, ALT level has been shown to have a positive association with the number of signs and symptoms of metabolic syndrome present^[22]. In addition, serum ALT level has been shown to have a positive correlation with serum triglyceride, serum dehydroepiandrosterone sulfate (DHEAS), plasma fasting glucose and BMI, and a negative correlation with hemoglobin (Hb), serum high-density lipoprotein and age^[23].

In some studies, NASH has been reported as the most prevalent cause of constant increases in serum ALT in asymptomatic blood donors^[24] and it has been demonstrated that 32% of people who had ultrasound examination have been diagnosed with steatohepatitis accompanying asymptomatic increases of transaminases level^[18]. In another study, intense fibrosis was shown to be independently predictable by ALT and two other indices (serum ferritin and diabetes)^[25]. Other studies have also indicated a relationship between presence of steatohepatitis and increased level of ALT^[26], and even ALT has been considered as a part of some steatohepatitis diagnostic panels, like the BAAT score^[27], FIB4 index^[28], FibroTest^[29], FibroMeter^[30], NashTest^[29] and NFS^[30].

In contrast, some studies have demonstrated that some patients suffering from NAFLD have normal serum level of ALT. In a study on a United States' population (the Dallas Heart Study), 79% of the adults with fatty liver had normal aminotransferase level^[31], which was the same as the 55% of the Italian adults suffering from this disease in another study (the Dionysos Study)^[32]. In a study of 2287 individuals from different tribes, almost one-third of the population had hepatic steatosis, among whom 79% showed normal ALT level^[33]. In another study, normal serum transaminases level was reported in 46% of patients with steatohepatitis^[34]. Of interest, a research study into the risk of NAFLD development in cases of normal serum levels of ALT (*i.e.*, lower than 35 U/L) indicated that the rise in serum ALT concentration - even within the normal range - was an independent predictor for NAFLD development^[35]. In a study on diabetic patients, it was also shown that high-normal ALT could increase the risk of nonalcoholic fatty liver by 2.7-times^[36].

There is no consensus on cutoff point and maximum normal concentration of ALT, and a broad value range, between 26 and 66 IU/L, has been suggested in different studies^[37]. Some studies have shown that risk of NAFLD increases as ALT rises to more than 19 and 30 in women and men, respectively. Accordingly, these values have been suggested as maximum probable normal values in

these groups^[38]. In a community-based study of 1346 Japanese patients with diagnostic criteria of NAFLD, the cutoff value of ALT for diagnosis of this disease was estimated to be 25 U/L in men and 17 U/L in women^[39]. For the BAAT score, a normal range of ALT is considered between 1 and 19^[27]. In another study that considered 19 and 30 (for women and men respectively) as the cutoff values for screening NASH among NAFLD patients, 99% and 8% sensitivity and specificity respectively were shown, while 40 U/L - which is in use in medicine - had 86% sensitivity and 32% specificity^[26].

Thus, some researchers have rejected considering a reduction of ALT level (within reference range) as a tool for increasing its sensitivity because it leads to the adverse effect of labeling large numbers of asymptomatic individuals as patients^[40]. Nevertheless, while some researchers believe that none of these values has enough sensitivity and specificity to support introduction of ALT as a reliable screening test for diagnosing steatohepatitis among patients with fatty liver^[26,41]. Some other researches have suggested this enzyme as the best individual marker for detecting fat infiltration in the liver^[42].

Riquelme *et al*^[43] showed that, in a Hispanic population, ALT greater than 14 had an odds ratio (OR) equal to 13.95 for fatty liver. Moreover, although some studies have mentioned that this enzyme cannot be considered as an index for prediction/diagnosis of steatosis or fibrosis^[44], in a study conducted on patients with type 2 diabetes, ALT was the only variable independently related to steatohepatitis in these patients^[45]. In another study, ALT was found to be considerably higher in patients with steatohepatitis, even when within normal ranges^[45]. And, a study on obese patients showed that elevated ALT level had a significant correlation with incidence of steatohepatitis and fibrosis^[34,46]. In contrast, a study conducted on 73 untreated patients - to investigate the relationship between changes in hepatic enzymes and histological liver changes in NAFLD - indicated absence of a clear relationship between the pattern of ALT levels and changes in steatosis, inflammation, hepatocyte ballooning or degree of fibrosis over time. Results of this study also demonstrated the level of hepatic enzymes as an insensitive tool for following histological changes of liver among patients with NAFLD^[47]. Regarding more advance stages of the disease, Kaneda *et al*^[48] showed ALT level to be significantly different in patients with cirrhosis, compared to patients with earlier stages (50 IU/L vs 87 IU/L).

AST

AST is another hepatic transaminase that plays a role in diagnosis of steatohepatitis. Up to 3.6% of people in the United States have asymptomatic increase in AST^[21], and it seems that this hepatic index is related to metabolic syndrome and BMI^[49]. In Asian studies, AST is considered as an independent marker for severity of hepatic fibrosis if it is at least twice as much

as the maximum normal value^[50]. This marker has been used in different diagnostic panels, such as the FibroTest^[12,29,51], NashTest^[52], NAFLD Fibrosis Score (NFS)^[53,54], FibroMeter^[55], FIB4 index^[28] and other different models^[56,57]. In addition, two common tests of AST to ALT ratio and the AST to platelet ratio index (APRI) have been generated using this enzyme.

The AST to ALT ratio, which is frequently used in medicine, is known as an independent index for predicting presence of advanced hepatic fibrosis and has been used as a part of different panels, such as the NAFLD Fibrosis Score^[26], ScoreBARD^[58] and other panels^[28,41,57]. The APRI is another non-invasive index for studying hepatic fibrosis in patients with NAFLD and it has been reported as preferred over the AST to ALT ratio for predicting advanced hepatic fibrosis^[59].

Alkaline phosphatase

Higher level of alkaline phosphatase (ALKP) can also be considered as a marker relating to hepatic fibrosis in patients with steatohepatitis. In a study conducted on NASH patients to investigate the association between serum levels of ALKP with the degree of hepatic fibrosis, it was shown that serum level of this enzyme was significantly higher in patients suffering from NASH as compared to those without it^[60]. Another study represented ALKP to be significantly higher in NASH patients, even when within normal range^[45]. In addition, the increased level of ALKP has been reported as an independent predictor for hepatic diseases-related death^[61].

Albumin

In different studies albumin has been identified as a factor related to steatohepatitis, septal and intensive fibrosis^[27,48,62] and cirrhosis^[48] and as an independent predictor of hepatic-related mortality^[61,63]. In a study on 73 untreated NAFLD patients, a significant decrease was reported in serum albumin level of patients during 2-year follow-up (from 0.5 ± 4.3 at the beginning of the study to 0.4 ± 4.2 after 24 mo)^[47]. In another study, serum level of this protein showed OR of 0.049 (95%CI: 0.003-0.879) and of 0.057 (95%CI: 0.007-0.477) for prediction of steatohepatitis and severe fibrosis, respectively^[62].

In an investigation by Suzuki *et al*^[64], serum albumin was reported significantly lower in patients with intensive hepatic fibrosis (stage 3-4) compared to those in earlier stages (4.1 ± 0.09 vs 4.4 ± 0.05). In another study, serum level of albumin was reported as 4.4 g/dL (range: 3.5-5.4 g/dL) in patients with mild fibrosis and 4.2 g/dL (range: 4.6-2.6 g/dL) in patients with intensive fibrosis, and the difference was statistically significant^[48]. In the same study, the serum level of this protein showed a significant difference in patients with and without cirrhosis (4 g/dL vs 4.4 g/dL)^[48]. However, in a study by Sumida *et al*^[65], although concentration of serum albumin showed diminishment when the

intensity of NAFLD increased, this decreasing trend was not statistically significant (4.51 g/dL in simple steatosis, 4.37 g/dL in mild steatosis and 4.29 g/dL in advanced steatosis).

INFLAMMATORY MARKERS

Platelets

Thrombocytopenia is a common finding in advanced stages of all chronic hepatic diseases, and platelet count is clinically important to predict status of hepatic fibrosis in patients with chronic hepatic diseases^[66]. In NAFLD, platelets are important in terms of absolute number as well as volume. In different studies, reduction of platelets to fewer than 160000 has been considered as an independent marker for severity of hepatic fibrosis^[17,50].

In a study by Suzuki *et al*^[64], platelet count was reportedly significantly lower in patients with intensive hepatic fibrosis (stage 3-4), compared to patients in earlier stages ($180 \pm 17 \times 10^3/\text{mm}^3$ vs $247 \pm 10 \times 10^3/\text{mm}^3$). The result of this study has been confirmed by other studies^[48]. In a study in 2006, when the cutoff point of $16 \times 10^4/\mu\text{L}$ was used, platelet count was shown to have 100% sensitivity, 95% specificity, 76% positive predictive value (PPV) and 100% negative predictive value (NPV) for diagnosis of cirrhosis^[48]. In this study, platelet count was determined as an independent predictor among different markers and it was shown that this index significantly decreased in cases of cirrhosis (from $241 \times 10^3/\mu\text{L}$ to $130 \times 10^3/\mu\text{L}$)^[48]. In addition, in another study conducted on NAFLD patients from 9 hepatology centers in Japan, it was shown that platelet count linearly decreased with the increase of histological intensity of hepatic fibrosis. The cutoff value of 19.2×10^4 was introduced and shown to provide a sensitivity of 62.7% and a specificity of 76.3% for diagnosis of the 3rd stage of hepatic fibrosis, while 15.3×10^4 was selected as the optimal cutoff value for diagnosis of the last stage of this disease (sensitivity: 80.5%, specificity: 88.8%)^[67].

Mean platelet volume (MPV) is considered as an indicator of platelet function^[68]. In 2010, Ozhan *et al*^[69] showed that patients with NAFLD have higher MPV than individuals without it (10.43 ± 1.14 vs 9.09 ± 1.25). This study also showed that MPV had a positive correlation with AST and ALT levels, and a negative correlation with platelet count. These results have been confirmed in another study conducted on obese patients which showed a significant association between NASH and MPV, and which indicated an increase in the prevalence of NASH by increasing the values of MPV after adjustment for all the confounding variables^[70].

In contrast, another study with 60 NAFLD cases and 54 healthy controls, the MPV did not show any difference between the two groups. This study also concluded that in the absence of other MPV metabolic risk factors, MPV does not play a role in the mechanism of increasing the risk of cardiovascular diseases in patients with

fatty liver^[71]. Moreover, in another study in 2012, which was conducted to investigate the relationship between MPV index and NAFLD, although MPV was increased in patients with NAFLD, no association was detected between degree of steatosis, lobular hepatitis, hepatocellular ballooning, NAFLD activity score and fibrosis with the values of MPV. In this study, it did not show a correlation with increase of resistance to insulin^[68].

Considering the novelty of this hypothesis (significant association between MPV and NAFLD) and remembering the scarcity of the studies conducted to examine this hypothesis so far, it seems that there is still need for more studies to make a clear conclusion. Furthermore, considering that platelets usually decrease in stage 4, it can be probably concluded that a decrease in platelets usually indicates the occurrence of more advanced stages of fibrosis, which is usually equivalent to cirrhosis^[70]. Since platelet count is a simple, cost-effective and accurate method, it can be considered as a suitable biomarker for diagnosis of fibrosis severity in these patients^[72].

C-reactive protein

C-reactive protein (CRP) is an acute phase reactant, which has many applications in clinic^[73]. It seems that serum concentration of CRP is a strong index for predicting the incidence of NAFLD^[74] and some studies have introduced increased serum level of CRP as an independent risk factor for development of NAFLD^[74,75]. In addition to routine measurement of CRP, another method has been invented to measure values of high-sensitivity CRP (hs-CRP). This method promises to make detection of even low degrees of inflammation possible^[76]. In some studies, hs-CRP has been called a diagnostic tool, which not only can be effective in differentiation of steatohepatitis but also can specify the severity degree of hepatic fibrosis in patients with NASH and is probably able to differentiate between advanced and mild fibrosis among these patients^[65,76]. This ability has been mentioned to persist even after adjusting for the effect of different confounding variables, such of age, sex, diabetes, dyslipidemia, BMI, subcutaneous fat and intra-abdominal visceral fat^[76]. Another research study has shown that hs-CRP level was higher in patients with more severe grades of steatohepatitis (grades 2 and 3), rather than in patients with mild or simple grades of NASH^[77].

Although several studies have shown a relationship between NAFLD and serum concentration of hs-CRP, results of some studies are in contrast to this hypothesis^[74]. It was concluded in one study that hs-CRP could be an index for determining steatosis in obese patients, but there is no association between this marker and NASH^[78]. In parallel, a cohort study could not find any relationship between hs-CRP level and severity of hepatic steatosis. The result of this study indicated that hs-CRP is not helpful in predicting histological intensity of NAFLD^[73]. Of note, some investigations

have also demonstrated increased serum levels of hs-CRP in disorders related to NAFLD, such as obesity, insulin resistance and manifestations of metabolic syndrome^[73,78-82]; although, there is doubt about the presence of a causal relationship between this marker and metabolic syndrome^[79].

CRP is mostly produced in liver, but it seems that it is also produced in the adipose tissue^[80]. Furthermore, adipose tissue can act as an endocrine organ and secretes some inflammatory cytokines, like interleukin (IL)-6, leading to stimulation of the liver for CRP production^[83]. So, generally, it seems that obesity is one of the strongest determinants of serum level of CRP. Of interest, serum level of CRP is higher in women than in men^[83-85], and the results of one study have shown that an increase in serum level of hs-CRP is significantly associated with intensity of NAFLD, only in women^[74]. One explanation for this could be related to the sex difference in correlation between CRP and obesity, which in turn can be justified by the difference between the two sexes in terms of amount and pattern of adipose tissue distribution^[83].

One of the disadvantages of this index is the effect of different factors, such as race, age, sex, smoking or alcohol consumption, on its serum concentration^[74,86,87]. On the other hand, it seems that an accurate and accepted cutoff point has not yet been determined for this marker. Median CRP value of about 1 has been introduced for diagnosis of steatohepatitis, while the suggested cutoff value for hs-CRP for diagnosis of metabolic syndrome, NASH and prediction of the risk of cardiovascular complications is 0.65 mg/L^[88].

In sum, considering most studies conducted in this field, it seems that this index can be regarded as a promising biomarker for screening steatohepatitis in the future^[77].

Iron (ferritin)

Iron is considered as an element that reacts to oxygen radicals. High rates of blood ferritin and increased iron accumulation in liver have been reported in steatohepatitis, which can be attributed to systemic inflammation, increase in iron storage, or both^[89]. Ferritin level usually increases in 20%-50% and transferrin saturation increases in 5%-10% of NAFLD patients^[90]. One study showed that elevation of serum ferritin level by 1.5-times as much as the maximum normal rate was related to accumulation of iron in liver, diagnosis of steatohepatitis and worsening of histological activity of this disease. It has been introduced as an independent index for diagnosis of advanced hepatic fibrosis among patients with fatty liver, which can probably be applied as a useful index for identifying the patients who are susceptible to steatohepatitis and fibrosis^[89].

In the NAFLC scoring method, serum ferritin levels of equal or more than 200 ng/mL and 300 ng/mL in women and men, respectively, have been used as an independent variable for diagnosis of NASH^[91]. Of note, although the study by Sumida *et al.*^[91] showed an

increase in concentration of ferritin by increasing the severity of NAFLD, the difference was not statistically significant (179 ng/mL in simple steatosis, 241 ng/mL in mild steatohepatitis and 278.1 ng/mL in advanced steatohepatitis). On the other hand, some researchers believe that homocystein level in serum may be able to independently predict steatohepatitis and could be applied as another noninvasive marker for evaluating NAFLD. Homocystein level of serum in NAFLD is not different from that in healthy people, while it is considerably decreased in patients with steatohepatitis^[92].

Malondialdehyde

In the two-hit theory - one of the most acceptable theories to justify the progression of NAFLD to NASH - an oxidative stress, which may lead to lipid peroxidation, is considered as the most probable mechanism for the second hit. During the process of lipid peroxidation, a wide range of pre-inflammatory and fibrogen products are produced as by-products, which result in progression of the disease. One of these by-products is Malondialdehyde (MDA)^[93]. MDA can stimulate hepatic stellate cells and result in fibrosis by producing collagen^[94].

In a study from 2010, MDA with a cutoff point of 11 had sensitivity of 60%, specificity of 92%, PPV of 81%, NPV of 81% and positive likelihood ratio (+LR) of 7.9 for predicting the presence of NASH^[65]. Another study which investigated the relationship between MDA level as index for oxidative stress, antioxidant vitamins A and alpha-tocopherol with presence of steatohepatitis showed that level of vitamin A and MDA increased in simple steatosis and steatohepatitis and alpha-tocopherol level significantly decreased in patients with steatosis and steatohepatitis compared with healthy people. In this study, although there was significant difference between patients with steatosis and steatohepatitis, the authors concluded that none of these indices had relationship with histopathologic severity of the disease^[93].

Plasma pentraxin 3

Pentraxin is a family of proteins divided into two short and long classes, based on the length of their structure. CRP and serum amyloid P are two short parts of this family. Plasma pentraxin 3 (PTX3) is one of the long proteins of this family, and it is significantly higher in patients with steatohepatitis than those without. Serum concentration of this protein is higher in patients suffering from more advanced stages of NAFLD and higher values of this protein are correlated with higher stages of the hepatic fibrosis. So, it seems that serum PTX3 level could be used as a marker for diagnosis of the severity of hepatic fibrosis, in addition to differentiating between steatohepatitis and simple steatosis^[95].

Adipocytokines

Adipocytokines are a large family of proteins produced

and secreted by adipose tissue and which have close relationship with the inflammation process^[87]. Adipokines and cytokines play a major role in regulation and orchestration of inflammatory processes all over the body and play an important role in insulin resistance pathogenesis and NAFLD through complex and mutual paracrine and endocrine mechanisms. Some adipocytokines reduce insulin resistance, like adiponectin and leptin, while others lead to increased insulin resistance, like IL-6 and tumor necrosis factor- α (TNF- α)^[96].

Some studies have shown that the concentration of serum adipokines in human can be used as an index for diagnosis of NAFLD, especially in its advanced stages^[46]; however, other studies have not been able to find a significant association between adipokines and histopathological intensity of this disease^[97] and it has been concluded that adipokines cannot differentiate between benign and advanced histological stages of NAFLD^[46]. It seems that there is a complex relationship between adipocytokines and pathogenesis of NAFLD^[98], and the balance between proinflammatory and anti-inflammatory function of adipocytokines may play an important role in the development of this disease^[99].

Adiponectin

Adiponectin is a collagen-like protein derived from adipocytes with anti-inflammatory and anti-lipogenic effects^[100]. This adipokine, which is circulating abundantly in human serum, protects against excessive accumulation of fat in the liver and subsequently protects liver against inflammation and fibrosis. Serum level of adiponectin decreases in obese people and shows greater decrease in patients with steatosis and steatohepatitis^[7,46,101], being higher in patients with lower degrees of steatosis^[102]. Hypoadiponectinemia has been demonstrated to be a predictive factor for necroinflammation and more severe grades of fibrosis, even after exclusion of the effect of variables such as age, BMI and waist circumference^[103]. It is among the variables which have a direct relationship with steatohepatitis as compared to simple steatosis^[98].

A study designed to measure liver fat, intraabdominal fat and subcutaneous fat, along with insulin resistance indices and adiponectin, showed that serum adiponectin had a reverse relationship with hepatic fat content^[9]. Hypoadiponectinemia is accompanied by increased risk of cardiovascular diseases and it seems to be a key factor in metabolic syndrome^[102], although some studies have shown that hypoadiponectinemia in steatohepatitis is independent from insulin resistance^[73,100]. It seems that hypoadiponectinemia is a primary finding in steatohepatitis, which is identifiable from a very long time before the appearance of diabetes or emergence of central obesity and which has a correlation with histological intensity of hepatic damage.

The serum level of adiponectin was shown to be significantly lower in patients with steatohepatitis than in

a control group (344 ± 5.476 vs 836 ± 11.548), while no difference was detected between the two groups in terms of other cytokines^[103]. In another study, the serum level of adiponectin was found to be significantly lower in patients with primary stages of steatohepatitis, compared to patients with simple steatosis (3.6 mug/mL vs 6.0 mug/mL). In this study, adiponectin showed higher differentiation power than serum level of type IV collagen 7s and the homeostasis model assessment of insulin resistance (HOMA-IR) and it had sensitivity of 68% and specificity of 79% for predicting primary stages of steatohepatitis^[104].

Considering the association between single nucleotide polymorphisms (SNPs) of the adiponectin gene and insulin resistance and increase of prevalence of type 2 diabetes, a study was conducted to determine the association between variations in this gene and NAFLD development, which indicated a positive association, especially with hepatic fibrosis^[105]. Another study showed a considerable decrease in the mRNA expression of adiponectin and RII receptors (adipoRII) in the liver of NASH patients as compared to patients with simple steatosis, which might be indicative for its pathophysiological relationship with NAFLD^[106].

Adiponectin is a powerful anti-inflammatory adipocytokine for neutralizing TNF- α ^[99]. Lack of a relationship between circulating levels of adiponectin and its hepatic expression, shown in the study by Kaser *et al.*^[106] was interpreted such that hepatic expression of adiponectin was probably regulated by different factors, such as TNF- α . Owing to the importance of adiponectin as a diagnostic marker, it has been used in different noninvasive panels for diagnosis of NAFLD^[104,107].

Leptin

Leptin is a 16 kD non-glycosylated protein which is usually secreted from adipocytes of white adipose tissue^[102,108]. Also, low amounts of leptin has been shown to be secreted by other tissues, such as placenta, skeletal muscles, stomach fundus and culture-activated hepatic stellate cells^[108]. Leptin plays the role of a peptide hormone, regulating food uptake and energy consumption of the body through central feedback mechanisms and relating eating to hypothalamus and adipose tissue mass (*i.e.*, it controls food uptake and increases energy consumption)^[102,108,109]. The importance of this regulatory role is such that recombinant leptin has been studied for treatment of all prevalent types of obesity in different clinical trials^[110-113].

Some researchers believe that serum leptin level is associated with NAFLD. It has been shown that increased serum leptin level in patients with steatohepatitis has no relation with BMI of these patients, and this increase could not be easily justified by the patient's sex, obesity or type 2 diabetes^[109,114]. In contrast, another study indicated a correlation between human serum level of leptin and body fat percent and BMI^[115]. A study which was conducted on young adults (18-year-old to 21-year-

old students) to determine the risk factors of NAFLD in this age group showed serum level of leptin to be associated with abdominal wall fat index (AFI) (as the only independent risk factor of fatty liver in this study) in women, while it had no correlation with AFI in men^[116].

Some studies have indicated a role for leptin in resistance of liver against insulin^[115], and some others have connected leptin to atherosclerosis and cardiovascular diseases in obese patients^[102]. However, a study designed to investigate the potential association between leptin with insulin resistance and histological changes in NAFLD patients was unsuccessful to show any association between serum level of leptin with fasting insulin level and severity of hepatic histological changes^[117]. Another study conducted in 2003 showed increase in the serum level of leptin as one of the presentations of patients with steatosis and normal serum level of transaminases. In this study, a negative correlation was detected between serum leptin and serum transaminase levels, as well as progression of hepatocytes damage^[118]. Another study which followed patients for 6 mo showed considerable decrease in the level of serum transaminase only in NASH patients with increased levels of serum leptin. This study concluded that leptin has a preventive effect on progression of hepatic damage in NAFLD^[117].

Different studies have been conducted so far on factors relating to leptin and its role in the pathogenesis of NAFLD^[119]. Some studies have shown that serum leptin level is directly associated with severity of hepatic steatosis and that increased levels of serum leptin may lead to increase of hepatic steatosis and steatohepatitis, but no association with emergence of inflammation and fibrosis was found^[109,114]. Some other studies, however, have failed to show a significant association between serum level of leptin and steatosis^[120] or steatohepatitis^[46]. Still other studies have concluded that leptin could be used for diagnosis of hepatic fibrosis, but not for staging of this disease^[121].

TNF- α

TNF- α plays roles in the progression of NAFLD through different mechanisms. TNF- α has pro-inflammatory effects and activates harmful pathogenic routes by decreasing HDL-cholesterol, increasing expression of cholesterologenic genes and suppressing cholesterol exclusion^[122]. It also stimulates synthesis of hepatic fatty acids, increases serum level of triglyceride^[123] and decreases sensitivity to insulin^[100,124]. In addition, TNF- α can also induce apoptosis in and proliferation of hepatic cells and play a role in pathogenesis of hepatic fibrosis^[124]. The importance of TNF- α in emergence of fatty liver disease (with genetic or nutritional origin) has been demonstrated in different studies, and it has been shown that neutralization of TNF- α activity leads to improvement of insulin resistance and fatty liver disease^[111].

Different studies have demonstrated that plasma level

of TNF- α is higher in patients with NAFLD and NASH, compared to control groups^[73,98,125-128], significantly or at least borderline significantly ($P = 0.052$)^[129]. In a study by Jarrar *et al.*^[98], TNF- α was introduced as the only independent predictor of fibrosis in patients with steatohepatitis. In another study conducted in 2005 to examine TNF- α as a potential noninvasive marker for studying histopathological intensity of NASH, serum level of TNF- α was higher in patients with steatohepatitis and cirrhosis than in the control group, but it did not show a significant association with histopathological severity of the disease^[126]. Another confirmatory study by Hui *et al.*^[100] showed that TNF- α level was higher in patients with fatty liver and steatohepatitis as compared to healthy individuals, but it was not efficient for differentiating steatohepatitis from fatty liver.

A study in 2010 conducted on NAFLD children, showed that serum concentration of TNF- α and its soluble receptors were significantly higher in obese children with fatty liver than those in the control group. But, again, the abilities of this cytokine and its receptors were insufficient in differentiating between different grades of steatosis. In this study, this marker was introduced as a suitable serum marker for predicting hepatic steatosis in obese children^[127]. However, another study conducted in 2005 did not find a significant difference between TNF- α level of patients with steatohepatitis as compared to the control group^[103].

Of note, TNF- α usually increases in visceral obesity and NAFLD, and it has been shown that this cytokine plays a role in regulation of the body's iron homeostasis. A study in 2008 which investigated these associations showed that the highest serum concentration of this cytokine was found in NAFLD patients with iron overload. It has also been shown that phlebotomy treatment can reduce concentration of TNF- α in addition to level of ferritin and transferrin saturation and can lead to improvements in hepatic function tests. This study concluded that TNF- α plays a regulatory role for iron that leads to accumulation of iron in the liver of patients with NAFLD^[125].

IL-6

IL-6 is a multifunctional cytokine which regulates immune responses, the acute phase reaction and homeostasis^[124] and is secreted from different cells in body, one of which is the adipocytes^[83,100]. This cytokine causes stimulation of liver and some acute phase proteins, like PTX3 and CRP, are then produced in liver in response to it^[129,130]. Serum level of IL-6 has considerable correlation with insulin-resistance, like TNF- α , which leads to increased resistance to insulin^[99,100]. Also, IL-6 stimulates hepatic lipogenesis related to obesity and insulin-resistance^[125]. It seems that this cytokine plays a critical role in pathophysiology of different aspects of NAFLD in humans^[99].

Zamora-Valdés *et al.*^[131] showed in 2007 that chronic alternating hypoxia resulting from obstructive

sleep apnea, as one of the risk factors for NAFLD, led to increased serum level of IL-6. IL-6 level has also been shown to be significantly different between NAFLD patients and non-afflicted people in some studies^[98]. Another study indicated a considerable increase in the serum level of TNF and IL-6 as well as of their soluble receptors in steatohepatitis, compared to simple steatosis, but no correlation was found between circulating levels of these cytokines and their receptors with either the degree of disease activity or fibrosis stage^[132]. However, another study did not find any increase in the hepatic mRNA transcription and expression of IL-6 in patients with steatohepatitis as compared to patients with simple steatosis^[76].

STRUCTURAL MARKERS

Cytokeratin-18

Different studies have indicated the key role of apoptosis in hepatic damage occurring in advanced stages of NAFLD (steatohepatitis) and, subsequently, use of hepatocyte apoptosis markers was suggested for diagnosis and staging of this disease^[133-135]. During the apoptosis process, cytokeratin-18 (CK-18) is fragmented by caspase 3^[135] and hepatocytes which leads to cell death through the apoptosis process and release fragments of CK-18 into the blood stream, so that the blood level of the fragmented CK-18 is associated with the presence of hepatic fibrosis^[133]. Indeed, this substance has been shown to be significantly higher in patients with NASH^[120].

Plasma CK-18 fragments have been studied and evaluated in large studies^[72,136,137]. In one study, for diagnosis of NASH, blood level of more than 395 U/L of cytokeratin had sensitivity, specificity, PPV and NPV values of 85.7, 99.9, 99.9 and 85.7, respectively^[134]. Younossi *et al.*^[107] reported sensitivity, specificity, PPV and NPV of cleaved CK-18 as 63.64, 87.23, 70 and 83.7, respectively, for the cutoff value of 174.1; however, when the cutoff point was increased to 261.35, specificity and PPV increased to 97.87 and 88.9, and when the cutoff decreased to 111.6, sensitivity and NPV changed to 81.82 and 77.8, respectively. In another study, sensitivity and specificity of this index were reported to be 78% and 87%, respectively, for diagnosis of steatohepatitis^[138].

In one study conducted to investigate the clinical utility of different serum markers such as CK-18, hyaluronic acid and tissue inhibitor of metalloproteinase 1 (TIMP1) for the diagnosis of steatohepatitis, CK-18 was the only biomarker which could be helpful, having PPV of 81% and NPV of 85%^[139]. In addition to the cleaved form of CK-18, its intact form, and also the difference between these two types (cleaved type and intact type), may play a role in evaluation of steatohepatitis. It has been reported that the serum level of intact CK-18 has higher predictive value than its cleaved form for the diagnosis of this disease. In the same study, intact CK-18, considering a cutoff point of

384.3, had sensitivity, specificity, PPV and NPV of 63.64, 89.36, 73.7 and 84.0, respectively; however, when the cutoff was increased to 545, its specificity and PPV increased to 95.74 and 80, respectively, and when the cutoff was decreased to 242.9, its sensitivity and NPV changed to 86.36 and 91.2, respectively^[107].

Considering cleaved CK-18 as an apoptosis marker, intact CK-18 as a total apoptosis marker and cleaved CK-18-intact CK-18 as a necrosis marker^[56], it seems logical that patients with steatohepatitis have higher levels of all of these 3 markers. Furthermore, it has been suggested that tissue polypeptide-specific antigen may be a serologic mirror for CK-18, so that it may also be also considered as an index for diagnosis of NASH (sensitivity, specificity, PPV and NPV of 95, 95.8, 22 and 0.09, respectively, with the cutoff value of 88 ng/mL)^[135].

In sum, considering the validation of use of plasma CK-18 fragment in different studies, it can be claimed that all pieces of evidence have favored the usefulness of this index to differentiate between steatohepatitis and simple steatosis^[140,141]. Therefore, some researchers believe that this biomarker can be applied as an ideal index for noninvasive diagnosis of steatohepatitis in the future, although it is not routinely accessible as a common laboratory test presently^[72].

Hyaluronic acid

Hyaluronic acid is a component of extracellular matrix that is produced by mesenchymal cells^[48]. Its serum level in hepatic diseases is affected by production of collagen in liver and reduction of endothelial function of hepatic sinusoids^[64]. It has been shown that serum level of hyaluronic acid is significantly higher in patients with steatohepatitis, compared to healthy controls, and that it is also higher in cirrhotic patients than in NASH patients^[127].

Another study conducted to investigate the possibility of using hyaluronic acid for diagnosis of steatohepatitis reported an association between this marker and stage of NAFLD^[140]. Yet another study has reported that cutoff value of greater or equal to 43 has sensitivity, specificity, PPV and NPV of 65.7%, 90.5%, 92% and 61.3%, respectively, for diagnosis of NASH^[142]. Moreover, hyaluronic acid has been introduced in different studies as a good and reliable index for diagnosis of hepatic fibrosis^[121,143,144]. A study which was conducted to investigate accuracy of serum hyaluronic acid in prediction of hepatic fibrosis intensity in NAFLD patients showed that this marker had a positive correlation with fibrosis degree. In this study, the cutoff point of 46.1 mg/L for serum hyaluronic acid demonstrated sensitivity, specificity, PPV and NPV of 85%, 80%, 51% and 96%, respectively, for diagnosis of fibrosis, and it was concluded that measurement of serum hyaluronic acid could be a useful tool for diagnosis of patients with severe fibrosis. Besides, the logarithm of hyaluronic acid in NAFLD patients was shown to be associated with the degree of hepatic fibrosis, age and serum albumin, and it was calculated as follows: $[40.1 + 0.333 \times (\text{degrees of hepatic fibrosis}) + 0.032 \times (\text{age})$

- $0.561 \times (\text{serum albumin})$].

Based on this model, an increase of fibrosis by one degree is accompanied by an increase of serum level of hyaluronic acid by 40%^[64]. In another study, hyaluronic acid was demonstrated to have NPV of 100% for the diagnosis of patients without fibrosis, when considering a cutoff value of 42 ng/mL. Sensitivity, specificity and PPV of this marker were calculated as 100%, 89% and 77%, respectively^[48]. Also, another investigation in this field has suggested hyaluronic acid and type IV collagen 7s as two independent predictors of severe fibrosis, with both capable of reflecting the degree and severity of hepatic fibrosis. In this study, the mean serum level of hyaluronic acid in patients with mild fibrosis was reported as 22 ng/mL, while it was 118 ng/mL in patients with severe fibrosis, and there was statistically significance^[48].

In another study by Palekar *et al.*^[57], hyaluronic acid of more than 45.3 mcg/L was introduced as a good index for predicting advanced hepatic fibrosis and was regarded as the strongest independent predictor of severe fibrosis among some variables such as age, sex, AST, BMI and AST/ALT ratio^[57,145]. In addition, because hyaluronic acid increases in stages 3 and 4 of hepatic fibrosis, compared to markers like platelet which usually only decreases in stage 4, hyaluronic acid can be applied for prediction of fibrosis in earlier stages^[72]. Owing to its importance, it has been used in different diagnostic panels^[13,143,146].

In contrast, in a study investigating the clinical application of this serum marker in diagnosis of steatohepatitis, hyaluronic acid was not approved to be suitable for diagnosis of this disease^[140]. In another study, this index did not show a correlation with degree of fibrosis, compared to age, TNF- α and serum concentration of type IV collagen, which had a weak but significant association; although, it could probably be used to differentiate between mild (degrees 1 and 2) and advanced fibrosis^[147].

In sum, it seems that serum level of hyaluronic acid is independently and significantly related to the presence of steatohepatitis and severe fibrosis^[143], and it probably can be used as a suitable marker for monitoring the progression of fibrosis toward cirrhosis in NAFLD patients^[126].

Collagen 7s

Collagen 7s is another diagnostic marker that has been evaluated in different studies. Sakugawa *et al.*^[143] conducted research on the utility of the fibrosis marker of type VI collagen 7s for the diagnosis of steatohepatitis and reported an association between this index and the stage of fatty liver. To our knowledge, this is the only study conducted on type VI collagen 7s that has been referred to in different publications^[141,148]. This marker has positive predictive value of 86% and 68.4%, negative predictive value of 61.8% and 83.6%, sensitivity of 70% and 81.3% and specificity of 81%

and 71.4%, respectively, for diagnosing steatohepatitis and severe fibrosis when the cutoff point of ≥ 5 ng/mL is considered.

Multiple studies have focused on type IV collagen 7s^[76,98,112,149]. A strong and stable association between type IV collagen 7s and advanced fibrosis (before progress to cirrhosis) has been shown in different studies of patients with steatohepatitis^[94]. A study by Yoneda *et al.*^[94] in 2007 showed that liver stiffness values had a suitable relationship with stage of hepatic fibrosis in patients with NAFLD and liver stiffness had a correlation with serum level of type IV collagen 7s. Type IV collagen 7s has been applied in different panels, such as NAFIC^[98,104].

It seems that this marker is independently and significantly related to the presence of steatohepatitis and severe fibrosis and can be useful for differentiating between mild and severe forms of NAFLD^[76,143].

OTHER MARKERS

Dehydroepiandrosterone

Dehydroepiandrosterone (DHEA) is one of the adrenal hormones with anti-oxidative stress effects that reduces resistance to insulin and is effective in mediating the expression of peroxisome proliferator-activated receptor alpha and mRNA of pro-collagen^[149,150]. This hormone represent the most abundant steroid hormone in the body, and its interchangeable sulfated form (DHEA-S) has different functions, and influences on obesity, diabetes, atherosclerosis and osteoporosis^[1,23,94].

Some studies have shown that body concentration of DHEA-S is independently and reversely correlated with death (resulting from all causes)^[1]. It has also been shown that serum levels of DHEA-S have positive correlation with Hb, platelet, ALT, cholinesterase, albumin and triglyceride, and have negative correlation with age, AST, AST/ALT ratio, ALP, HDL-cholesterol, hyaluronic acid and type IV collagen 7s^[65,149,150]. Thus, some researchers believe that DHEA may play a role in the pathophysiology of NAFLD and also in its progression toward more advanced stages of the disease *via* different mechanisms^[23].

A study on menopausal women by Saruç *et al.*^[151] showed that women suffering from NAFLD had higher levels of DHEA and DHEAS than the control group (menopausal women with normal histology). In this study, DHEA and DHEAS showed positive correlation with BMI and waist circumference. A cross-sectional study conducted in 2010 on 1912 men demonstrated that steatohepatitis was associated with higher levels of serum DHEA-S, and the highest risk of NASH development was reported for those who had the highest serum level of DHEA-S (OR = 1.59, 95%CI: 1.04-2.43)^[152]. Subsequently, a study conducted to investigate the clinical significance of serum level of DHEA-S in NAFLD patients showed that the serum level of this hormone was significantly higher in patients than in controls^[23].

The study by Sumida *et al.*^[65] reported the same results, although the difference did not reach statistical significance (128.7 µg/dL in NAFLD patients vs 113.6 µg/dL in control group). However, this study demonstrated that patients suffering from severe stages of NAFLD (steatohepatitis with fibrosis of stage 3 or 4) had lower serum level of DHEA-S than patients with mild stages of the disease (simple steatosis or steatohepatitis with fibrosis of stage 0-2)^[65].

In parallel, some other studies have also shown that NASH with advanced fibrosis is strongly associated with low concentration of circulating DHEA-S^[149,153]. This association persisted even after adjustment for variables such as age, sex and resistance to insulin^[65]. By applying single-variable analysis of serum level of DHEA in another study, a significant difference was detected between mild fibrosis and advanced stages of steatohepatitis (95 ± 9 µg/dL vs 72 ± 28 µg/dL); however, this marker was unable to differentiate between patients and non-patients^[153]. These results confirmed the results of another study conducted 4 years earlier which had shown that the mean DHEA-S level in patients with degree 2-4 of steatohepatitis was significantly lower than in those suffering from milder stages (0-1) and that the mean of DHEA-S level decreased stage by stage with increase in the fibrosis stages. In this study, a DHEA-S value of more than 1.0 µg/mL showed sensitivity of 95% and specificity of 58% for diagnosis of advanced stages of NAFLD^[149]. In another study, sensitivity, specificity, PPV and NPV were 76.5%, 73.3%, 29.5% and 95.5%, respectively, considering a cutoff point of equal or less than 66 mg/dL^[65]. In the same direction, Sumida *et al.*^[11] in another article suggested that patients with DHEA-S serum level of more than 66 µg/dL are unlikely to suffer from advanced stages of NAFLD.

It can be generally concluded that increased level of serum DHEA-S may be a part of the pathophysiology of NAFLD and could play a role in the development of this disease^[23]. However, considering metabolic and intracellular effects reported for this hormone, such as its protective effect against oxidative damage in animal models which is applied by reducing concentration of MDA and increasing activity of superoxide dismutase in hepatocytes and total concentration of glutathione, it may have an important role in prevention of histological progress of NAFLD; its blood level variations may also be well correlated with different histological patterns of patients with similar metabolic profile, age and sex^[149]. Considering these findings, it is not surprising if serum level of DHEA developed into a successful predictor for diagnosis and staging of hepatic fibrosis in NAFLD patients in the future.

Fibrinogen-like protein 2

Fibrinogen-like protein 2 (FGL2) is a new member of the fibrinogen-like protein family. A study investigating this protein in patients with NAFLD showed that plasma

level of this protein was considerably higher in patients with steatohepatitis, but there was no difference between patients with simple steatosis and the control group. Results of this study indicated a potential role for FGL2 level in the diagnosis of severe forms of fatty liver diseases and differentiated between simple steatosis and steatohepatitis^[154].

CONCLUSION

Although some of the reported markers such as adiponectin, hyaluronic acid, CK-18 and DHEA have shown promising results, at present, it does not seem that any of these individual markers are reliable enough to be considered as a marker for diagnosis and staging of NAFLD. An ideal biomarker should have specific characteristics such as simplicity, accessibility, accuracy, repeatability and cost-effectiveness, some of which are not applicable to any of these markers^[72,155]. Also, a reliable marker should be able to provide physicians with clear information, have high diagnostic power for differentiation between different stages of the disease, and should have been validated in large prospective trials^[156,157]. It should be noted, as well, that selection of a suitable test depends on the clinical demand of the healthcare provider.

If ruling out of the diagnosis of NAFLD and/or its staging is the goal, applying the markers with higher sensitivity and NPV is recommended; however, if the main aim is diagnosis of the disease in suspected patients, the test's specificity and PPV would be the most important factors that should be taken into consideration. Furthermore, determining a suitable cutoff point for a test is usually based on a tradeoff between sensitivity (true positive) and specificity (true negative) results of the test. In fact, the optimal cutoff point has the highest sensitivity and specificity, but this point can hardly be obtained for a marker. Therefore, based on different cutoff values, the diagnostic power of a test and its productivity will be different. Paying attention to different cutoff values suggested for each marker while comparing them is important.

Considering the weaknesses of every individual marker for diagnosis and staging of the disease, it seems that the present trend would be toward combining some of these serum markers together, and even adding some other parameters, such as demographic variables or radiologic results, and developing a mathematical model which has higher diagnostic power and accuracy^[72,158].

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P- Reviewer: Chan CH, Satapathy SK, Sugawara I **S- Editor:** Ji FF
L- Editor: A **E- Editor:** Wu HL



Celiac disease: From pathophysiology to treatment

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Conflict-of-interest statement: The authors declare that they have no competing interests.

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Manuscript source: Invited manuscript

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Received: April 24, 2016

Peer-review started: April 25, 2016

First decision: June 6, 2016

Revised: March 8, 2017

Accepted: March 23, 2017

Article in press: March 24, 2017

Published online: May 15, 2017

Abstract

Celiac disease, also known as "celiac sprue", is a chronic inflammatory disorder of the small intestine, produced by the ingestion of dietary gluten products in susceptible people. It is a multifactorial disease, including genetic and environmental factors. Environmental trigger is represented by gluten while the genetic predisposition has been identified in the major histocompatibility complex region. Celiac disease is not a rare disorder like previously thought, with a global prevalence around 1%. The reason of its under-recognition is mainly referable to the fact that about half of affected people do not have the classic gastrointestinal symptoms, but they present nonspecific manifestations of nutritional deficiency or have no symptoms at all. Here we review the most recent data concerning epidemiology, pathogenesis, clinical presentation, available diagnostic tests and therapeutic management of celiac disease.

Key words: Celiac disease; Epidemiology; Diagnosis; Treatment; Pathogenesis

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Core tip: Celiac disease is a chronic inflammatory disorder of the small intestine, produced by the ingestion of dietary gluten products in susceptible people. It is a multifactorial disease, including genetic and environmental factors. Thanks to advanced understanding of its pathogenesis, numerous therapeutic strategies have been devised for the treatment of celiac

disease. But there is need of further basic research studies and randomized clinical trials to introduce them into usual management of this disease.

Parzanese I, Qehajaj D, Patrinicola F, Aralica M, Chiriva-Internati M, Stifter S, Elli L, Grizzi F. Celiac disease: From pathophysiology to treatment. *World J Gastrointest Pathophysiol* 2017; 8(2): 27-38 Available from: URL: <http://www.wjgnet.com/2150-5330/full/v8/i2/27.htm> DOI: <http://dx.doi.org/10.4291/wjgp.v8.i2.27>

INTRODUCTION

Celiac disease defined an autoimmune disorder originating by an aberrant adaptive immune response against gluten-containing grains in susceptible individuals. Celiac disease was first described in 1888 by Samuel Gee, but only in 1953 it became clear the importance of the gluten in the origin of this pathology^[1-3]. In celiac subjects the ingestion of gluten leads to an enteropathy with an impairment of the mucosal surface and, consequently, abnormal absorption of nutrients^[4-7]. Celiac disease might be considered a syndrome, because of the wide spectrum of clinical manifestations and the involvement of various human systems. Celiac disease shows peculiar features in comparison to others autoimmune disorders, including the complete recovery of the mucosal damage as well as the reversibility of its progression and chronic dynamics, with a total avoidance of gluten. Conversely, it is now ascertained that undiagnosed celiac disease, might have severe consequences in children as well as in adult subjects^[8-10]. Besides celiac disease and wheat allergy, a new entity has been included, apparently not driven by an immune response: The non-celiac gluten sensitivity (NCGS). The pathogenesis of NCGS remains largely unknown, although it is now ascertained that it includes a set of factors^[3,11]. Here, we review the epidemiology, pathogenesis, clinical presentation, diagnostic tests and therapeutic management of celiac disease.

EPIDEMIOLOGY

In the last decades a high number of epidemiological data have been reported. Nowadays celiac disease is one of the most frequent genetically based disorder in humans, although it was thought that some country, including United States, were exempt from this disease^[12]. Europe is historically considered a geographical area at high frequency, with a prevalence of 1%-2%, although it has been recently shown a similar prevalence in United States^[13,14]. Despite the advances in diagnosis, the overall prevalence of this disease remains still unclear. A variable frequency has been reported between European countries, although it is still uncertain whether it depends on the different screening tool, sample size or a real variability of celiac disease prevalence^[15]. What

is known is that many cases remains undiagnosed, as idealized with the "iceberg model" (Figure 1). Typical cases of celiac disease are diagnosed because of suggestive symptoms. The submerged part of the iceberg represents all the undiagnosed cases that usually show atypical, minimal, or even absent symptoms^[16]. A multicenter study reported a prevalence of 1 out of 133 (0.75%) in healthy people in the United States, and similar frequency is confirmed by studies on European and Australian populations^[12]. The overall prevalence of celiac disease ranges from 4.5% among high-risk subjects to 0.75% in not-at-risk subjects^[12]. High-risk subjects include the relatives of patients with celiac disease, children or adults with celiac disease-associated symptoms (*i.e.*, diarrhea, abdominal pain and constipation) and children or adult subjects with celiac disease-associated disorders (*i.e.*, Diabetes Mellitus type-1, Down syndrome, anemia, infertility, osteoporosis)^[17]. It has been shown that celiac disease is not exclusive of industrialized countries, but includes North Africa, Middle East and India with an incidence overlapping those of European countries^[18-20]. However, given the worldwide distribution of the causal factors this heterogeneous diffusion is not surprising. It has been shown that the Saharawi, an Algerian population has the highest prevalence of celiac disease (nearly, 6%) among all of the worldwide populations^[16,21,22].

GENETIC SUSCEPTIBILITY

The best-characterized genetic risk factor for celiac disease, accounting for 35% of the total genetic risk, is the presence of genes encoding for MHC class II proteins including human leukocyte antigen (HLA) DQ 2 and HLA-DQ8^[23-25]. Over 90% of affected subjects express HLA-DQ2 molecules; the remainder express HLA-DQ8. The frequency of celiac disease risk HLA genotypes is about 30%, whereas only 1%-3% develops the disease^[26]. It is now accepted that HLA is one of the main but not sufficient factors involved in the onset of celiac disease, but a multitude of genetic factors are responsible in celiac disease susceptibility, as demonstrated by studies on monozygotic twins^[27].

Recently, genome-wide association studies have identified 39 non-HLA loci that also predispose to celiac disease^[28]. One of these genes may relate to genetic variants on chromosome 19, in the myosin IXB gene (*i.e.*, MYO9B), and may potentially predict responsiveness to a gluten-free diet (GFD)^[29,30]. Both HLA-DQ2 and HLA-DQ8 codified for heterodimers located on Antigen-Presenting Cells (APCs)^[31]. It has been ascertained that they present gluten peptides to antigen-specific T-lymphocytes in the intestinal mucosa, inducing their proliferation as well as cytokine production. In particular, tTG2 may transform non-charged glutamine into negatively charged glutamic acid^[32].

Environmental factors

Feeding patterns in the first year of life and potential

viral infections (*i.e.*, rotavirus)^[33] might be involved in the development of celiac disease^[34]. A prospective study has investigated the role of a specific infectious agent in celiac disease and the authors have found that an increased frequency of rotavirus infection predicts increased risk of celiac disease autoimmunity in children^[35]. Gluten, one of the most common ingredients in human nutrition^[36], is mainly composed by prolamines and glutenin. The prolamines in wheat are gliadins, in rye secalines and in barley hordeins. Catassi *et al.*^[37] proposed that 50 mg gluten/day is the minimum amount able to determine evident alterations to the small-intestinal mucosa in celiac disease subjects. Others pivotal “environmental factors” as the milk-feeding type and the duration of breast-feeding, can also play a role, influencing the intestinal microenvironment^[38]. In addition, increased intestinal Gram-negative and reduced Bifidobacteria, has been found in celiac disease subjects^[39]. Other debated environmental factors could be represented by heavy metals^[40] and bacterial TG present in food stuff^[41].

IMMUNE SYSTEM AND THE CELIAC DISEASE

Celiac disease resembles a systemic immune-mediated disorder^[34,42-44]. The primary mechanism involved in celiac disease is related to an inappropriate adaptive immune response to gluten-derived peptides. It has been ascertained that prolamines contain critical epitopes presented by either HLA-DQ2 or HLA-DQ8 induce a CD4⁺ T-lymphocytes response. In celiac disease pathogenesis the role exerted by the intestinal epithelia barrier, physiologically impermeable to macromolecules such as gliadin is actually recognized. In people with a genetic susceptibility to develop celiac disease, gliadin interacts with the intestinal cells to trigger the disassembling of the inter-enterocyte tight junctions (TJs). The impairment of the TJs determines the up-regulation of zonulin, a peptide involved in TJ regulation and responsible for the increased gut permeability. Gliadin peptides pass through the epithelial barrier and activate T-lymphocytes located in the lamina propria. Activated CD4⁺ T-lymphocytes produce high levels of pro-inflammatory cytokines, inducing either a T-helper 1 pattern dominated by IFN- γ , and a T-helper 2 pattern, which causes a clonal expansion of B-lymphocytes that subsequently differentiate in plasma-cells secreting anti-gliadin and anti-tissue-transglutaminase antibodies^[45]. Some gliadin peptides that are not recognized by T-lymphocytes activate both APCs and intestinal epithelial cells; in particular, CD8⁺ T-lymphocytes may be stimulated by interleukin (IL)-15. An increased density of CD8⁺ intraepithelial cells is considered as a hallmark of celiac disease^[34]. Gliadin-specific T-cell responses have been found to be enhanced by the action of tissue transglutaminase, an enzyme located in the extracellular space of the sub-

epithelial region or at the epithelial brush border^[46].

HISTOLOGICAL FEATURES OF THE CELIAC DISEASE

Physiologically the height of enterocytes ranges between 29-34 μ m. T-cells are usually located in the *lamina propria*; however, the number of intraepithelial lymphocytes (IELs) is highly variable. A large number of subjects without disease have less than 20 lymphocytes per 100 epithelial cells; based on the experiences of Hayat *et al.*^[47] and Veress *et al.*^[48], a density of IELs between 25 and 29/100 epithelial cells is considered borderline and pathological over 30/100 epithelial cells. Tissue samples taken from subjects affected by celiac disease mainly show: (1) Decreased enterocyte height; (2) Crypt hyperplasia; (3) Villous atrophy; (4) Increased intraepithelial T lymphocytes.

Although still debated, recently, Mubarak *et al.*^[49] have shown that staining for CD3 has an additional value in the histological detection of celiac disease lesions^[49,50]. Immunohistochemical stains for CD3 and CD8 do not improve detection of gluten-sensitive enteropathy in duodenal biopsies. A different grading has been proposed for the evaluation of the gluten free diet in duodenal mucosal healing^[51].

Table 1 reports Marsh classification of histologic findings in celiac disease^[52]. Modifications to this scoring system have been proposed^[53,54]. Oberhuber *et al.*^[55] suggested that Marsh III lesions should be included into *a*, *b*, and *c* categories. However, Figure 2 shows two prototypical tissues classified as Marsh II (A) and Marsh IIIA (B).

CELIAC DISEASE: CLINICAL PRESENTATION

Celiac disease is greatly heterogeneous, at least in part depending on the patient's age, the duration and extent of disease, and the presence of extra-intestinal comorbidities.

Although celiac disease was originally thought as a pediatric disorder, the diagnosis is increasingly made in adults^[16]. Various subtypes of celiac disease have been described^[56].

Classical or typical form. It is characterized by common clinical symptoms related to abnormal intestinal absorption. Generally occurs between 6 and 18 mo of age, after the introduction of weaning foods containing prolamines^[57]. Histology shows villous atrophy and crypt hyperplasia.

Atypical form

It is characterized by a prevalence of extra-intestinal symptoms with few or no gastrointestinal symptoms. Usually, atypical forms are encountered in older children and adults and the common features of abnormal

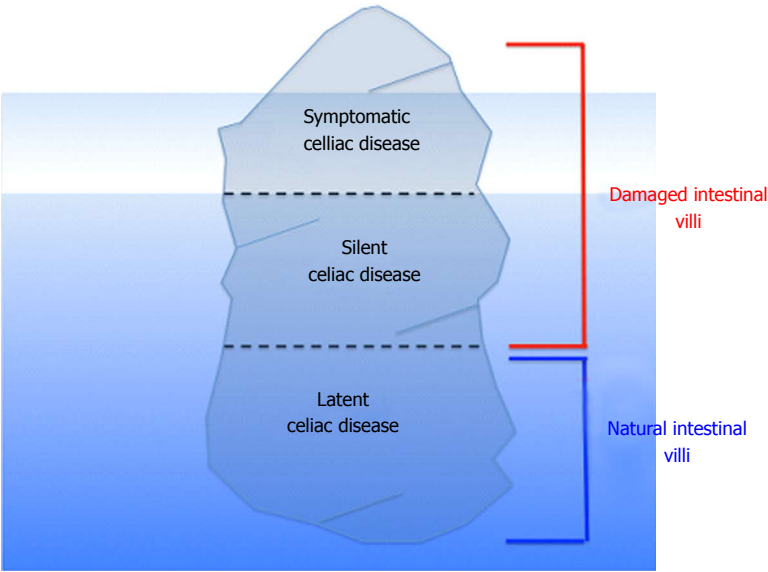


Figure 1 The “iceberg model” idealizing the interplay between celiac disease genetic makeup and exposure to gluten, the environmental trigger of the disease.

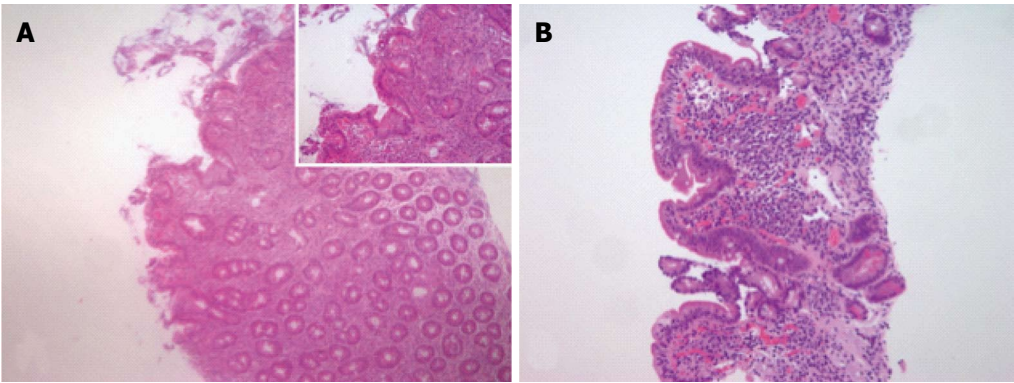


Figure 2 Histological features of celiac disease. A: Example of tissue scored as Marsh 2 characterized by lymphocytic enteritis with crypt hyperplasia: Intraepithelial lymphocytosis and elongation and branching of crypts in which there is an increased proliferation of epithelial cells; B: Example of tissue scored as Marsh 3A characterized by partial villous atrophy, the villi are blunt and shortened. Arbitrarily, samples are classified as partial villous atrophy if the villus-crypt ratio was less than 1:1 (Objective magnification $\times 4$, inset $\times 10$).

Table 1 Marsh classification of histologic findings in celiac disease	
Marsh 0	Normal mucosal architecture without significant intraepithelial lymphocytic infiltration.
Marsh I	Lymphocytic enteritis: Normal mucosal architecture with a marked infiltration of villous epithelium by lymphocytes; arbitrarily defined marked as more than 30 lymphocytes per 100 enterocytes
Marsh II	Lymphocytic enteritis with crypt hyperplasia: intraepithelial lymphocytosis and elongation and branching of crypts in which there is an increased proliferation of epithelial cells
Marsh III	Intraepithelial lymphocytosis, crypt hyperplasia, and villous atrophy. There are 3 distinct stages of villous atrophy
Marsh IIIA	Partial villous atrophy, the villi are blunt and shortened. Arbitrarily, samples are classified as partial villous atrophy if the villus-crypt ratio was less than 1:1
Marsh IIIB	Subtotal villous atrophy, villi are clearly atrophic, but still recognizable
Marsh IIIC	Total villous atrophy, villi are rudimentary or absent, and the mucosa resemble colonic mucosa.

Modifications to this scoring system have been proposed^[53,54]. Oberhuber *et al*^[55] suggested that Marsh III lesions should be included into a, b, and c categories. However, Marsh *et al*^[52] examined these subdivisions by means of correlative light and scanning electron microscopy, and demonstrate that Oberhuber’s classification is untenable. In their view, this categorization reflects misinterpretations of the real architectural contours of flat mucosae.

absorption are absent.

Silent or asymptomatic form

It is characterized by serological and histological

abnormalities without evidence of clinical symptoms. This subtype is often observed in subjects with a family history of celiac disease, patients with associated autoimmune (*i.e.*, type 1 diabetes) or genetic disorders

(i.e., Down, Turner, or Williams syndrome).

Latent form

It is characteristic of subjects with previous asymptomatic celiac disease, although a gluten-containing diet. Positive serology but no villous atrophy or others tissue abnormalities are recognized. Troncone *et al.*^[58] postulated that the presence of elevated endomysial antibodies in these patients might be one of the most important predictor of progression.

Potential form

The term "potential" is used in individuals who have never had diagnosis of celiac disease, but show presence of appropriate genetic background (HLA-DQ2/DQ8), positive serology, with normal or mildly abnormal histology.

Refractory form

It is defined by the presence of malabsorptive symptoms and villous atrophy that persist 1 year after a strict gluten-free diet. Several refractory patients (roughly, 5%-30%) never respond to a gluten-free diet^[59], others initially responded but have a recurrence of symptoms and intestinal damage. Two different subtypes of refractory celiac disease have been recognized: "Type 1", showing a normal intraepithelial lymphocytes count and "type 2" presenting aberrant intraepithelial lymphocytes^[29].

Celiac disease can affect individuals from any age, but two peaks can be seen: In the childhood (< 6-year-old) and 4th-5th decade^[46]. Classical presentation is more frequent in pediatric and tends to occur early in life (6-24 mo), whereas atypical presentation generally occurs at a later age (> 5-year-old) and in adults^[60].

DERMATOLOGICAL CELIAC DISEASE

Dermatitis herpetiformis is an inflammatory cutaneous disease, presenting with diffuse, symmetrical, polymorphic lesions consisting of erythema, urticarial plaques, papules, herpetiform vesiculae and blisters followed by erosions, excoriations and hyperpigmentation. It is characterized by typical histopathological and immunopathological findings. Rarely it is diagnosed in childhood but commonly appears in the third decade.

MAIN CONDITIONS ASSOCIATED TO CELIAC DISEASE

A set of conditions can be associated with celiac disease. The term "associated conditions" refers to states that are found more frequently in patients affected by celiac disease^[61]. These conditions include "genetic disorders" such as Down syndrome, Turner syndrome and Williams syndrome, and "autoimmune" or "neurological" disorders.

Type 1-diabetes

One of the most recognized and widely investigated disorders associated with celiac disease is type 1-diabetes^[62]. Ludvigsson *et al.*^[63] reported that type 1-diabetes constitutes a 5- to 10-fold risk increase for celiac disease in a very large cohort of children. This increasing of risk may partly be explained by shared genetic risk represented by HLA. A percentage approximately of 5%-10% of patients affected by type 1-diabetes presented celiac disease related antibodies with up to 75% having abnormalities on small intestinal biopsy tissue^[64]. The prevalence of celiac disease ranges between 1% to 19% in patients with type 1 diabetes mellitus^[65]. Prospective studies of high-risk infants for type 1 diabetes and celiac disease have been shown that early introduction of gluten is associated with an increased risk for autoimmunity^[66-68]. However, the relationship between the two conditions is still debated^[69].

Autoimmune thyroid disorders

In patients affected by celiac disease it has been reported an increased prevalence (nearly, 2%-5%) of thyroid disorders (i.e., hyperthyroidism-Graves's disease or hypothyroidism-Hashimoto's thyroiditis), diagnosed either before than after the diagnosis of gluten-enteropathy^[65]. These two conditions share genetic risk factors represented by HLA-DQ2 and DQ8. HLA-DQ2 and DQ8 haplotypes have been association with Hashimoto's thyroiditis, while HLA-DQ2 association is less clear in Graves' disease. This difference between hyper- and hypothyroidism is reflected also by the greater risk of celiac disease in patients with Hashimoto's disorder than patients with Graves^[70]. In addition to HLA, it has been reported an association with the gene encoding cytotoxic T-lymphocyte-associated antigen-4^[65]. Another mechanism related to the association between these two conditions is represented by abnormal absorption with consequent selenium deficiency induced by celiac disease. Stazi *et al.*^[70] highlighted that the abnormal selenium absorption in celiac disease could be the factor directly leading to thyroid and intestinal damage, since thyroid is particularly sensitive to selenium deficiency. In the setting of autoimmune thyroid disease it should be useful to pay attention to celiac disease marker and to monitor growth and pubertal status. In patients with celiac disease, a screening for thyroid abnormalities has been also suggested in some cases^[61].

Autoimmune hepatitis and other forms of liver involvement

The involvement of liver is common among patients affected by celiac disease^[71]. Hypertransaminasemia has been reported in about 40% of adults and in 54% of children with a classical presentation of celiac disease at the time of diagnosis^[72]. Conversely, celiac disease is present in about 9% of patients with chronic unexplained hypertransaminasemia^[73,74]. It has been

postulated that the mechanism leading to hepatic damage is related to entry of toxins, inflammatory molecules and antigens in the portal circulation. Volta *et al*^[75] pointed up about the need of a serological screening for celiac disease in all patients with persistent hypertransaminasemia of unknown cause. Moreover, exclusion of a gluten-related liver damage is necessary for all patients affected by autoimmune liver disorders or by those forms of severe liver disease, whose etiology remains unknown, and for patients enrolled for liver transplant. A prolonged exposition to gluten in a patient with an overlooked celiac disease, in fact, can causes chronic hepatitis and liver cirrhosis^[75,76].

Neurological disorders

It has been reported a potential link between celiac disease and different neurological disorders^[77]. Data concerning the association between neurological conditions and celiac disease remain poor. Although ataxia is a neurological disorder indicated in some patients with celiac disease^[78], the most frequent neurologic condition in celiac disease subjects is epilepsy, showing a prevalence between 1.2% and 5%^[79]. Its clinical spectrum in association with celiac disease varies from focal to generalize with variable outcome and response to gluten avoidance. In 1985 it has been described a more specific and rare syndrome characterized by the co-presence of celiac disease, epilepsy, and occipital calcifications (CEC). Gobbi suggested that the HLA genotype predisposing to CEC is the same to that predispose to celiac disease^[79].

COMPLICATIONS ASSOCIATED WITH UNTREATED CELIAC DISEASE

Evidences that celiac disease in adults, especially if diagnosed late is burdened by complications have been reported. Among them: (1) Osteoporosis: It represents the most common complication resulting from abnormal calcium absorption secondary to defective calcium transport by the diseased small intestine, but also due to vitamin D deficiency. With an early gluten free diet in children it is possible to prevent the bone disease in adult life; (2) Enteropathy-associated intestinal T cells lymphoma: It represents one of the most important complication; (3) Collagenous sprue: Patients do not react to diet and histology shows extra-cellular matrix components in the intestinal wall at the level of the superficial sub-epithelial layer. This morphological pattern is very similar to the condition of collagenous colitis described in the colon, where the thickness of the connective band best highlighted with Masson's trichrome is more than 15 nanometers, although this is a very rare event described in the literature; (4) Refractory sprue: This condition is depicted as collagenous sprue although can be identified by immunohistochemical staining, demonstrating that T lymphocytes, which in normal conditions express CD3 and CD8, in this case

present only the expression of CD3 and not of CD8; (5) Ulcerative jejunoileitis: Extensive ulceration of the intestinal mucosa, and involving ileum and jejunum. It presents around 50 years old with chronic diarrhea, steatorrhoea and complications of intestinal ulceration (perforation, haemorrhage or obstruction)^[59]; (6) Non-Hodgkin lymphoma: Recent data suggests an association between celiac disease and Non-Hodgkin lymphoma^[80]; (7) Small bowel adenocarcinoma: Even rare, a connection between carcinoma of the small bowel and celiac disease is known since 1958^[81]. The etiologic factors predisposing to malignancy in celiac disease are uncertain. Possibilities include immunologic disturbance associated with mucosal lymphocyte infiltration, pre-malignant changes in the damaged surface epithelial cells, increased permeability to oncogenic factors and abnormal absorption of protective substances such as vitamins A and E; and (8) Reproductive disorders: Celiac disease might be associated with decreased fertility in both males and females^[82].

DIAGNOSTIC TESTS OF CELIAC DISEASE

Serological tests

Anti-tissue transglutaminase antibodies: the best strategy for serological diagnosis is the blood detection of IgA anti-tissue transglutaminase antibodies (tTGA) by enzyme-linked immunosorbent assay (ELISA). These antibodies show a sensitivity up to 97%, a specificity around 96%, and an accuracy of 98%, whereas IgA anti-endomysial (IgA EMA) antibodies are employed as a confirmatory test in tTGA positive cases due to their higher specificity (about 100% vs 91% of tTGA). In concomitance of IgA deficiency and celiac disease, found in around 2%-10% of the patients, it is recommended to detect celiac disease testing tTG-IgG. "False negative" occurs, as previously reported, in case of IgA deficiency. The IgA EMA represents the most specific test (approximately 100%), with a sensitivity around 94% and a diagnostic accuracy of 97%^[83]. However, EMA are routinely detected by indirect subjective immunofluorescence^[84]. These antibodies can also result falsely negative in case of IgA deficiency and in children aged > 2 years.

Anti-gliadin antibodies: The antigliadin (AGA) antibodies (IgG and IgA) are today no longer recommended because of their low sensitivity and specificity and inferior accuracy, except in younger children^[85].

Deamidated gliadin peptides: Actually detection of antigliadin antibodies have been replaced by the more recently developed immunoassays employing antibodies to deamidated gliadin peptides, IgA and IgG. To increase the diagnostic accuracy, in the last years the clinicians tend to prescribe serial testing.

Histology

The gold standard to diagnose celiac disease in adulthood

is the intestinal biopsy sampled by endoscopy^[54,86]. Histology of celiac disease consists of an integrated assessment of different entities: villous atrophy, crypt hyperplasia, decreased enterocyte height, inflammatory infiltrates in small-bowel mucosal biopsies. Based on one or more of these elementary lesions the histopathology of celiac disease is subdivided into different diagnostic categories according to the Marsh classification^[86] (Table 1).

The open question remains the early lesions, *i.e.*, normal villi with a pathologic increase in intraepithelial T-lymphocytes, that it is possible to find in many others conditions^[87]. Villanacci *et al.*^[86] pointed out that three conditions deserve specific mention: (1) "Autoimmune enteritis"; (2) Damage by drugs: especially non-steroidal anti-inflammatory drugs (NSAID) that cause morphological modifications likely to those of celiac disease; and (3) The co-infection with *Helicobacter pylori* in the stomach^[86].

Recently, have been introduced methods to quantify pathologic features of the small intestinal mucosa in celiac patients^[88].

Genetic analyses

HLA testing should not be routinely performed in all celiac disease cases, but it is indicated only when diagnosis is controversial. Large multicenter studies have shown that only 0.4% of celiac disease patients are both DQ2 and DQ8 negative^[89]. In the absence of HLA-DQ2/8, it is consented to rule out predisposition to celiac disease in family members of celiac patients^[86]. HLA test represents a helpful tool particularly for potential celiac disease, to suggest (if positive) or reject (if negative) the diagnosis. Furthermore, HLA negativity in patients with villous atrophy and negative serology should direct towards other possible causes of these histological alterations. Many disorders, such as lactose intolerance, bacterial overgrowth, Crohn's disease, infectious diseases (*i.e.*, *Giardia lamblia*, *Cryptosporidium*, *Microsporidium*, Cytomegalovirus, Herpes virus, Whipple's disease), characterized by mal-absorption share the same histological findings as celiac disease.

In vitro gluten challenge test

Currently, several studies have proposed that gluten-sensitive immunological activation in celiac disease can be reproduced by *in vitro* gluten challenge test using culture cells from the duodenal mucosa^[90].

CURRENT TREATMENTS OF CELIAC DISEASE

Life-long gluten-free diet

The current available treatment for celiac disease is life-long gluten-free diet^[91-93]. Generally clinical improvement is achieved within a few weeks and the mucosal damage recovers in 1-2 years^[16]. As patients with celiac disease

may have accompanying brush border lactase deficiency secondary to damage to surface epithelial cells, milk and dairy products should be avoided in the first period of treatment too. As vitamin B deficiency is common after an extended period on a GFD, all patients are advised to take a gluten-free multivitamin^[84]. Early diagnosis and treatment are fundamental in pediatric celiac disease particularly, as some of the complications may be irreversible: Growth retardation, abnormal dentition, osteoporosis^[29]. Several observational studies suggest that prolonging breast-feeding and delaying and gradually introducing gluten in the first year of life may reduce the risk of celiac disease development during childhood^[60]. As described above, even products specifically targeted to dietary treatment of celiac disease may contain tiny amounts of gluten proteins^[16]. Another cause of uncertainty is represented by the differences in the labeling rules for food products existing among countries. Specific considerations about gluten free diet and refractory celiac disease should be taken. Even with respect of dietary restriction, actually, in the minority of patients affected by refractory celiac disease (RCD), GFD is ineffective. It has showed a higher mortality compared with RCD type 1, explainable by the more severe malnutrition combined with the higher risk of developing overt lymphoma^[94]. These forms of the disease can require corticosteroids and other immunosuppressant, like azathioprine or cyclosporin, which can improve transiently clinical symptoms in most patients. But till date, it has not yet been possible to design effective treatments for the both form of RCD^[94]. Furthermore, it is important remember that these drugs may enhance the risk of progression into an overt T-cell lymphoma, so they require caution particularly in RCD type 2 patients, at risk of developing this complication. Recently, have been used with some success chemotherapy agents such as the anti-T cell nucleoside analogues Cladribine and Pentostatine, and it has been proposed also as a therapeutic option the stem cell transplantation^[95,96].

Other drug-based therapies are in under investigations although patients indicate GFD as a good and well-tolerated therapy^[97,98].

Gluten-degrading enzymes

Enzyme supplement therapy with bacterial prolyl-endopeptidasis expressed by various microorganism has been proposed to accelerate gluten digestion in the gastrointestinal tract and thus to destroy T cell epitopes^[99]. Prolyl-endopeptidasis are proline-specific enzymes capable to cleave gluten peptides. Actually, there are introduced into clinical trials two drug candidates, ALV003 and AN-PEP (*Aspergillus niger* prolyl-endoprotease). Recent data on results of two phase 1 clinical trials have revealed that pre-treating gluten with ALV003 eliminates the peripheral blood T cell response in celiac disease patients, suggesting the potential therapeutic utility of gluten-specific enzymes to treat celiac disease^[100]. Currently is undergoing clinical phase IIa testing showing a significantly reduce gluten-

related T-cell responses compared with placebo but without a significantly reduction of symptoms typically induced by the gluten^[101].

AN-PEP is an enzyme that degrades gluten peptides efficiently in a pH compatible with that found in the stomach. Therefore this enzyme might be suitable for oral supplementation but further studies are necessary^[102].

Modified grains

Can be developed either through selective breeding of early wheat species or using small interfering RNA (siRNA) technology to mutate or silence immunostimulatory sequence^[103].

Blocking gluten entry across the intestinal epithelium

Zonulin inhibitor larazotide (AT-1001) corrects intestinal barrier defects. It has been explored in an animal model^[99]. AT-1001 is currently the best-studied pharmacologic agent to treat patients with celiac disease, actually undergone in phase II clinical trials^[104]. It has been shown that patients treated with AT-1001 had an improved symptom score, a less pronounced autoantibody response and pro-inflammatory production, and lower urinary nitrate excretion when compared with the placebo controls^[105].

Rho/Rho kinase inhibition

It has been clarified that the increase in intestinal permeability is dependent on Rho kinase (ROCK) activity^[106]. In addition to regulating tight junction structure and function, ROCK is known to regulate axon growth^[107,108]. The drug could be used to establish whether ROCK inhibition can reverse gluten-dependent increase in intestinal permeability in these patients^[104].

Immunotherapy

The first observation of the occurrence of celiac disease following allogeneic bone marrow transplantation performed in a patient with acute leukemia, made evident the involvement of T-lymphocyte in pathogenesis of this condition^[109]. Since then, a lot of acknowledgments have been acquired such that many efforts are being made to develop immunologic therapeutic tools. Cytokine therapies based either on amplification of regulatory cytokines or on blockage of inflammatory cytokines expression are largely diffused for management of the severe autoimmune disorders. Also for celiac disease resistant to dietary approach and especially for refractory celiac disease, the use of immunomodulators is developing. IL-15 blocking antibodies have shown the capability to induce intra-epithelial lymphocytes apoptosis in the intestinal epithelium of human IL-15 transgenic mouse models^[110]. These antibodies have been only investigated on patients with rheumatoid arthritis and a human study for celiac disease is still awaited. Only human recombinant IL-10 was tried and it showed suppression of gluten-dependent T-cell activation

in celiac disease cultured intestinal mucosa^[111]. But later, a pilot study conducted on patients affected by refractory celiac disease did not show any pharmacological efficacy of this monoclonal antibody^[110]. Anti-IFN- γ antibodies demonstrated a good tolerance among patients with inflammatory bowel disease, but the use in patients with celiac disease needs of more investigations. Equal considerations can be assumed also for the use of antibodies anti-TNF- α , commonly recognized for the treatment of inflammatory bowel disease but described only in few case reports of patients with refractory celiac disease^[112,113]. Another possible target of immunotherapy is represented by chemokines and their receptors, which play a significant role in the pathogenesis of celiac disease through the recruitment of lymphocytes in the gut. T-lymphocytes that home to the small intestine express CCR9, which binds to the CCL25 secreted by intestinal epithelial cells, and integrin $\alpha_4\beta_7$, which binds to mucosal vascular addressin cell 1 (MAdCAM-1). Therefore both molecules are necessary for migration of T-cells to the intestinal mucosa and could represent potential therapeutic targets. Furthermore, a monoclonal antibody anti-integrin α_4 (Natalizumab) has revealed effectiveness in Crohn's disease suggesting a possible effectiveness also in celiac disease^[114] as well as a CCR9 inhibitor (CCX282-B) and integrin $\alpha_4\beta_7$ blocking antibody (LDP-02), both already studied in patients affected by Crohn's disease but still under investigation for celiac disease^[115]. Some concern could be raised about the benefit of blocking lymphocyte homing to the intestine, and potential long-term adverse consequences, also because beneficial immunosuppressive regulatory T cells are equally inhibited. Since its demonstration, the possibility to develop tolerance to gluten in certain patients affected by celiac disease has gained more attractiveness as another potential immunomodulatory approach^[116,117]. An alternative method studied for induction of tolerance to gluten is oral administration of a genetically modified *Lactococcus lactis* bacterium, capable of secreting deamidated DQ8-restricted gliadin epitope. This experiment was conducted on previously sensitized transgenic mice and demonstrated the induction of Foxp3⁺ regulatory T-lymphocytes and a significant suppression of local and systemic T-cell responses to the corresponding gliadin peptide^[104,118]. Also this approach still needs of human trials, although others clinical trials for non-specific immunomodulation have been carried out to test the role of parasitic infection in inhibition of immune response to gluten^[119]. In fact, the disappearance of intestinal parasites from humans in developed countries has created a predisposition to develop autoimmune disease, a phase I clinical trial using larvae from the hookworm *Necator Americanus* has been initiated with the purpose of establishing a potential shift of T-cells response toward a suppressive T-regulatory response, however to date, any significant effect was revealed by this infection on gluten-induced enteropathy^[120].

Vaccines

Clinical trials have been started with a prototypical vaccine based on a set of gluten peptides that are recognized by HLA-DQ2 in an immunodominant manner^[104], and phase I has been recently completed. A great interest is focused on vaccination, especially for raising the compliance of many patients which could benefit with a single-dose administration rather than a daily intake of other treatment options^[110]. The risk of immune system activation related to the vaccine therapy and its side effects represent a still open question that need of further investigations. There is still need of more studies also for the most part of the previously mentioned alternative treatment options, with large long-term clinical trials that could answer to the unresolved questions relating to their real clinical effectiveness, their safety, and their affordability.

CONCLUSION

It is indubitable that celiac disease remains a still controversial and complex human disorder. There is need of further research studies and randomized clinical trials to introduce them into usual management of this disease. A number of unanswered questions remain to clarify including the real associations of celiac disease with other conditions, and the impact of some environmental factor in its pathogenesis. There are still controversial opinion about the effective role of the breast feeding in celiac disease, what is to be elucidated if it could offer a permanent protection or if only delays the appearance of the disease. In addition, an improvement in non-invasive diagnostic tests could allow avoidance of endoscopy, especially in pediatric patients, and a more defined efficacy of the new therapeutic tools could improve quality of life either in term of reduction of complications and physical health either in term of social life.

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P- Reviewer: Krupa-Kozak U, Vasudevan A **S- Editor:** Song XX

L- Editor: A **E- Editor:** Wu HL



Embryonary way to create a fatty liver in portal hypertension

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Author contributions: Aller MA, Arias N and Arias J wrote the manuscript; Peral I, García-Higarza S and Arias JL contributed to the manuscript by providing intellectual input, and have participated in preparing the manuscript and approved its final version.

Supported by FICYT FC-15-GRUPIN 14-088 and Alfonso Martín Escudero Foundation.

Conflict-of-interest statement: The authors declare that there are any conflict of interest.

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Manuscript source: Invited manuscript

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Received: December 5, 2016

Peer-review started: December 7, 2016

First decision: January 16, 2017

Revised: February 3, 2017

Accepted: February 28, 2017

Article in press: March 2, 2017

Published online: May 15, 2017

Abstract

Portal hypertension in the rat by triple partial portal vein ligation produces an array of splanchnic and systemic disorders, including hepatic steatosis. In the current review these alterations are considered components of a systemic inflammatory response that would develop through three overlapping phenotypes: The neurogenic, the immune and the endocrine. These three inflammatory phenotypes could resemble the functions expressed during embryonic development of mammals. In turn, the inflammatory phenotypes would be represented in the embryo by two functional axes, that is, a coelomic-amniotic axis and a trophoblastic yolk-sac or vitelline axis. In this sense, the inflammatory response developed after triple partial portal vein ligation in the rat would integrate both functional embryonic axes on the liver interstitial space of Disse. If so, this fact would favor the successive development of steatosis, steatohepatitis and fibrosis. Firstly, these recapitulated embryonic functions would produce the evolution of liver steatosis. In this way, this fat liver could represent a yolk-sac-like in portal hypertensive rats. After that, the systemic recapitulation of these embryonic functions in experimental prehepatic portal hypertension would consequently induce a gastrulation-like response in which a hepatic wound healing reaction or fibrosis occur. In conclusion, studying the mechanisms involved in embryonic development could provide key results for a better understanding of the nonalcoholic fatty liver disease etiopathogeny.

Key words: Inflammation; Non-alcoholic fatty liver disease; Hepatic steatosis; Extraembryonic functions; Fibrosis; Portal hypertension

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Core tip: The current hypothesis proposes that the re-expression of two embryonic systemic functional axes in the rat after partial portal vein ligation produces a non-alcoholic fatty liver disease. These axes, a coelomicamniotic axis and a trophoblastic yolk-sac or vitelline axis, would then integrate in the interstitial liver space of Disse. If so, these recapitulated embryonic functions would produce firstly, the evolution of liver steatosis. In this way, this fat liver could represent a yolk-sac-like in portal hypertensive rats. After that, these embryonic functions would induce a gastrulation-like response in which liver fibrosis occurs. For that reason, studying the mechanisms involved in embryonic development could provide key results for a better understanding of the non-alcoholic fatty liver disease pathophysiology.

Aller MA, Arias N, Peral I, García-Higarza S, Arias JL, Arias J. Embryonic way to create a fatty liver in portal hypertension. *World J Gastrointest Pathophysiol* 2017; 8(2): 39-50 Available from: URL: <http://www.wjgnet.com/2150-5330/full/v8/i2/39.htm> DOI: <http://dx.doi.org/10.4291/wjgp.v8.i2.39>

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a pathological condition derived from a wide spectrum of liver damage^[1-3]. NAFLD covers from nonalcoholic fatty liver (NAFL) to nonalcoholic steatohepatitis (NASH). While NAFL is characterized by hepatic steatosis without hepatocellular injury such as ballooning of the hepatocytes, NASH condition shows hepatic steatosis, inflammatory state accompanied with hepatocyte injury and ballooning, with or without fibrosis^[4]. Although NAFLD is strongly associated with obesity, diabetes mellitus and dyslipidemia, its pathogenesis remains poorly understood and therapeutic options are limited^[3,4].

The concept that this range of related hepatic disorders is an evolutive inflammatory condition could simplify the integration of the different etiopathogenic mechanisms involved in each one of its evolutive phases. Even, NAFLD has been already considered like the hepatic manifestation of a systemic auto-inflammatory disorder^[5]. The earliest inflammatory phase of NAFLD consists in hepatic steatosis, which is characterized by the deposition of triglycerides inside of hepatocytes^[3]. This early evolutive period of the disease is usually clinically silent, which is why its clinical diagnosis is delayed and why it is difficult to identify the risk contribution of the splanchnic and systemic etiopathogenic factors^[2].

Using experimental liver steatosis models can be very useful to prevent this gap in etiopathogenic knowledge during the early phase of this disease^[6,7]. Therefore, the partial portal vein ligation of the rat can be considered one of the experimental surgical models

of hepatic steatosis^[8]. We have shown an increase in triglycerides, diacylglycerol and cholesterol in the liver of these animals, together with a microvesicular hepatocytic fatty infiltration. Moreover, the presence of megamitochondria at short (1 mo) and long-term (1 year)^[8-10] has been found. Liver steatosis was described in rats with portal hypertension by Izzet *et al.*^[11] in 2005, a pathological association suggested in humans 35 years ago^[12]. Moreover, the liver parenchyma in portal hypertensive rats, due to the hepatocytic fatty infiltration, is progressively converted into fat^[8,10] (Figure 1).

Portal hypertension is a type of vascular pathology due to the pressure of mechanical energy on splanchnic venous circulation^[13]. In mammals there are five cellular pathways able to translate mechanical forces into biological programs, such as integrin-matrix interactions, cytoskeletal strain responses, stretch ion channels, cell traction forces and G-protein-coupled receptors^[14,15]. Therefore, mechanical energy may act after TPVL in the rat on the vascular splanchnic endothelium as a stressor stimulus triggered by mechanotransduction^[16].

Tissue injury caused by this process can produce a systemic inflammatory response in the body. This response is developed by three successive and overlapping phenotypes: The neurogenic, the immune and the endocrine^[17,18]. Similar overlapping could also be expressed in the body through the action of the intrinsic endogenous mechanical energy on the splanchnic venous system after the partial PVL^[19]. Therefore during its evolution, prehepatic portal hypertension would induce a low-grade inflammatory response which could be developed during the above mentioned phenotypes, the neurogenic, the immune and the endocrine^[19] (Table 1).

THE NEUROGENIC INFLAMMATORY PHENOTYPE

The systemic inflammatory response in prehepatic portal hypertension could begin with an immediate neuromuscular response including the splanchnic and systemic vascular smooth muscle with vasoconstriction and vasodilation producing an ischemia-reperfusion phenomenon. This pathologic vasomotor response induces local and systemic hemodynamic impairments, *i.e.*, hyperdynamic splanchnic and systemic circulation^[20]. Within systemic and splanchnic alterations, hyperdynamic circulation is pointed out in relation to prehepatic portal hypertension^[21]. Regarding this, the hyperdynamic or progressive vasodilator syndromes could be triggered by splanchnic and systemic vasodilation. Moreover, multiorgan failure in chronic liver disease could be attributable to this syndrome^[21,22]. Once developed, the hyperdynamic syndrome induces tissue hypoxia, stress (oxidative and nitrosative) accompanied by mild edema, and inflammation in tissues and organs causing their dysfunction^[19]. Multiple studies

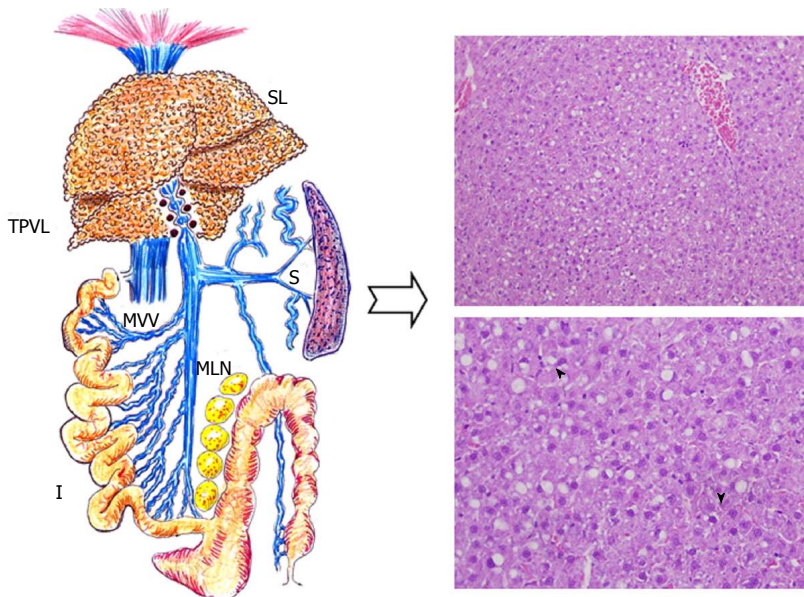


Figure 1 Liver histopathological changes of a rat after three months with triple partial portal vein ligation. Hepatic steatotic areas are mainly distributed in zone 1 of the liver acinus, but typically hepatocyte ballooning is more apparent near zone 3. Both macrovesicular and microvesicular steatosis are evident as well as scattered necroinflammatory foci (arrowheads) (H and E stain, $\times 200$). I: Intestine; MVV: Mesenteric venous vasculopathy; S: Spleen; SL: Steatotic liver; TPVL: Triple partial portal vein ligation.

Table 1 Similarities between the inflammatory changes developed after triple partial portal vein ligation in the rat and the extra-embryonic functions in mammals

Inflammatory phenotype		Extra-embryonic and embryonic functions
Neurogenic	Portal ischemia	Coelomic-amniotic functions
	Hepatic arterial reperfusion	Neurogenic potential
	Neuroendocrine response	Interstitial edema
	Hyperdynamic circulation	Bacteriostasis
	Mild edema of space of Disse	Anti-inflammation
Immune	Kupffer cell and hepatic stellate cell activation	Trophoblastic-yolk sac functions
	Leukocyte infiltration	Digestive (phagocytic) functions
	Acute phase response	Acute phase proteins
	Angiogenesis	Angiogenic switch
	Hepatic steatosis	Lipid and protein nutrients (vitellum)
Endocrine	Sinusoidal remodeling	Gastrulation-like functions
	Capillarization of hepatic sinusoids	Epithelial-mesenchymal transition
	Perisinusoidal fibrosis	Intra-embryonic mesenchyma
	Steatohepatitis	

have shown that the central nervous system plays a key role in the pathophysiology of the hyperdynamic circulation^[23,24]. In particular c-Fos is detected in the brain stem and hypothalamic nuclei of rats following portal vein ligation^[23]. Then, hyperdynamic circulation leads to decreased mean arterial pressure thus stimulating the sympathetic nervous system and the multiple neuro-endocrine axes, including the renin-angiotensin-

aldosterone system. This results in sodium retention and volume expansion^[20,25,26]. Sodium retention seems to be critical for inflammatory response. Under inflammatory circumstances, endothelium tends to increase permeability in tissues and organs affected. This strategy will allow a selective diffusion of circulating substances from the blood into the interstitial space^[27].

Stress of the neuro-endocrine system by portal hypertension could be induced by three mechanisms: The hypothalamic-pituitary-adrenal axis, the sympathetic-adrenal medullary and the sympathetic nervous system^[28] (Figure 2). Moreover, the localization of the early inflammatory response could also favor the posterior storage of substances derived from the acute phase response^[29]. Therefore, in the liver of the rats with prehepatic portal hypertension, the space of Disse could represent the anatomically definable compartment where the early inflammatory response is expressed (Figure 2).

The main and immediate effect on the liver vascular flow after the partial portal vein ligation is greatly reduced portal blood flow, which normally represents the 70% of its blood supply^[30]. Nevertheless, initial portal ischemia is followed by arterial reperfusion. It has been described that decreased portal venous flow is able to induce an increase in hepatic arterial blood flow or "hepatic arterial buffer response"^[31]. In turn, this vascular compensatory mechanism arterializes the liver and produces a capillarization of hepatic sinusoids^[30]. This phenomenon is defined by fenestrae loss and the formation of a continuous basement membrane^[32]. Although the mechanism of defenestration induced in the arterialized liver is not well known, it has been demonstrated that liver sinusoidal endothelial cell phenotype in capillarized

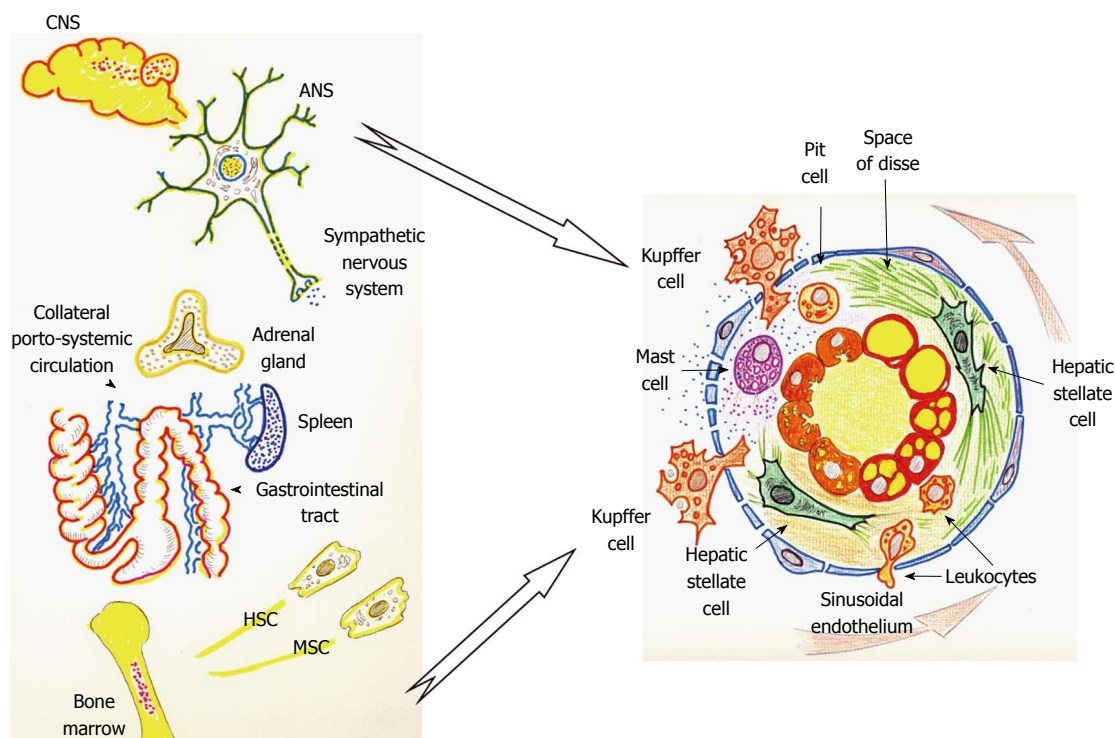


Figure 2 Inflammatory phenotypes, neurogenic and immune in prehepatic portal hypertensive rat. ANS: Autonomic nervous system; CNS: Central nervous system; HSC: Hematological stem cell; MSC: Mesenchymal stem cell.

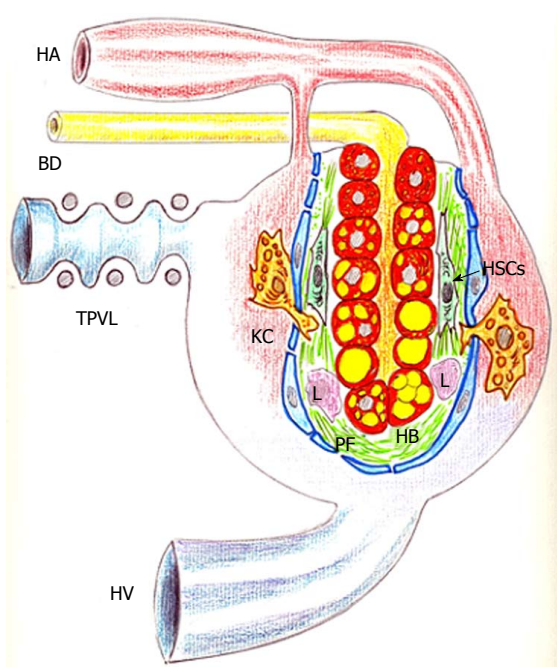


Figure 3 Schematic representation of the liver parenchyma after partial portal vein ligation in the rat. The deposit of lipids within the hepatocytes and the liver arterialization, associated with defenestration of the sinusoidal endothelium, and the perisinusoidal fibrosis stand out. BD: Bile duct; HA: Hepatic artery; HV: Hepatic vein; HB: Hepatocyte ballooning; HSCs: Hepatic stellate cells; KC: Kupffer cell; L: Leukocyte; PF: Perisinusoidal fibrosis; TPVL: Triple partial portal vein ligation.

liver promotes Kupffer cell^[30] and hepatic stellate cell^[33] activation (Figure 3). Moreover, it has been suggested

that intrasinusoidal crawling behavior could represent a form of immune surveillance. So, this mechanism could explain the reduction in CD8 T cell surveillance by infected or transformed hepatocytes. Furthermore, intrasinusoidal crawling behavior could participate in the hepatocarcinoma's development and progression^[34]. This liver ischemia-reperfusion phenotype with progressing interstitial edema in the space of Disse could activate lymphatic circulation^[35]. As a consequence, increased hepatic lymph flow and the transmigration of free cells, such as dendritic cells and lymphocytes, have been shown^[34]. In addition, the edematous space of Disse may also serve as a stem cell niche for substances derived from the neuro-endocrine response to portal hypertensive stress, for mediators of the acute phase response and cytokines, and for activated hepatic stellate cells and lymphocytes^[35-37] (Figure 4).

THE IMMUNE INFLAMMATORY PHENOTYPE

The immune inflammatory phenotype by the splanchnic system after ischemia/reperfusion, and therefore suffer oxidative and nitrosative stress, is coupled with the activation of intracellular signaling pathways including the nuclear factor- κ B (NF- κ B) pathway, and the inflammasome^[38]. Reactive oxygen species are central in the regulation of NF- κ B, although their role is not completely understood on account of the contrasted outcome reported: Activation vs inhibition. Most likely,

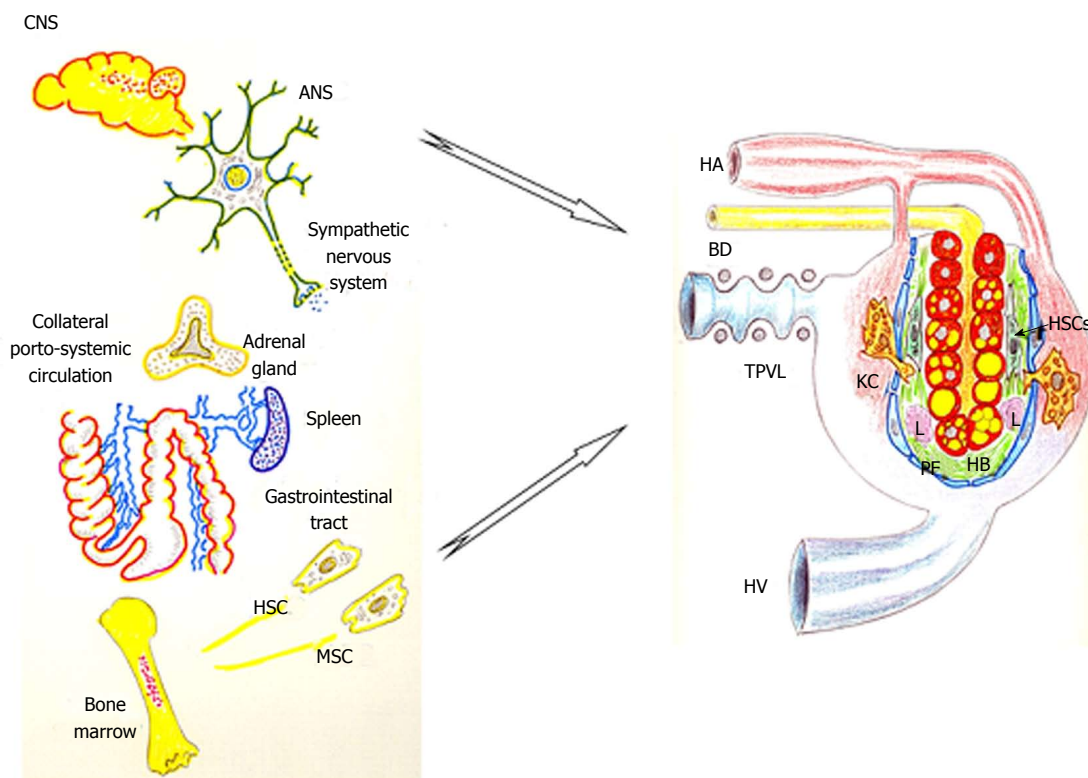


Figure 4 The preferential expression of the neurogenic and immune inflammatory phenotypes into the space of Disse induces hepatic steatosis, steatohepatitis and fibrosis in portal hypertensive rats by triple partial portal vein ligation. ANS: Autonomic nervous system; BD: Bile duct; CNS: Central nervous system; HA: Hepatic artery; HB: Hepatocyte ballooning; HSC: Hematological stem cell; HSCs: Hepatic stellate cell; HV: Hepatic vein; KC: Kupffer cell; L: Leukocyte; MSC: Mesenchymal stem cell; PF: Perisinusoidal fibrosis; TPVL: Triple partial portal vein ligation.

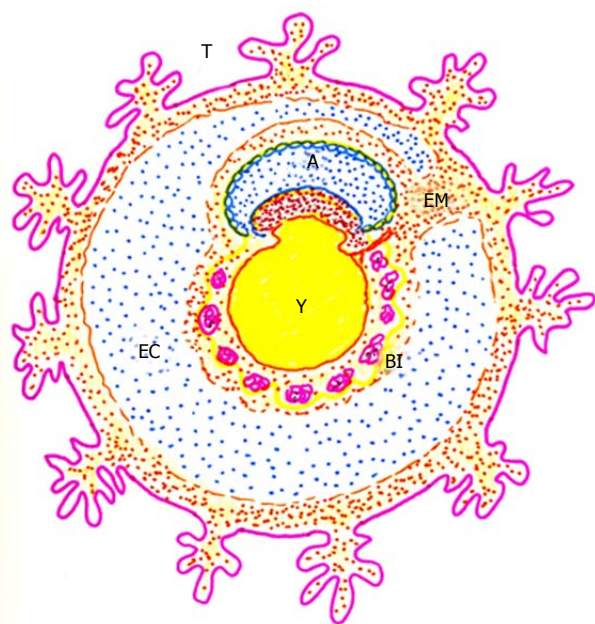


Figure 5 Schematic representation of early embryo development. The extra-embryonic mesoderm and the exocoelomic cavity connect the trophoblast with the amnion and the yolk sac. A: Amnion; BI: Blood islands; EC: Exocoelomic cavity; EM: Extra-embryonic mesoderm; T: Trophoblast; Y: Yolk sac.

reactive oxygen species could promote NF- κ B responses in the early stages of the inflammatory response instead of inhibiting these responses at later stages.

Reactive oxygen and nitrogen species could also favor the induction of tissue repair or remodeling^[38-40].

During this phase the oxygen is creating enzymatic stress^[19]. Along this acute phase the compensatory mechanisms include production of proteins which bind proteolytic enzymes, inhibitors of leukocyte and lysosomal proteolytic enzymes^[41,42]. Likewise, anti-enzymatic stress could be promoted by the natural inhibitors of matrix metalloproteinases^[19]. The immune phenotype could be coupled with bacterial intestinal translocation to mesenteric lymph nodes, increased mast cells in the splanchnic area, an acute phase response, dyslipidemia and hepatic steatosis^[43]. We have shown that splanchnic and systemic inflammatory changes develop in TPVL-rats, including portal hypertensive enteropathy^[43,44], mesenteric adenitis^[45,46], portal hypertensive encephalopathy^[47], liver steatosis^[8-10], aortic atherosclerosis-like disease^[48-50] and metabolic syndrome^[51].

Hepatic steatosis and visceral adipose tissue are metabolic risk factors in accumulation of visceral fat. Due to their anatomical position, the venous blood from there is drained directly into the liver through the portal vein^[52]. We speculate that the induction of intraabdominal fat deposits around the portal venous system could represent ontogenic reminiscences, associated with yolk sac, or phylogenetic reminiscences, related to vitellogenesis^[53,54] (Figure 5). Regarding the ontogenic origin, the liver, and in particular the omentum, could

be mimicking the yolk sac, in which pathological lipid deposition takes place. Under this scenario, the liver and the omentum would be regressing to evolutive phases with suitable metabolic conditions, supported by the expression of inflammatory markers such as tumor necrosis factor (TNF)- α , IL-6, C reactive protein and leptin^[19,29]. In terms of phylogenetic approach, the body would be adopting the molecular mechanisms related to the vitellogenesis^[53,54], in which oviparous species provide a glycolipoprotein yolk storage called vitelline to the egg as food-source for the embryo^[54]. Lipoprotein transport through the circulatory system by eukaryotes has been an important function for the existence^[53]. Thus, the evolutionary perfection of energy accumulation in fat has provided organisms an advantage in adapting to environmental and developmental changes^[54].

The increased uptake of free fatty acids derived from the hydrolysis of adipose-tissue triglycerides results in hepatic steatosis. Moreover, the contribution of dietary chylomicrons, hepatic biogenesis and insulin resistance increased the uptake^[3,4,55]. Hepatic steatosis is a key concept in the two-hit NAFLD hypothesis, which postulates that hepatic steatosis sensitizes fatty liver to secondary hits, such as oxidative and nitrosative stress or inflammatory cytokines^[56]. In addition, cholesterol sensitizes fatty livers to secondary hits, particularly when trafficking to mitochondria as it has been recently recognized^[57,58]. In portal hypertensive rats by TPVL the distinction between simple steatosis and steatohepatitis (NASH) includes hepatocyte ballooning, leukocyte infiltration (lobular inflammation) and perisinusoidal (zone 3) fibrosis^[9] (Figures 3 and 4). In long-term portal hypertensive rats, the plasmatic increase of low density lipoprotein and lipopolysaccharide binding protein as well as high-density lipoprotein reduction has been associated with NASH^[8-10]. These findings could suggest a NASH role in developing cardiovascular disease by two ways: Systemic release of inflammatory mediators and/or the production of insulin resistance and atherogenic dyslipidemia^[49,50]. In this line, recent evidence supports a relationship between NAFLD and cardiovascular disease^[50,55]. Particularly in TPVL-rats NASH, this association could be considered a risk factor for a wound-like inflammatory aortic response^[49], with increased expression of NF- κ B, TNF- α , IL-1 β and IL-6 in the aortic wall^[48,49].

THE ENDOCRINE INFLAMMATORY PHENOTYPE

During the expression of the endocrine inflammatory phenotype in TPVL-rats there is a splanchnic remodeling by angiogenesis, which implies growth of new vessels from pre-existing ones^[59], and fibrosis. Moreover, an abnormal splanchnic and systemic angioarchitecture, such as portosystemic collaterals have been observed in chronic liver diseases^[60-62]. This vascular alteration found in the gastrointestinal tract under portal hyper-

tension conditions is called "hypertensive portal intestinal vasculopathy"^[63,64]. However, not only vascular alterations have been described, even histological changes have been found^[64]. So, angiogenesis is key role in portal hypertension and represents a potential therapeutic target^[65]. Splanchnic hyperemia which is followed by increased splanchnic vascularization, together with portal-systemic collateral circulation in experimental portal hypertension have angiogenic origins partly driven by the vascular endothelial growth factor (VEGF)^[65,66]. Mast cells are able to release VEGF among others^[67], being involved in promoting hypertensive portal vasculopathy and portal systemic collateral circulation^[43].

Fibrosis is a scarring response that seems to be reversible in rats with long-term TPVL. Hepatic stellate cells (HSCs) play a central role in liver fibrosis and homeostasis^[68]. In particular, perisinusoidal/pericellular fibrosis is typically found in NASH. Excess deposits of the extracellular matrix are primarily found in the spaces of Disse surrounding sinusoids or groups of hepatocytes. This leads to "capillarization of sinusoids" or "chickenwire pattern", respectively^[69] (Figures 3 and 4). HSCs express both mesenchymal markers and neuronal or glial markers^[70]. The activation of HSCs is needed to develop hepatic fibrogenesis^[68,69]. HSCs activate resident immune cells such as Kupffer and Pitt cells which trigger hepatic inflammation^[71], as well as mono- and polymorphonuclear leukocytes infiltration^[70].

In addition, HSCs, once in their myofibroblastic phenotype, respond to vasoactive substances by contracting being important in the pathogenesis of portal hypertension^[72]. They also respond to sinusoidal morphogenesis since direct hepatic stellate cell-endothelial cell contact inhibits endothelial cell capillary/sinusoidal formation^[73]. Dynamic interplay between HSCs and endothelial cells could be important for understanding how the sinusoidal remodeling process is regulated in NAFLD in portal hypertensive rats^[73] (Figure 3).

RECAPITULATED EXTRA-EMBRYONIC FUNCTIONS RELATED TO NAFLD

The inflammatory response associated with NAFLD in portal hypertensive rats could be an ontogenic process based on the re-expression of two speculative extra-embryonic axes (exocoelomic-amniotic and trophoblastic-yolk sac) in the space of Disse. If so, the NAFLD concept which vary from steatosis and NASH to advanced fibrosis and cirrhosis^[58] could be representing a normal embryonic development (Table 1).

The hypothetical recapitulation of these initial embryonic phases during the evolution of NAFLD would be accompanied by functions similar to the extra-embryonic membranes that surround the embryo. The extra-embryonic coelom or exocoelomic cavity surrounds the blastocyst, which is composed of two structures, the amnion and the primary yolk sac (Figure 5). Coelomic

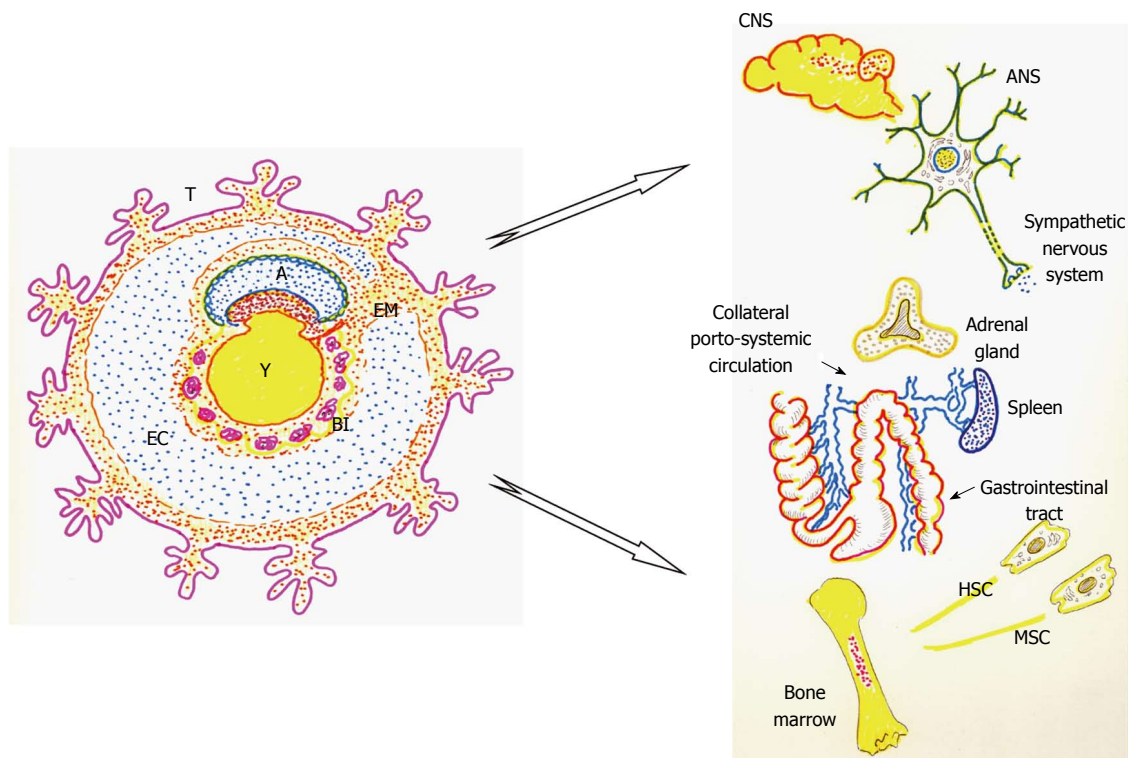


Figure 6 The coelomic-amniotic functions could be hypothetically recapitulated by the systemic neurogenic phenotype activation, while the trophoblastic-yolk sac functions could be carried out by the systemic immune inflammatory phenotype activation. A: Amnion; ANS: Autonomic nervous system; BI: Blood islands; CNS: Central nervous system; EC: Exocoelomic cavity; EM: Extra-embryonic mesoderm; HSC: Hematological stem cell; MSC: Mesenchymal stem cell; T: Trophoblast; Y: Yolk sac.

fluid results from an ultrafiltrate of maternal serum with the addition of specific placental and secondary yolk sac byproducts^[74]. Accordingly, the embryonic phenotype could be adopted by the inflamed liver interstitium, which will induce fluid accumulation with low pH and oxygen environment as coelomic fluid^[74,75]. This interstitial edema seems to occur secondary to hepatic ischemia-reperfusion. Moreover, the edema shows proinflammatory characteristics due to the high content in proteins such as albumin, electrolytes, metals, amino acids, antioxidants, cytokines and cholesterol-derived hormones^[75,76]. So the edema will be leading liver trophism.

Biological and anatomical data of the exocoelomic cavity stand it out as a nutritional pathway before placental circulation is established^[76]. The strong neural potential of the amnion, an embryonic functional axis, has been previously stated^[77]. Moreover, from the amnion is secreted the “amnio-derived cellular cytokine solution” which highlighting a connection between mesenchymal and epithelial cells during embryo development^[78]. Furthermore, the amniotic fluid could be understood as an extension of the extracellular space of the fetal tissues^[79]. Finally, pluripotent stem cells within the amniotic fluid could also be a new source for stem cell research^[80,81]. Because of these characteristics, the amnio-like phenotype could favor nutrition by diffusion, transport, excretion, and bacteriostatic and antiinflam-

matory protection^[78,79] (Figure 5).

The development of hematopoiesis and angiogenesis^[81] occurs in the mesenchymal layer which builds the wall of the secondary yolk sac in mammals^[74]. This sac appears from the sixth week of gestation and is covered by superficial small vessels^[82]. Others layers of the secondary yolk sac include, mesothelial and endodermal layers which are active in endocytosis/digestion and absorptive functions^[81,82], and the endodermal layer which produces acute phase proteins, such as transferrine, α 1-antitrypsin, and α -fetoprotein (produced by both the adult and fetal liver)^[74,83] (Figure 5). So, the yolk sac provides lipids, carbohydrates, proteins and vascular integrity to the embryos^[84] and could be involved in lipid metabolism gene regulation^[85], immune cells recruitment and angiogenic switch^[86]. Phagocytosis has been associated to trophoblast differentiation as well^[87].

THE RECAPITULATED SYSTEMIC EXTRA-EMBRYONIC FUNCTION AND THE INTERSTITIAL INFLAMMATORY SPACE OF DISSE IN NAFLD

NAFLD systemic inflammatory pathophysiology shares mechanisms with the pluripotential extra-embryonic pathways. It is hypothesized that during NAFLD evolu-

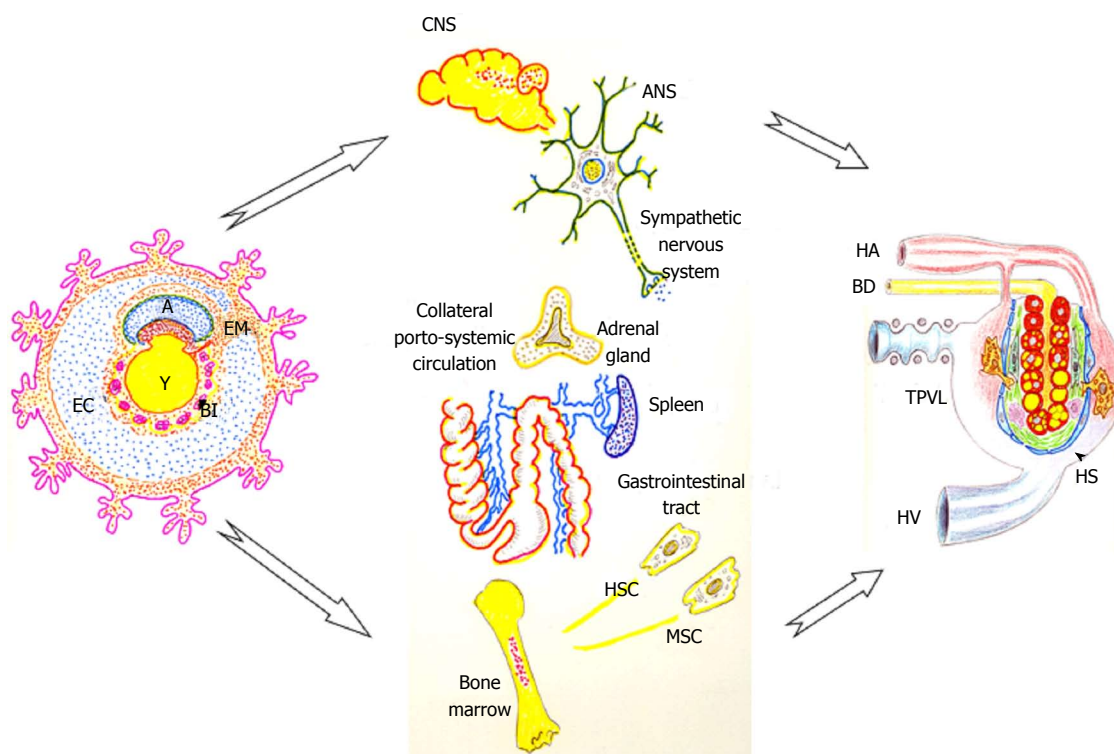


Figure 7 The recapitulated systemic extra-embryonic functions, that is, the coelomic-amniotic and the trophoblastic-yolk sac (or vitellum), are located into the space of Disse. This fact results in the development of hepatic steatosis and steatohepatitis in prehepatic portal hypertensive rats. A: Amnion; ANS: Autonomic nervous system; BD: Bile duct; BI: Blood islands; CNS: Central nervous system; EM: Extraembryonic mesoderm; HA: Hepatic artery; HS: Steatotic liver; HSC: Hematological stem cell; HV: Hepatic vein; MSC: Mesenchymal stem cell; TPVL: Triple partial portal vein ligation; Y: Yolk sac.

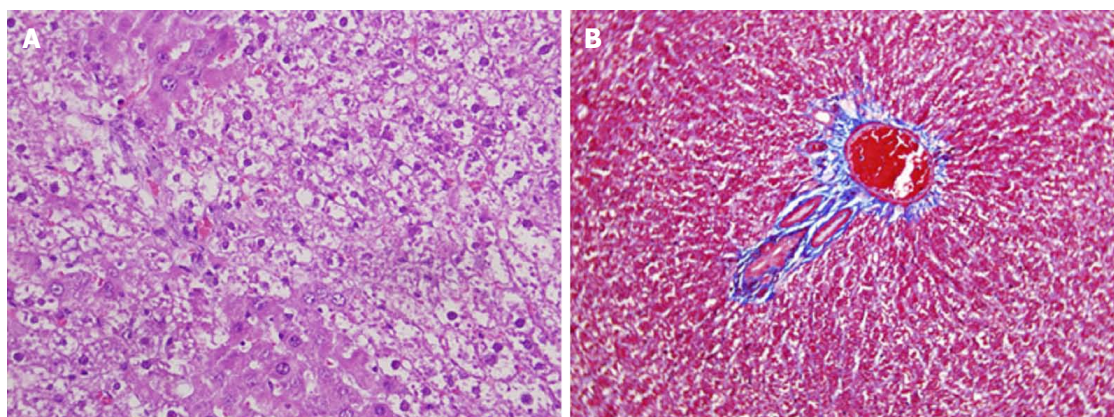


Figure 8 Histological appearance of non-alcoholic steatohepatitis in portal hypertensive rats at three months of postoperative evolution. It's shown the hepatocyte ballooning (A) with lobular inflammation and perisinusoidal fibrosis (B) (H and E stain, × 200).

tion, the coelomic-amniotic and trophoblastic-yolk sac functions are integrated into the interstitial space of Disse, thereby activating embryonic programs in the acinus. In this line, coelomic-amniotic functions would be embodied by the systemic neurogenic inflammatory phenotype, while the trophoblastic-yolk sac functions could be represented by the immune inflammatory phenotype^[88] (Figure 6). Furthermore, the polarization of neurogenic and immune-related phenotypes in the interstitial space of Disse would condition the evolution of NAFLD, including a wound-healing response producing fibrosis in the rat^[88,89] (Figure 4 and Table 1).

Essentially, the recapitulation of the extra-embryonic functions when focused on the space of Disse^[88] would produce a gastrulation-like process which is a recapitulation of the intra-embryonic mesenchyme formation process^[89]. Therefore, mesoderm-derived cells, particularly fibrocytes and hepatic stellate cells have shown a main role in liver restore (Figure 5) after TPVL. The involution or dedifferentiation of the liver in portal hypertensive rats could be exemplifying a prenatal specialization^[88] which involves or angiogenic regenerative or fibrotic/scarring repairing processes^[89].

The systemic complications of the prehepatic portal

hypertensive syndrome in rats could be connected to similar metabolic functions of the extra-embryonic coelomic-amniotic and trophoblastic-yolk sac axes^[88]. For example, hydroelectrolytic decompensation and its effects, including hyperdynamic circulation, multiple neuroendocrine axis activation, edema due to sodium and water accumulation, increase interstitial hepato-intestinal lymph flow and stimulation of the sympathetic nervous system, might be associated to the upregulation of a systemic coelomic-amniotic axis after TPVL in the rat (Figure 7). In this line, an upregulation of a systemic trophoblastic-yolk sac or vitellogenic-like axis could be represented through the immune phenotype activation of NF- κ B and inflammasome, acute phase response, splanchnic infiltration by mast cells, bacterial intestinal translocation which implies hepatic dyslipidaemia and the excessive splanchnic angiogenic response. The convergence of these systemic extraembryonic axes in the interstitial space of Disse could favor a gastrulation-like response in which NAFLD develops (Figure 7).

Recently, it has been established that human NAFLD is commonly associated with hypertension, type 2 diabetes, obesity, dyslipidemia, metabolic syndrome and cardiovascular abnormalities leading to death^[90-92]. The array of alterations associated with the evolution of NAFLD make up a syndrome of great pathophysiological complexity. Studying this syndrome would help the experimental model of liver steatosis in the rat after TPVL. Also, the results obtained from studying multiple splanchnic and systemic alterations produced in the surgical experimental model of NAFLD suggest that the systemic inflammatory response could condition the evolution of hepatic steatosis. Thus, when the animals are isolated in individual cages after TPVL, accelerating the development of the liver histopathological changes typical of steatosis is possible (Figure 8). This early appearance of liver steatosis in TPVL-rats could be attributed to stress induced by isolation when rats are separated from other rats, and to their limited space for movement when kept in individual holders. If the stress related to isolation and movement limitation favors liver steatosis development, studying the influence of systemic neuroendocrine alterations associated with stress in NAFLD development in rats with TPVL would be an attractive goal for the future.

Finally, if our hypothesis presented in the current review of the NAFLD model secondary to TPVL in the rat represents the focus of two systemic functional axes that recapitulate extra-embryonic functions in the interstitial space of Disse, then modulating the evolution of experimental liver steatosis is possible by modifying the re-expression of these functions. For that reason, studying the mechanisms involved in embryonic development could provide key results for a better understanding of the NAFLD etiopathogeny.

ACKNOWLEDGMENTS

The excellent secretarial support of Mrs. Maria-Elena

Vicente is gratefully acknowledged. We would also like to thank Elizabeth Mascola for translating it into English and Javier de Jorge and Maria-José Valdemoro, Director and librarian, respectively of the Complutense University Medical School Library. This review was made, in part, thanks to FICYT FC-15-GRUPIN 14-088 and MINECO PSI2013-45924-P grants. Finally, Natalia Arias is now granted for Alfonso Martin Escudero Foundation.

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P- Reviewer: Dourakis SP, Manenti A, Popovic DD, Zapater P

S- Editor: Ji FF **L- Editor:** A **E- Editor:** Wu HL



Non-alcoholic fatty liver disease and cardiovascular risk

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Author contributions: Patil R and Sood GK were involved in designing and writing the manuscript; Patil R was responsible for a thorough literature search; Sood GK was involved in editing the manuscript.

Conflict-of-interest statement: Patil R and Sood GK have no conflicts of interest to report.

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Manuscript source: Invited manuscript

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Received: August 27, 2016

Peer-review started: August 31, 2016

First decision: December 13, 2016

Revised: December 30, 2016

Accepted: March 12, 2017

Article in press: March 13, 2017

Published online: May 15, 2017

gression to cirrhosis. Compelling evidence over the past several years has substantiated a significant link between NAFLD and cardiovascular disease ranging from coronary artery disease to subclinical carotid atherosclerosis. Close follow up, treatment of risk factors for NAFLD, and cardiovascular risk stratification are necessary to predict morbidity and mortality in this subset of patients.

Key words: Non-alcoholic fatty liver disease; Cardiovascular risk; Outcomes; Coronary artery disease; Steatosis

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Core tip: Non-alcoholic fatty liver disease (NAFLD) is often associated with insulin resistance and is strongly associated with type 2 diabetes mellitus and obesity. In addition to being at risk for nonalcoholic steatohepatitis, cirrhosis and its complications, NAFLD patients are also at higher risk of cardiovascular diseases (CVD), including coronary heart disease and stroke. NAFLD confers increased cardiovascular disease risk independent of traditional cardiovascular risk factors and metabolic syndrome. Close followup of patients with NAFLD may be indicated to prevent major vascular events. Risk stratification scores are needed that address both the risk for advanced liver disease and CVD.

Patil R, Sood GK. Non-alcoholic fatty liver disease and cardiovascular risk. *World J Gastrointest Pathophysiol* 2017; 8(2): 51-58 Available from: URL: <http://www.wjgnet.com/2150-5330/full/v8/i2/51.htm> DOI: <http://dx.doi.org/10.4291/wjgp.v8.i2.51>

Abstract

Non-alcoholic fatty liver disease (NAFLD) is a chronic liver disease associated with insulin resistance and metabolic syndrome. The spectrum of disease ranges from simple steatosis to steatohepatitis and pro-

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the upcoming leading cause of chronic liver disease in the United States and its prevalence is increasing world-

wide. It is a spectrum of liver diseases that ranges from simple steatosis to a progressive form of liver disease called nonalcoholic steatohepatitis (NASH)^[1]. It may progress to advanced fibrosis, cirrhosis, and hepatocellular carcinoma in some individuals. NAFLD is often associated with insulin resistance and is strongly associated with type 2 diabetes mellitus and obesity. NAFLD patients are at risk of progressing to NASH and ultimately cirrhosis; they are also at higher risk of cardiovascular diseases (CVD), including coronary heart disease and stroke^[2]. NAFLD confers increased cardiovascular disease risk independent of traditional cardiovascular risk factors and metabolic syndrome (MetS). In this review, we have discussed the association of NAFLD with cardiovascular disease, the likely mechanisms underlying this association, proper risk assessment of patients with NAFLD for cardiovascular diseases, and treatment options for modification of CVD morbidity and mortality in patients with NAFLD.

EPIDEMIOLOGY OF CVD IN NAFLD

Adverse CVD events in NAFLD subjects compared with the general population are described through a description of several recent epidemiological studies (Table 1). The diagnosis of NAFLD can be based either on histology or imaging studies. Abnormal liver enzymes are used as biochemical surrogates of NAFLD in several recent studies. The prospective cohort study by Dunn *et al.*^[3], using data from the Third National Health and Nutrition Examination Survey (NHANES III), showed that subjects with NAFLD identified based on elevated alanine aminotransferase (ALT) had a higher mortality from CVD. After adjusting for cardiovascular risk factors, several large population studies have shown an association between an elevated ALT and increased cardiovascular mortality^[4,5]. Elevated GGT has also been reported as a marker of NAFLD in described prospective studies. A meta-analysis of ten pooled studies confirmed the independent association between elevated GGT and adverse CV events^[6].

Liver imaging may be a more reliable method for diagnosing NAFLD. In three large population studies, ultrasound imaging suggestive of NAFLD was independently associated with cardiovascular events^[7-9]. Although these studies did show that NAFLD may be a predictor of CVD, ultrasound may not be as sensitive for a diagnosis of NAFLD and therefore this was a major limitation for these studies. Hepatic fat concentration as measured by MRI has been used for diagnosis of NAFLD. Quantity of liver fat has been reported to be predictive of metabolic syndrome and CVD risk. In a recent study Loomba *et al.*^[10] quantified liver fat in patients with NAFLD and controls using a magnetic resonance imaging and measuring proton-density-fat-fraction (MRI-PDFF). In patients with NAFLD high proton-density-fat-fraction on MRI was a predictor of metabolic syndrome and increased cardiovascular risk^[10].

Liver biopsy is considered to be the gold standard for diagnosis of NAFLD and some studies have shown that patients with biopsy-proven NAFLD have higher total mortality rates compared to the general population. Söderberg *et al.*^[11] reported increased mortality from cardiovascular disease in patients with biopsy proven NASH. Forty-three percent of patients had NASH at initial biopsy. The median follow up in their study was 28 years. Overall survival was reduced in subjects with NASH compared to the general population due to increased mortality by cardiovascular disease. Importantly in this study, only subjects with NASH had significantly reduced survival^[9-11].

MECHANISM (PATHOGENESIS)

Several mechanisms have been postulated for development of accelerated atherosclerosis in patients with NAFLD, including genetic predisposition, insulin resistance and atherogenic dyslipidemia, oxidative stress, chronic inflammation, reduced levels of the adiponectin and altered production of pro and anticoagulant factors^[12]. All these mechanisms are present at the same time. NAFLD, regardless of its stage, is strongly associated with hepatic and adipose tissue insulin resistance (IR). In fact, liver fat content can be used as an independent predictor of insulin resistance. These mechanisms work synergistically^[13].

NAFLD, especially in its necroinflammatory form (NASH), may cause atherogenic dyslipidemia^[14]. In addition there is an increase of pro-coagulant factors like fibrinogen, plasminogen activator inhibitor-1 and tumor growth factor, which all increase the risk of atherosclerosis^[15]. NAFLD is considered to have chronic sub-clinical inflammation and associated with many inflammatory markers. Increased vascular risk has been linked to increased levels of inflammatory cytokines and markers such as IL-6, TNF, CRP, and fibrinogen. Oxidative stress may also play a role. This stress is thought to trigger changes in endothelial function leading to formation and deposition of oxidized LDL in the sub-intimal space^[16].

Visceral adipose tissue is also thought to play a role in NAFLD. Visceral fat is metabolically active and secretes several hormones that help regulate inflammation; tissue distribution is affected by an alteration in cellular free fatty acid transport. These alterations are possibly caused by hyperinsulinemia and ultimately divert accumulated triglycerides away from adipose tissue and towards other metabolic organs such as the liver^[17,18].

Nonalcoholic fatty liver disease, abdominal obesity, and insulin resistance all play a role in increased cardiovascular risk, though the exact causal relationship is still unclear. Hepatic necroinflammation, as seen in NASH, is an atherogenic mechanism that may explain why patients with NASH have greater CV risk than patients with simple steatosis. In the liver, a signal of hepatic necroinflammation is elevated liver enzymes which may

Table 1 Recent epidemiological studies evaluating cardiovascular risk in non-alcoholic fatty liver disease

Ref.	Study characteristics	Years of follow-up	Diagnosis of NAFLD	Study outcomes	Main findings
Ekstedt <i>et al</i> ^[30] (2015)	Retrospective cohort study <i>n</i> = 229 Swedish patients with NAFLD and elevated liver enzymes (49% NASH); mean age 49 yr, 66% men	26.4 (mean)	Histology	<i>n</i> = 96 total deaths, 41 CVD related deaths	Increased rates of all-cause, liver-related and CVD mortality with NAFLD compared with general control population. Fibrosis stage on histology significantly predicted the risk of all-cause, liver-related and CVD mortality
Ekstedt <i>et al</i> ^[31] (2006)	Cohort study 129 consecutively enrolled patients diagnosed with biopsy-proven NAFLD were reevaluated. Survival and causes of death were compared with a matched reference population. Living NAFLD patients were offered repeat liver biopsy and clinical and biochemical investigation	13.7 (mean)	Histology	Mortality was not increased in patients with steatosis. Survival of patients with nonalcoholic steatohepatitis (NASH) was reduced. These subjects more often died from cardiovascular and liver-related causes. At follow-up, 69 of 88 patients had diabetes or impaired glucose tolerance. Progression of liver fibrosis occurred in 41%. These subjects more often had a weight gain exceeding 5 kg, they were more insulin resistant, and they exhibited more pronounced hepatic fatty infiltration at follow-up	Increased total mortality which was primarily CV related (only in NASH patients but not in simple steatosis) compared with matched reference population
Soderberg <i>et al</i> ^[11] (2010)	Retrospective cohort study 256 subjects (61% men, mean age of 45 ± 12 yr) This study was undertaken to determine the frequency of NAFLD in a cohort of subjects who underwent liver biopsy from 1980 to 1984 because of elevated liver enzymes, and to assess mortality among subjects with NAFLD in comparison with the general Swedish population. Liver biopsies were blindly scored for NAFLD and NASH	24 yr (mean)	Histology	During the follow-up period, 113 (44%) of the total population and 47 (40%) of the 118 subjects diagnosed with NAFLD died. Of the 113 deaths, 37 were of cardiovascular disease and 16 of liver diseases. NAFLD exhibited a 69% increased mortality, subjects with bland steatosis, a 55% increase, and subjects with NASH, 86%	Increased total mortality in NAFLD was predominantly CV related, compared with matched reference population
Pickhardt <i>et al</i> ^[32] (2014)	Retrospective cohort study United States adults undergoing abdominal CT selected among 4412 consecutive adults scanned with CT for clinical reasons over a 12-mo period: 282 NAFLD patients and 786 non-steatotic controls after exclusion of those with known liver diseases or < 1 yr of follow-up; mean 51 yr, 46% men	7.5 (mean)	Unenhanced CT	Non-fatal CVD events (myocardial infarction, stroke, TIA or coronary bypass or stent); <i>n</i> = 73 CVD events	NAFLD was not independently associated with non-fatal CVD events
Zeb <i>et al</i> ^[12] (2016)	Prospective cohort study <i>n</i> = 4119 United States participants aged 45-84 yr (mean 62 yr, 45% men) who were free of CVD and known liver diseases at baseline	7.6 (mean)	Unenhanced CT	All-cause mortality and non-fatal CVD events (myocardial infarction, resuscitated cardiac arrest, angina, or coronary revascularization procedures), <i>n</i> = 253 deaths and 209 non-fatal CVD events	NAFLD was independently associated with a composite endpoint inclusive of all-cause death and non-fatal CVD events

Kim <i>et al</i> ^[33] (2013)	Population-based cohort <i>n</i> = 11154 United States adults; mean age 43 yr, 48% men	14.5 (median)	Ultrasound	All-cause and CVD mortality <i>n</i> = 1795 total deaths (673 CVD deaths)	NAFLD was not associated with increased all-cause and CVD mortality in the whole cohort however NAFLD with advanced fibrosis (defined by the NAFLD fibrosis score) was independently associated with increased all- cause and CVD mortality
Emre <i>et al</i> ^[34] (2015)	Retrospective cohort study <i>n</i> = 186 Turkish, non- diabetic patients undergoing PCI for ST- elevation MI; patients with known liver disease were excluded; mean age 58 yr, 78% men	In-hospital cardiac events	Ultrasound	In-hospital CVD events (MI, acute heart failure, cardiac arrest), <i>n</i> = 32 CVD events and <i>n</i> = 8 CVD deaths	Moderate-severe NAFLD was independently associated with increased in-hospital CVD events but not with increased CVD death

NAFLD: Non-alcoholic fatty liver disease; CT: Computed tomography; CAC: Coronary artery calcification; CIMT: Carotid intima-media thickness; CP: Carotid plaque; DM: Diabetes mellitus; NASH: Nonalcoholic steatohepatitis.

serve as a marker for those at increased risk of CVD^[19]. Patients with NASH are also noted in several studies to have a greatly increased carotid-artery intimal medial thickness which further supports the necroinflammation hypothesis.

Further research is required to uncover other specific mechanisms by which nonalcoholic fatty liver disease and nonalcoholic steatohepatitis may contribute to the development and progression of cardiovascular disease^[20].

EVIDENCE FOR CORONARY ARTERY DISEASE IN NAFLD

Atherosclerosis is the main trigger of overall vascular disease and different methods are used to detect it in its subclinical stage. Endothelial dysfunction is the first stage of subclinical atherosclerosis. Carotid intima-media thickness (CIMT) and the presence of carotid plaques are important markers of vascular disease. Other markers of atherosclerosis are coronary artery calcification (CAC), as determined by multi-slice CT scan. CAC represents the atherosclerotic burden in arterial beds and is known to correlate strongly with the presence of coronary artery disease (CAD) and increased risk of poor cardiovascular outcomes.

Several studies have demonstrated the association of coronary artery calcium score (CACS) with NAFLD (Table 2). A recent, large, population-based study reported a strong relationship between NAFLD and CAC. Importantly, this association was independent of the traditional risk factors for coronary artery disease^[21]. Assy *et al*^[21] described that the presence of NAFLD was associated with increased prevalence of non-calcified coronary plaques, independent of metabolic syndrome, in a case-controlled study. Another study showed a significant association in NAFLD patients and the appearance of vulnerable plaques on coronary artery imaging^[22,23].

Though definitions of "significant" coronary artery disease may vary from study to study, a strong corre-

lation exists between NAFLD and the prevalence of CAD as determined by coronary angiography.

STUDIES EVALUATING SUBCLINICAL ATHEROSCLEROSIS IN NAFLD

Measuring carotid intima-media thickness (CIMT) by ultrasound is a widely accepted screening tool for the prediction of cardiovascular disease in patients with NAFLD who may be asymptomatic. Several studies show an association between NAFLD and carotid disease, some independently and other weakly after adjusting for metabolic syndrome. There seems to be a correlation histologically in severity of NAFLD when compared to increasing CIMT.

There is also evidence to support the association of NAFLD with subclinical atherosclerosis independent of traditional risk factors and metabolic syndrome. In a recent comprehensive systematic review there was strong evidence that NAFLD is associated with subclinical atherosclerosis^[24]. The presence of NAFLD was associated with the increased severity of CIMT, coronary calcification, endothelial dysfunction and arterial stiffness. These were independent of traditional risk factors and metabolic syndrome.

CARDIOVASCULAR RISK ASSESSMENT IN PATIENTS WITH NAFLD

All patients with NAFLD should be evaluated for CVD disease risk; this assessment can be repeated every 1-2 years. Patients should be evaluated for traditional CVD risk factors including obesity, diabetes, dyslipidemia and hypertension. Fasting glucose or glycosylated hemoglobin level should be done on the initial visit to diagnose DM. MetS is frequent in individuals with NAFLD and is associated with increased CVD and all-cause mortality. Therefore, assessment for the MetS is an important component of CV risk stratification. The MetS as defined by the National Cholesterol Education

Table 2 Studies evaluating coronary artery disease and carotid disease in non-alcoholic fatty liver disease

Ref.	Study characteristics	Modality to assess CV risk	Diagnosis of NAFLD Ultrasound	Main findings
Sinn <i>et al</i> ^[16] (2016)	Retrospective cohort study - 8020 men (average age, 49.2 yr) without carotid atherosclerosis at baseline and with proven NAFLD	CIMT on carotid ultrasound		NAFLD was associated with an increased risk of subclinical carotid atherosclerosis development. This association was explained by metabolic factors that could be potential mediators of the effect of NAFLD. Markers of liver fibrosis also were associated with subclinical carotid atherosclerosis development
Pais <i>et al</i> ^[35] (2016)	Longitudinal cohort study - 1871 subjects (mean age 53 yr; 65% males). Half of cohort had steatosis while half did not	CIMT on carotid ultrasound	Fatty Liver Index	Steatosis occurred in 12% and CP in 23% of patients. C-IMT increased in patients with steatosis occurrence whereas it did not change in those that stayed free of steatosis. Steatosis at baseline predicted CP occurrence independent of age, sex, type-2 diabetes, tobacco use, hsCRP, hypertension and C-IMT
Park <i>et al</i> ^[36] (2016)	Longitudinal cohort study - 1732 subjects underwent serial CAC evaluation. Half the cohort had NAFLD and half did not	Calcium scoring CT to assess CAC	Ultrasound	More subjects with NAFLD than without showed CAC development or progression. In subjects without calcification at baseline, NAFLD significantly affected the development of calcification after adjusting for traditional metabolic risk factors. The severity of NAFLD was dose-dependently associated with the development of CAC
Kim <i>et al</i> ^[15] (2012)	Retrospective chart review- 4 023 subjects (mean age, 56.9 ± 9.4 yr; 60.7% males) without known liver disease or a history of ischemic heart disease	Calcium scoring CT to assess CAC	Ultrasound	Patients with NAFLD are at increased risk for coronary atherosclerosis independent of classical coronary risk factors, including visceral adiposity. These data suggest that NAFLD might be an independent risk factor for coronary artery disease
Fracanzani <i>et al</i> ^[20] (2016)	Longitudinal cohort study - 125 NAFLD patients and 250 age and gender matched Controls at baseline and 10 yr later were followed. Incidence of cardiovascular and cerebral events was recorded	CIMT on carotid ultrasound	Ultrasound	Major cardiovascular events were observed in 19% of NAFLD patients, with an estimated cumulative risk significantly higher in NAFLD than in Controls. Presence of plaques and of steatosis were the strongest predictors for cardiovascular events. Grade of steatosis, ALT and GGT levels were higher in NAFLD patients who developed cardiovascular events. CIMT value after 10 years was significantly higher in NAFLD than in Controls. NAFLD should be included among risk factors for cardiovascular damage and underline the utility to evaluate, once it is diagnosed, the presence of atherosclerotic lesions
Nahandi <i>et al</i> ^[17] (2014)	Case control study - 151 patients in three groups: group I including 49 patients with NAFLD and DM; group II including 50 non-diabetic NAFLD patients; and the control including 52 normal subjects as group III	CIMT on carotid ultrasound	Ultrasound	There is a significant association between the presence of NAFLD and atherosclerosis, but this association was independent of DM. The grade of NAFLD and elevated liver function tests had no effect on severity of atherosclerosis

NAFLD: Non-alcoholic fatty liver disease; CT: Computed tomography; CAC: Coronary artery calcification; CIMT: Carotid intima-media thickness; CP: Carotid plaque; DM: Diabetes mellitus.

Program (NCEP) requires the presence of 3 or more of the following components: (1) increased triglyceride levels (≥ 150 mg/dL); (2) low HDL level (< 40 mg/dL in men, < 50 mg/dL in women); (3) increased fasting glucose level (≥ 110 mg/dL); (4) hypertension ($\geq 130/85$ mm Hg or on antihypertensive medication); and (5) abdominal obesity (waist circumference: > 102 cm in men, > 88 cm in women).

Several different methods are used in the general population to estimate CVD risk including the Framingham Risk Score (FRS). The FRS is a validated measure of CV risk in the general population. The FRS predicts an individual's 10-year risk of myocardial infarction or CV death and incorporates age, sex, cholesterol, HDL, smoking status, and hypertension. Furthermore, the

FRS has been validated as a predictor of CVD in NAFLD and should be used to risk-stratify individuals and guide treatment of risk factors including dyslipidemia. Recently, the American Heart Association recommended a new cardiovascular assessment tool for prediction of atherosclerotic cardiovascular disease. This score incorporates the usual risk factors for CVD but needs to be validated in patients with NAFLD^[25].

ASSESSMENT OF DYSLIPIDEMIA IN NAFLD

Dyslipidemia is frequent in individuals with NAFLD. The dyslipidemia in NAFLD is characterized by increased

serum triglycerides, increased small, dense low-density lipoprotein (LDL non-type A) particles, and low high-density lipoprotein (HDL) cholesterol. Recently, the value of non-HDL-C has been demonstrated in predicting coronary heart disease. Non-HDL-C is superior in predicting incidence of cardiovascular events and cardiac death in NAFLD patients compared to the traditional marker low-density lipoprotein^[25]. The Adult Treatment Panel III of the National Cholesterol Education Program has added non-HDL-C to its recommended screening algorithm for assessing cardiovascular disease risk.

Non-HDL-C is a calculated value derived by subtracting HDL cholesterol from the total cholesterol (TC) level, both available on traditional lipid panels, and requires no additional cost. In addition, because it is derived from TC and HDL levels, which are not impacted by fasting, non-HDL-C does not require fasting for accuracy.

Because patients with NAFLD have a high prevalence of cardiovascular disease, the use of non-HDL-C provides an important value in cardiovascular risk stratification and as a target for lipid-lowering therapy.

Non-HDL-C levels are increased in patients with NASH compared with those with steatosis, particularly in those persons who are not receiving lipid-lowering medications.

MANAGEMENT OF CARDIOVASCULAR DISEASE RISK IN NAFLD

All patients with NAFLD, irrespective of their body weight, should be advised lifestyle modifications in the form of regular exercise. Those who are overweight or obese are advised weight reduction. Regular exercise has been shown to improve the insulin sensitivity even without weight reduction. An exercise regimen should aim to achieve a target heart rate of 60%-70% of maximal heart rate through exercises such as brisk walking, jogging, or other aerobic exercises for at least 30 min, 5 d per week. Initial weight reduction in patients who are overweight or obese should be 10% of the body weight to be achieved in 6-8 mo. Overall, these overweight and obese patients need to create a negative balance by consuming fewer calories and burning more calories through regular exercise.

TREATMENT OF DYSLIPIDEMIA

Lifestyle modification, encompassing weight loss and increased physical activity, is the cornerstone of dyslipidemia management in NAFLD. However, for groups with increased CVD risk, lifestyle modification should be accompanied by lipid-lowering therapy. Guidelines set forth by the NCEP Adult Treatment Panel III provide guidance on which groups should be targeted for lipid-lowering therapy and outlines treatment goals^[26]. These guidelines were not designed specifically to address dyslipidemia in individuals with NAFLD; however, they can be applied safely to individuals with NAFLD.

In this context, the most attractive group of lipid-lowering agents for cardiovascular protection are the statins. In addition to a major effect in lowering LDL, they have modest effects on increasing HDL and lowering serum triglycerides, as well as non-cholesterol-lowering effects on vascular endothelium by inducing endothelial nitric oxide synthase.

Statin hepatotoxicity has not been shown to be of increased risk in NAFLD. The Liver Expert Panel stated in a report in 2014 that statins can be safely used in NAFLD and NASH, and routine liver enzyme monitoring need not be done. Statins can be safely used in patients with decompensated liver disease. There is always concern for using high dose statins in patients with elevated liver enzymes; in such circumstances adding ezetimibe has a synergistic effect with statins^[26,27].

Ezetimibe is less effective as a single agent to lower serum cholesterol and does not have the same vascular protective effects as statins. Ezetimibe is also useful when patients experience partially dose-dependent statin adverse effects such as myopathy.

Evidence that cholesterol lowering with statins reduces cardiovascular risk comes from the Greek Atorvastatin and Coronary Heart Disease Evaluation study, in which atorvastatin reduced the incidence of new cardiovascular events to a greater extent in patients with NAFLD (assumed by raised liver enzymes) than among those with normal liver enzymes^[28]. Patients without serum cholesterol elevation benefited as well, and there was a 40% reduction in serum triglyceride.

Triglyceride elevations are also a risk factor for cardiovascular disease, albeit less so than cholesterol. Attempts to reduce raised serum triglyceride levels center around weight reduction, improving insulin resistance (physical activity) and diabetic control, with use of polyunsaturated fatty acids (fish oil) as the first-line pharmacologic approach. Ezetimibe, has been compared with placebo for the treatment of NASH in the MOZART randomized clinical trial. In secondary analysis of the MOZART trial, FRS and CAC score improved in a greater proportion of patients with ezetimibe but did not reach statistical significance^[29].

TREATMENT OF DIABETES MELLITUS

DM is associated with an increased risk of CVD. Because DM is highly prevalent among individuals with NAFLD, comprehensive management is essential for CVD risk reduction. A detailed discussion of the management of DM in individuals with NAFLD is beyond the scope of this review. However, primary and secondary prevention of CVD events in individuals with DM should focus on multifactorial risk reduction, including treatment of hypertension and dyslipidemia. In addition, specific treatments of DM, including metformin, may decrease CVD events.

CONCLUSION

In the past several years, compelling evidence has

substantiated a strong link between NAFLD and increased risk of cardiovascular disease in individuals with or without coexisting metabolic syndrome. NAFLD is now recognized as a risk factor for poor cardiovascular outcomes including mortality and morbidity from major vascular events. As a whole, NAFLD patients may benefit from more careful surveillance and early treatment interventions. However, despite evidence linking increased cardiovascular risk with NAFLD, there is still uncertainty regarding the prognostic role of NAFLD in risk stratification for CHD. Additional, large follow-up studies are needed to establish whether adding NAFLD to the currently available risk scoring systems will improve cardiovascular disease risk prediction. Furthermore, the question of whether the prognostic value of NAFLD in the development and progression of cardiovascular disease only applies to NASH or also is associated with simple steatosis remains unresolved. Finally, more research is needed to understand the pathophysiology linking NAFLD with cardiovascular disease and to better elucidate whether genetic traits in NAFLD carry the same cardiovascular risk as metabolic syndrome-associate NAFLD.

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P- Reviewer: Ding WJ, Zhu X S- Editor: Qi Y L- Editor: A
E- Editor: Wu HL



Basic Study

Rectification of oxygen transfer through the rat colonic epithelium

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Supported by The Secretaría de Ciencia, Técnica y Posgrado, National University of Cuyo, Mendoza 5500 Argentina to Saraví FD, No. 06/J457.

Institutional review board statement: This study was reviewed and approved by the Secretaría de Ciencia y Técnica, National University of Cuyo, institutional review board.

Institutional animal care and use committee statement: All procedures involving the care and use of animals were approved by the Committee for Animal Care and Use of the Faculty of Medical Sciences, National University of Cuyo, Mendoza 5500, Argentina.

Conflict-of-interest statement: There is no conflict of interest.

Data sharing statement: There are no additional data available.

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Manuscript source: Invited manuscript

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Received: January 26, 2017

Peer-review started: February 8, 2017

First decision: March 9, 2017

Revised: April 12, 2017

Accepted: April 23, 2017

Article in press: April 24, 2017

Published online: May 15, 2017

Abstract

AIM

To assess whether higher sensitivity of colonic epithelium to hypoxia at the serosal side is associated with oxygen transfer asymmetry.

METHODS

Rats were fed either with normal chow or a low-sodium diet. Tissues were mounted as flat sheets in a modified, airtight Ussing chamber with oxygen meters in each hemichamber. Mucosal samples from normal diet animals were studied under control conditions, in low-chloride solution and after adding chloride secretion inhibitors and chloride secretagogues. Samples from sodium-deprived rats were studied before and after ouabain addition. In separate experiments, the correlation between short-circuit current and oxygen consumption was analyzed. Finally, hypoxia was induced in one hemichamber to assess the relationship between its oxygen content and the oxygen pressure difference

between both hemichambers.

RESULTS

In all studied conditions, oxygen consumption was larger in the serosal hemichamber than in the mucosal one ($P = 0.0025$ to $P < 0.0001$). Short-circuit current showed significant correlation with both total oxygen consumption ($r = 0.765$; $P = 0.009$) in normoxia and oxygen consumption in the serosal hemichamber ($r = 0.754$; $P = 0.011$) during mucosal hypoxia, but not with oxygen consumption in the mucosal hemichamber. When hypoxia was induced in the mucosal hemichamber, an oxygen pressure difference of 13 kPa with the serosal hemichamber was enough to keep its oxygen content constant. However, when hypoxia was induced in the serosal hemichamber, the oxygen pressure difference with the mucosal hemichamber necessary to keep its oxygen content constant was 40 kPa ($P < 0.0001$).

CONCLUSION

Serosal oxygen supply is more readily available to support short-circuit current. This may be partly due to a rectifying behavior of transepithelial oxygen transfer.

Key words: Colonic epithelium; Hypoxia; Oxygen diffusion; Short-circuit current; Ussing chamber

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Core tip: The physiological dependence of the colonic epithelium on oxygen provided from the serosal side is not only due to the structure of its blood supply and the low oxygen pressure of colonic intraluminal contents, since it is also observed in isolated mucosa preparations. This study demonstrates for the first time that a much larger partial pressure difference is needed for oxygen transfer from the mucosal side to the serosal side of the epithelium than for transfer in the opposite direction, a phenomenon that may be considered a rectifying behavior.

Saraví FD, Carra GE, Matus DA, Ibáñez JE. Rectification of oxygen transfer through the rat colonic epithelium. *World J Gastrointest Pathophysiol* 2017; 8(2): 59-66 Available from: URL: <http://www.wjgnet.com/2150-5330/full/v8/i2/59.htm> DOI: <http://dx.doi.org/10.4291/wjgp.v8.i2.59>

INTRODUCTION

Hypoxia is considered the earliest factor causing organ damage in intestinal ischemia^[1], caused, for example, by hypoperfusion associated with septic shock^[2], general anesthesia^[3], or ischemic colitis; the latter condition is the most frequent form of ischemic injury to the gastrointestinal tract^[4,5].

The intestinal epithelium is very sensitive to hypoper-

fusion. Epithelial ion transport is closely coupled to aerobic metabolism^[6,7]. However, the intestinal epithelium normally has relatively low oxygen partial pressure (P_{O_2}), a condition characterized as "physiologic hypoxia"^[8]. Hypothetically, oxygen might be provided to the epithelium from both the luminal and the serosal side. However, the composition of intraluminal gas is variable and, as a rule, its P_{O_2} is lower than that of either arterial or venous blood^[9,10].

While oxygen consumption by intraluminal bacteria may partially account for the differences, it has been reported that intracolonic P_{O_2} was less than 2 mmHg higher in germ-free rats than in control rats^[11]. Luminal oxygen is an unlikely source for epithelial consumption, since intraluminal P_{O_2} is lower than mucosal P_{O_2} ^[12]. In the intact colon *in vivo*, oxygen reaches the epithelial cells through their basolateral membranes by diffusion from the capillary network which surrounds the crypts^[8,13].

In most studies of epithelial biology employing Ussing chambers, both sides of the samples are oxygenated. If unremoved, in the intestinal epithelium both the adherent mucus gel layer and the submucosal tissue restrain oxygen diffusion^[14]. Hypoxia induced in the serosal hemichamber while keeping a high oxygen pressure at the mucosal side lowers short-circuit current (I_{sc}) just as effectively and with the same time-course as hypoxia simultaneously induced in both sides. On the other hand, hypoxia induced in the mucosal hemichamber does not reduce I_{sc} as long as the serosal side remained oxygenated^[15]. Furthermore, non-everted colonic sacs oxygenated from the serosal side showed a 127% higher I_{sc} than everted sacs oxygenated from the mucosal side^[15]. One possible cause of the observed asymmetry is higher serosal than mucosal oxygen consumption (QO_2); another is some kind of barrier, present at the mucosal but not at the serosal side, which hinders oxygen diffusion.

Our working hypotheses were, first, that the observed asymmetries are due to an intrinsic property of the epithelium, and therefore they should be found under different experimental treatments. Second, that the epithelial hindrance to oxygen diffusion may be different for transfer from lumen to interstitium than in the reverse direction. This may be deemed a rectifying behavior by analogy to electrical devices. Rectification is a property of diodes, which are electrical devices that allow current flow in one direction far more easily than in the other. Some membrane ion channels display rectifying behavior, since they show a nonlinear relationship between the driving force (potential difference) and the resulting current^[16].

The aims of this paper are, first, to assess the differences in oxygen consumption provided from the apical side of the epithelium and from its serosal side during different experimental treatments and, second, to measure the partial pressure difference needed for oxygen transfer from the mucosal to the serosal side and for oxygen transfer in the opposite direction.

MATERIALS AND METHODS

Animals

Wistar-Hokkaido male rats weighing 250-300 g were housed and managed according to the guidelines for animal care and biosafety of our Medical School. The animals were kept at an environmental temperature of $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$ with a 12-h light-dark cycle.

All animals were given food and drink *ad libitum*. A group of rats drank tap water and ate a standard rat chow containing 1.77 mg of sodium per gram of food (76.9 mEq/kg; Cargill Co.). A second group was given distilled water and a low-sodium diet with 0.161 mg of sodium per gram of food (7 mEq/kg; ICN Flow Catalog # 902902) for 10 d. The low-sodium diet was purchased from ICN, Inc. (Costa Mesa, California, United States). Daily food consumption was recorded in both groups.

All procedures involving the care and use of animals were approved by the Committee for Animal Care and Use of the Faculty of Medical Sciences, National University of Cuyo. This study was reviewed and approved by the Secretaría de Ciencia y Técnica, National University of Cuyo, institutional review board.

Serum aldosterone determination

Blood samples from all rats fed with the sodium-deficient diet and an equal number of rats fed with standard chow were obtained during the surgical procedure. Blood was allowed to clot; serum was extracted and frozen at -70°C . Aldosterone concentration in stored sera was afterwards measured with a coated-tube radioimmunoassay (Diagnostic Products Corporation, Los Angeles, California, United States).

Dissection and mounting

Under ether anesthesia, the entire colon was excised. A 3-cm segment was cut from the descending portion just above the pelvic brim and dissected to obtain an isolated mucosa preparation, as previously described^[15]. The segment was cut open along its mesenteric border, the adherent mucus gel layer was gently removed with a cotton tip soaked in dissecting solution and the segment was mounted as a flat sheet in a modified Ussing chamber (described below).

Solutions and gas mixtures

The composition of the standard Ringer solution employed was as follows (in mmol/L): Na 132.8; K 4.5; Ca 1.25; Mg 1.0; Cl 114.0; HCO_3 24.0; H_2PO_4 1.0; SO_4 1.0; D (+) glucose 10.0. In the low-chloride solution, most chloride was replaced by sulfate, with the addition of mannitol to compensate for the difference in osmolality^[11], as follows (in mmol/L): Na 136.6; K 4.5; Ca 1.25; Mg 1.0; Cl 2.5; HCO_3 24.0; H_2PO_4 2.0; SO_4 58.1; D (+) glucose 10.0; mannitol 93.4. The osmolality of both solutions was 280 mOsm/kg H_2O .

Two gas mixtures were used: 950 mL/L O_2 - 50

mL/L CO_2 (normoxic mixture) and 950 mL/L N_2 - 50 mL/L CO_2 (hypoxic mixture). The gas mixtures were purchased from Air Liquide, Inc. (Buenos Aires, Argentina). Their compositions were certified by the company. When gassed with either gas mixture, the pH of both solutions was 7.40.

Drugs

Bumetanide, 3-methyl-1-isobutylxanthine (IBMX), serotonin, and amiloride were purchased from Sigma-Aldrich (St Louis, Missouri, United States), and diphenylamine-2-carboxylate (DPC) from ICN, Inc (Costa Mesa, California, United States). The appropriate drug solutions were prepared just before each experiment. The standard Ringer solution was employed to dissolve serotonin and carbachol; dimethylsulfoxide was used for IBMX and bumetanide, and absolute ethanol was used for amiloride and DPC. As indicated, bumetanide, IBMX and serotonin were added to the serosal hemichamber, while DPC and amiloride were added to the mucosal hemichamber to achieve the following concentrations (in mmol/L): bumetanide 0.1; DPC 0.5; IBMX 0.1; serotonin 0.1, and amiloride 0.1. At the start of each experiment, 91 $\mu\text{g/mL}$ gentamycin (Schering-Plough, Buenos Aires, Argentina) was added to each hemichamber, to prevent bacterial overgrowth.

Oxymetric Ussing chamber and electrophysiological measurements

For this report, a modified Ussing chamber which has already been described and validated was used^[17]. The chamber is airtight and it has a 1 cm^2 window between the hemichambers. Polarimetric oxygen meters (CellOx 325, WTW GmbH, Weilheim, Germany) are attached to each hemichamber. This arrangement allows measurement of the oxygen concentration and its time course in both the hemichamber facing the basolateral (serosal) side of the epithelium and the hemichamber facing its apical (mucosal) side. The chamber content was kept at 37°C with a water jacket fed from a thermostatic reservoir.

The transepithelial potential difference was recorded with calomel electrodes connected to each hemichamber through saline bridges (agar-in-Ringer, 30 g/L). Ag-AgCl electrodes were used to supply current to the chamber from an amplifier, in order to clamp the transepithelial potential difference (TPD) at 0 mV. The output of the amplifier took into account corrections for solution resistance and bridge asymmetry. The short-circuit condition was kept throughout all experiments, except for the brief periods of release needed to measure open circuit TPD. The transepithelial resistivity was calculated, according to Ohm's law, as the quotient between TPD and I_{sc} .

Experimental procedures

Before each experiment, oxygen probes were calibrated according to the user's manual and had their

slopes checked and recorded. At the beginning of each experiment, the hemichambers were gassed with the normoxic mixture until a plateau of their oxygen concentration was reached. Afterwards both hemichambers were hermetically closed. QO_2 was measured under baseline conditions for 30-min periods, starting 60 min after the closure of the hemichambers.

Solution replacement was carried out without opening the chamber with a gravity driven system which allowed passing from a water-jacketed reservoir (37 °C) through the chamber a volume of low-chloride solution 10-fold larger than the chamber volume, with the overflow being drained through siphons. In experiments involving replacement of Ringer with low-chloride solution or addition of chloride secretion blockers, I_{sc} was allowed to stabilize during 20 min before QO_2 was measured for the next 30 min. On the other hand, the 30-min QO_2 measurement was started immediately after addition of IBMX or serotonin.

The change in oxygen concentration in each hemichamber during the measurement period was used to calculate QO_2 , as detailed elsewhere^[11]. In experiments assessing oxygen transfer between the hemichambers, a baseline QO_2 was obtained after attaining the same oxygen level in both hemichambers. Then hypoxia was induced in one hemichamber by gassing its contents with 950 mL/L N_2 - 50 mL/L CO_2 for various times while bubbling 950 mL/L O_2 - 50 mL/L CO_2 in the other hemichamber, to obtain graded differences in oxygen pressure (ΔP_{O_2}) between the hemichambers. Afterwards the chamber was closed and the rate of change in oxygen concentration (ΔCO_2) in the hypoxic hemichamber was plotted against the ΔP_{O_2} between both sides.

After a 15-min period of reoxygenation of both hemichambers, the procedure described above was repeated for inducing hypoxia in the other hemichamber. The ΔP_{O_2} was calculated as the difference in mean P_{O_2} of each hemichamber during each hypoxic period. The order in which hypoxia was induced in each hemichamber was switched between experiments to avoid a possible effect derived from the hemichamber which was made hypoxic first. Finally, both hemichambers were reoxygenated and a second control QO_2 measurement was performed.

After each experiment, the mucosa was replaced with a polyethylene membrane for a blankrun. The experiment was discarded if the decrease in oxygen concentration was above 5% of that observed with the biological sample.

Statistical analysis

QO_2 in the serosal and mucosal hemichambers and I_{sc} under each tested condition were compared with a paired, two-sided Student's *t* test. An unpaired, two-sided Student's *t* test was employed for analysis of food intake, sodium intake, and serum aldosterone in rats fed with normal sodium diet and in those submitted to the low-sodium diet. During analysis, significant

deviations from a Gaussian distribution were ruled out with the Kolmogorov-Smirnov test.

A one-way analysis of variance with Geisser-Greenhouse correction was used to assess differences in the magnitude of the serosal vs mucosal difference in QO_2 under all conditions tested. Simple linear regression was used to assess the relationships between I_{sc} and ΔP_{O_2} , and between ΔP_{O_2} and ΔCO_2 . Checks for significant deviation from linearity and outliers were performed for all comparisons.

A commercial software was employed for statistical analyses (GraphPad Prism version 5.1 for Windows, GraphPad Software, San Diego, California, Δ). Values are reported as means $\pm$ SEM, unless otherwise stated. The significance level was set at $P < 0.05$.

RESULTS

Mean daily food intake of rats fed with the low-sodium diet (80.0 ± 1.4 g per kilogram body weight; $n = 6$) was not significantly different from that of rats given standard chow (82.1 ± 0.9 g per kilogram body weight; $n = 20$). The respective mean daily sodium intakes per kilogram body weight were 12.9 ± 0.3 mg and 145.3 ± 1.6 mg ($P < 0.0001$). As expected, serum aldosterone concentration was significantly higher in rats with low sodium intake (10.49 ± 2.1 nmol/L; $n = 6$) than in controls (1.42 ± 0.26 nmol/L; $n = 6$, $P = 0.0016$).

Values of I_{sc} , total QO_2 , serosal QO_2 , and mucosal QO_2 for epithelial samples are shown in Table 1. Compared with controls, I_{sc} and QO_2 were higher in the presence of chloride secretagogues and in epithelial samples from sodium-deprived rats. Conversely, I_{sc} and QO_2 were lower in low-chloride solution, in the presence of chloride secretion blockers or, in tissues from sodium-deprived animals, after addition of amiloride. Under all conditions tested, serosal oxygen consumption was higher than mucosal oxygen consumption (Figure 1). The magnitude of this difference was similar for all treatments ($P = 0.0847$ according to one-way analysis of variance).

I_{sc} was correlated with total QO_2 and serosal QO_2 both under baseline condition and during mucosal hypoxia (Figure 2), but those correlations were lost during serosal hypoxia. On the other hand, I_{sc} was not correlated with mucosal QO_2 under any condition (Table 2).

When different oxygen tension differences were imposed between both hemichambers, the oxygen content of the hypoxic hemichamber increased with time when ΔP_{O_2} was high but decreased when it was low (Figure 3). The relationship was linear when hypoxia was induced in either hemichamber. The slope for hypoxia induced in the mucosal hemichamber was $0.0535 \pm 0.009 \mu\text{mol} \times \text{cm}^{-2} \times \text{h}^{-1}$ per kPa. The slope for hypoxia induced in the serosal hemichamber was $0.0494 \pm 0.014 \mu\text{mol} \times \text{cm}^{-2} \times \text{h}^{-1}$ per kPa ($P = 0.8244$ for the comparison of both slopes). However, the calculated ΔP_{O_2} at which

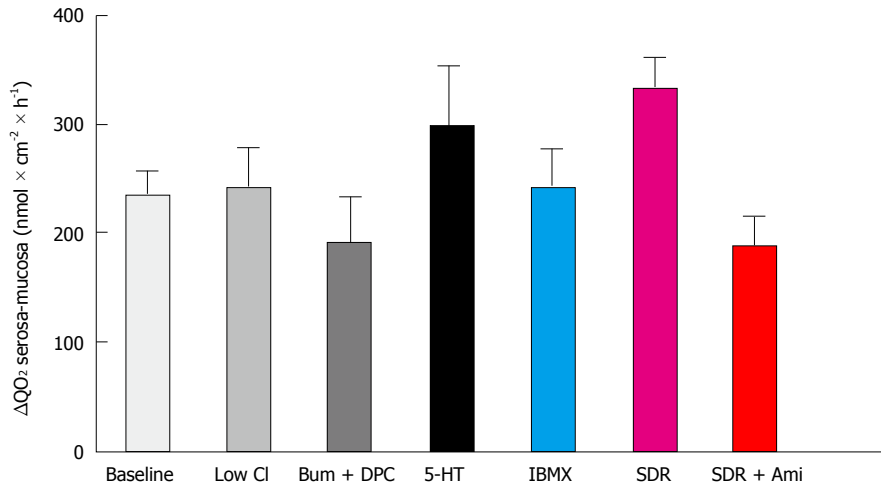


Figure 1 Differences in serosal vs mucosal oxygen consumption of rat sigmoid colon epithelium under several conditions. No significant difference between groups was found, as assessed by one-way analysis of variance ($P = 0.0849$). ΔQO_2 : Serosa-mucosa, difference between serosal and mucosal oxygen consumption; Bum: Bumetanide; DPC: Diphenylamine-2-carboxylate; IBMX: 3-methyl-1-isobutylxanthine; SDR: Sodium-deprived rats; Ami: Amiloride.

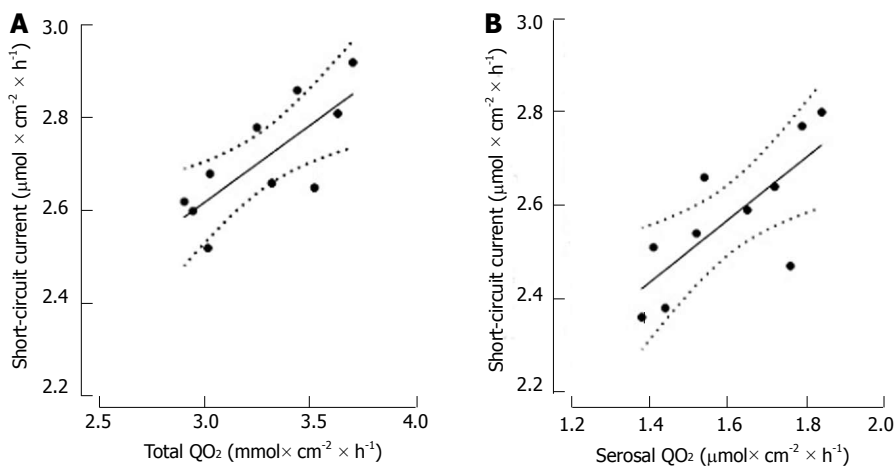


Figure 2 Linear regression of short-circuit current vs total oxygen consumption during normoxia (A), and vs serosal oxygen consumption during mucosal hypoxia (B). Regression coefficients were 0.765 for A ($P = 0.009$) and 0.754 for B ($P = 0.011$). No outliers were detected.

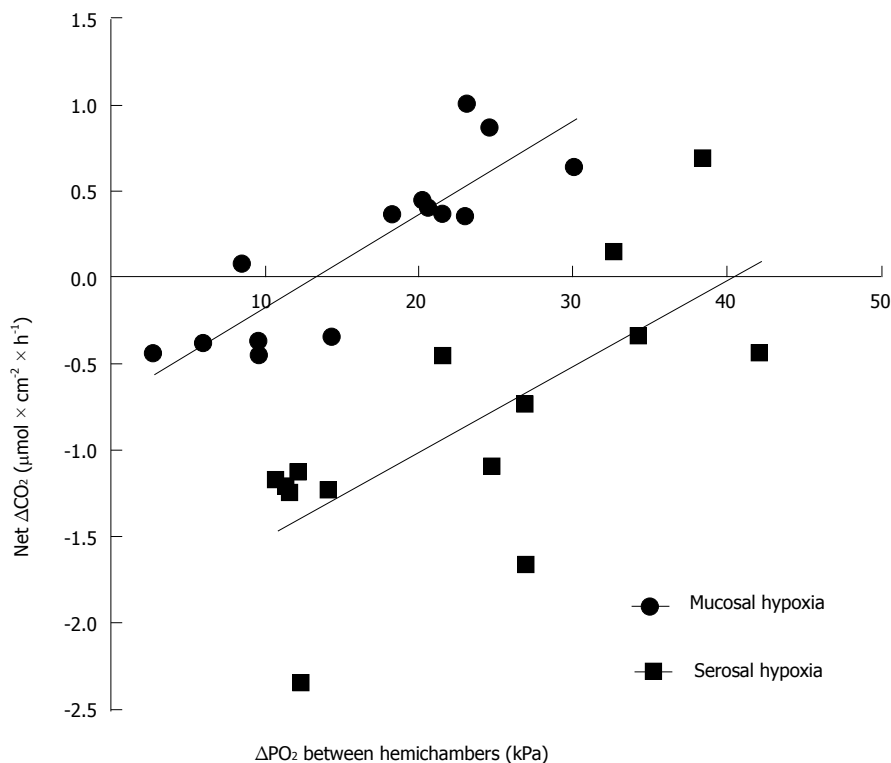


Figure 3 Rate of change in oxygen content of the hypoxic hemichamber as a function of the difference in oxygen partial pressure between hemichambers. Various degrees of hypoxia were induced in either the serosal or the mucosal hemichamber while keeping the opposite hemichamber fully oxygenated, and the change in oxygen content was plotted as a function of the mean difference in oxygen pressure between both hemichambers during a 30-min observation period. The slope of the relationships was the same when either the serosal or the mucosal hemichamber was hypoxic ($P = 0.8244$), but the oxygen pressure difference at which there was no net change in oxygen content in the hypoxic hemichamber was larger when hypoxia was induced in the serosal hemichamber ($P < 0.0001$). ΔCO_2 : Change in oxygen content of the hypoxic hemichamber; ΔPO_2 : Oxygen pressure difference between hemichambers.

Table 1 Short-circuit current and oxygen consumption of the isolated mucosa of rat sigmoid colon

Treatment	<i>n</i>	I _{sc} μmol × cm ⁻² × h ⁻¹	Total QO ₂ μmol × cm ⁻² × h ⁻¹	Serosal QO ₂ μmol × cm ⁻² × h ⁻¹	Mucosal QO ₂ μmol × cm ⁻² × h ⁻¹
None	20	3.33 ± 0.21	2.79 ± 0.06	1.52 ± 0.04	1.28 ± 0.08 ^b
Low Cl	6	0.66 ± 0.07	2.56 ± 0.13	1.41 ± 0.08	1.14 ± 0.06 ^d
Bum + DPC	6	0.63 ± 0.08	2.47 ± 0.15	1.35 ± 0.07	1.15 ± 0.08 ^d
Serotonin	6	5.30 ± 0.29	3.64 ± 0.13	1.97 ± 0.08	1.67 ± 0.06 ^d
IBMX	6	3.98 ± 0.24	3.22 ± 0.09	1.75 ± 0.07	1.47 ± 0.06 ^d
SDR	6	8.50 ± 0.45	3.71 ± 0.35	2.02 ± 0.15	1.68 ± 0.12 ^b
SDR + Ami	6	0.33 ± 0.05	2.88 ± 0.12	1.53 ± 0.07	1.35 ± 0.08 ^d

^b*P* < 0.0001 and ^d*P* < 0.01 for the difference between serosal and mucosal oxygen consumption. I_{sc}: Short-circuit current; QO₂: Oxygen consumption; Bum: Bumetanide; DPC: Diphenylamine-2-carboxylate; IBMX: 3-methyl-1-isobutylxanthine; SDR: Sodium-deprived rats; Ami: Amiloride.

Table 2 Relationship between short-circuit current and epithelial oxygen consumption of the isolated mucosa of rat sigmoid colon

Condition	Comparison	R ²
Baseline	I _{sc} vs total QO ₂	0.586 ^b
	I _{sc} vs serosal QO ₂	0.620 ^c
	I _{sc} vs mucosal QO ₂	0.299
Mucosal hypoxia	I _{sc} vs total QO ₂	0.633 ^d
	I _{sc} vs serosal QO ₂	0.569 ^b
	I _{sc} vs mucosal QO ₂	0.388
Serosal hypoxia	I _{sc} vs total QO ₂	0.164
	I _{sc} vs serosal QO ₂	0.315
	I _{sc} vs mucosal QO ₂	0.361

^b*P* = 0.009; ^c*P* = 0.018; ^d*P* = 0.006; ^e*P* = 0.011; *n* = 10. I_{sc}: Short-circuit current; QO₂: Oxygen consumption.

there was no change in oxygen content of the hypoxic hemichamber ($\Delta\text{CO}_2 = 0$) was three times higher for serosal hypoxia ($\Delta\text{PO}_2 = 40$ kPa) than for mucosal hypoxia ($\Delta\text{PO}_2 = 13$ kPa): *P* < 0.0001.

DISCUSSION

Present results show that, when oxygen is available from both the serosal and the mucosal sides to distal colonic epithelium, the tissue consumes more oxygen supplied from the serosal than from the mucosal side. Since blood supply plays no role in oxygen availability under present *in vitro* conditions, the observed preference may not be explained by the path through which oxygen is normally provided to the epithelium. Furthermore, the higher serosal QO₂ was observed under a variety of conditions, including those in which chloride secretion is the major electrogenic phenomenon and those in which the major electrogenic phenomenon is sodium absorption. This suggests that the asymmetry is also not due to the specific ion being transported but represents a physiological characteristic of the colonic epithelium. It should be noted that this asymmetry has also been demonstrated in epithelial samples from human sigmoid colon^[18].

The importance of oxygen supply from the serosal side to sustain electrogenic transport was further corroborated by the correlation between I_{sc} and serosal

QO₂ under baseline condition and during hypoxia induced in the mucosal hemichamber, while no such correlation was present during hypoxia induced in the serosal hemichamber. On the other hand, no correlation was found between I_{sc} and mucosal QO₂ in any condition tested.

When graded hypoxia is induced in one hemichamber, while the other is saturated with oxygen, theoretically the oxygen content of the hypoxic hemichamber could decrease, remain constant, or increase with time. It would decrease if the hypoxic chamber still provides part of the oxygen consumed by the epithelium. It would remain constant if all oxygen consumed by the epithelium is provided by the oxygenated chamber and there is no net oxygen transfer between the hemichambers. Finally, it would actually increase if the oxygenated hemichamber, apart from providing all the oxygen that the epithelium consumes, transfers part of its oxygen to the hypoxic hemichamber. The relationship between ΔCO_2 in the hypoxic hemichamber and ΔPO_2 between the hemichambers was linear and showed the same slope for serosal-to-mucosal transfer than for mucosal-to-serosal transfer. Remarkably, however, the ΔPO_2 at which there was no net oxygen transfer between the hemichambers was three-fold higher for mucosal-to-serosal transfer than for transfer in the reverse direction, indicating that a larger ΔPO_2 between hemichambers is needed for mucosal-to-serosal transfer than for serosal-to-mucosal transfer. This may be characterized as a rectifying behavior of oxygen transfer.

One explanation for the larger contribution of oxygen supply from the serosal side may be that the oxygen permeability of the basolateral membrane to oxygen is larger than the oxygen permeability of the apical membrane. While it is classically accepted that biological membranes are very permeable to gases, low permeability of some membranes to gases such as ammonia or carbon dioxide has been reported, for example in gastric glands^[19] and colonic epithelium^[20]. This issue has been recently reviewed, and the existence of gas channels has been postulated^[21,22]. There are few reports on membrane oxygen permeability^[21] and the results are contradictory^[22].

Even if apical and basolateral epithelial membranes

had different oxygen permeability, this would not explain *per se* the apparent rectifying behavior of oxygen transfer. We are not aware of reports on this topic for oxygen, or for any other gas. Since gases generated in the colon as products of bacterial fermentation are partly transferred into the bloodstream^[10], it is of physiological and clinical interest to assess whether their rate of transfer from the lumen to the interstitium is selectively limited, as it seems to be the case for oxygen.

In conclusion, the sigmoid colon epithelium *in vitro* in a rat model preferentially consumes oxygen supplied from the serosal side. I_{sc} correlates with serosal oxygen consumption, but not mucosal oxygen consumption. The ΔP_{O_2} for inducing net mucosal-to-serosal transfer is higher than the ΔP_{O_2} for inducing serosal-to mucosal transfer, therefore indicating a rectifying behavior for transepithelial oxygen transfer.

COMMENTS

Background

The colonic epithelium has a high oxygen consumption, a large part of which is needed to sustain electrogenic ion transport. *In vivo*, oxygen is mostly supplied from the serosal side, since the luminal environment has a very low oxygen partial pressure. When the epithelium is placed in an Ussing chamber, oxygen is usually available from both sides of the epithelium, but even then, the serosal supply is more important to sustain electrogenic ion transport than the mucosal supply.

Research frontiers

The intestinal epithelium is normally submitted to a relatively low oxygen pressure when compared with other tissues, a condition known as physiological hypoxia. This makes the epithelium susceptible to hypoxic injury when the oxygen supply is further compromised by disease.

Innovations and breakthroughs

In this paper, it is shown that serosal supply provides more oxygen than mucosal supply to the colonic epithelium under several different conditions, suggesting that the difference is an intrinsic property of the tissue. For the first time, evidence is provided suggesting that transepithelial oxygen diffusion from serosa to mucosa needs a lower gradient of oxygen pressure than diffusion from mucosa to serosa.

Applications

Since epithelial hypoxia seems to be an important cause of injury and dysfunction in several conditions affecting the intestinal epithelium, a deeper knowledge of the factors influencing epithelial oxygen supply may help to understand their pathophysiology and to devise better management strategies.

Terminology

Rectification: A term borrowed from electronics, referring to devices such as diodes, which allow electric current to flow more easily in one direction than in the opposite one. In the present context, it is applied to the observation that oxygen diffuses more easily from the serosal to the mucosal side of the epithelium than in the opposite direction. Short-circuit current: The electrical current passing through the epithelium needed to keep the transepithelial potential difference at 0 mV. It is a measure of electrogenic ion transport.

Peer-review

The aim of this study was to assess whether higher sensitivity of colonic epithelium to hypoxia at the serosal side is associated with oxygen transfer asymmetry. It is a very well design study and the issue is elegantly developed.

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P- Reviewer: Huerta-Franco MR, Koch TR **S- Editor:** Song XX
L- Editor: A **E- Editor:** Wu HL



Basic Study

Combination curcumin and vitamin E treatment attenuates diet-induced steatosis in *Hfe*^{-/-} mice

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Author contributions: Heritage M, Jaskowski L and Briskey D performed all experiments for the study; Campbell C undertook histological scoring; Heritage M, Bridle K, Britton L, Fletcher L, Vitetta L, Subramaniam VN and Crawford D were responsible for study design, data analysis and manuscript editing; Heritage M and Bridle K wrote the manuscript; Subramaniam VN and Crawford D were the joint senior authors.

Institutional review board statement: No human subjects were used in this study.

Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the

Animal Ethics Committees of QIMR Berghofer Medical Research Institute (P829) and the University of Queensland (313/11).

Conflict-of-interest statement: The authors report no conflicts of interest.

Data sharing statement: No additional data are available.

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Manuscript source: Invited manuscript

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Received: August 28, 2016

Peer-review started: September 1, 2016

First decision: October 31, 2016

Revised: December 1, 2016

Accepted: February 28, 2017

Article in press: March 2, 2017

Published online: May 15, 2017

Abstract**AIM**

To investigate the synergistic hepato-protective properties of curcumin and vitamin E in an *Hfe*^{-/-} high calorie diet model of steatohepatitis.

METHODS

Hfe^{-/-} C57BL/6J mice were fed either a high calorie diet or a high calorie diet with 1 mg/g curcumin; 1.5 mg/g vitamin E; or combination of 1 mg/g curcumin + 1.5 mg/g vitamin E for 20 wk. Serum and liver tissue were collected at the completion of the experiment. Liver histology was graded by a pathologist for steatosis, inflammation and fibrosis. RNA and protein was extracted from liver tissue to examine gene and protein expression associated with fatty acid oxidation, mitochondrial biogenesis and oxidative stress pathways.

RESULTS

Hfe^{-/-} mice fed the high calorie diet developed steatohepatitis and pericentral fibrosis. Combination treatment with curcumin and vitamin E resulted in a greater reduction of percent steatosis than either vitamin E or curcumin therapy alone. Serum alanine aminotransferase and non-alcoholic fatty liver disease (NAFLD) activity score were decreased following combination therapy with curcumin and vitamin E compared with high calorie diet alone. No changes were observed in inflammatory or fibrosis markers following treatment. Epididymal fat pad weights were significantly reduced following combination therapy, however total body weight and liver weight were unchanged. Combination therapy increased the mRNA expression of *AdipoR2*, *Ppar-α*, *Cpt1a*, *Nrf-1* and *Tfb2m* suggesting enhanced fatty acid oxidation and mitochondrial biogenesis. In addition, combination treatment resulted in increased catalase activity in *Hfe*^{-/-} mice.

CONCLUSION

Combination curcumin and vitamin E treatment decreases liver injury in this steatohepatitis model, indicating that combination therapy may be of value in NAFLD.

Key words: Non-alcoholic fatty liver disease; Hemochromatosis; Iron overload; Steatosis; High calorie diet

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Core tip: The high prevalence of obesity and the metabolic syndrome suggests that many patients with liver disease of varying etiologies will have co-existent non-alcoholic fatty liver disease. Our model of co-toxic liver disease incorporates increased hepatic iron in combination with steatosis and was associated with necroinflammation and early hepatic fibrosis. Because of the beneficial effect of combination therapy, we believe vitamin E and curcumin should be investigated in other animal models of non-alcoholic steatohepatitis. Should beneficial effects be demonstrated, combination treatment with the development of appropriate dosing strategies could be rapidly moved to human studies allowing for an effective treatment strategy.

Heritage M, Jaskowski L, Bridle K, Campbell C, Briskey D, Britton L, Fletcher L, Vitetta L, Subramaniam VN, Crawford D. Combination curcumin and vitamin E treatment attenuates diet-induced steatosis in *Hfe*^{-/-} mice. *World J Gastrointest Pathophysiol*

2017; 8(2): 67-76 Available from: URL: <http://www.wjgnet.com/2150-5330/full/v8/i2/67.htm> DOI: <http://dx.doi.org/10.4291/wjgp.v8.i2.67>

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease in Western countries^[1] and encompasses a range of hepatic pathologies from simple steatosis to the more aggressive non-alcoholic steatohepatitis (NASH)^[2]. Homozygosity for the C282Y mutation in the *HFE* gene is the most common cause of hereditary hemochromatosis (HH)^[3]. Steatosis is common in patients with HH^[4] and is associated with increased hepatic fibrosis; conversely, heterozygosity for the C282Y mutation in *HFE* is common in patients with NAFLD^[5]. Even mild increases in hepatic iron concentration (HIC) may play an important role in the transition from simple steatosis to NASH as *Hfe*^{-/-} mice fed a high calorie diet develop NASH, impaired anti-oxidant activity and accelerated liver injury^[6]. This relationship between HFE, altered iron metabolism and the development of NAFLD could be the target for therapeutic strategies that may attenuate disease progression in NASH. Curcumin, the yellow pigment of the plant *Curcuma longa* (turmeric), has potent antioxidant and chemo preventative effects^[7]. Curcumin treatment produces beneficial effects in many animal models of liver diseases^[8,9]. Curcumin ameliorates the early stages of experimental steatohepatitis and limits the development and progression of fibrosis in mice fed a methionine choline deficient diet^[10,11]. In addition, a recent randomised controlled trial of curcumin in fatty liver patients demonstrated a reduction in NASH features^[12]. The anti-oxidant, vitamin E has been shown to be beneficial in animal models of liver disease and in subjects with NASH^[13-15].

Curcumin and vitamin E have been used with therapeutic benefit in experimental models of NASH^[10,11,14]. To date, these agents have mostly been used at the initiation of injury which does not validate their utility in NASH in the usual clinical situation of already established injury and ongoing exposure to excess calorie intake. We hypothesized that these two agents may have synergistic activity in attenuating disease progression in NASH. Thus, in the present study, we investigated combination use of curcumin and vitamin E in the treatment of *Hfe*^{-/-} mice with established liver injury induced by a high-fat, high-carbohydrate diet, and with ongoing exposure to the diet. Our results show that combination treatment of curcumin and vitamin E decreased liver injury and hepatic steatosis suggesting that this treatment may be of therapeutic value in NAFLD.

MATERIALS AND METHODS**Animals**

All animals received humane care under the guidelines and approval of the QIMR Berghofer Medical Research

Institute and The University of Queensland Animal Ethics Committees. Eight-week-old male *Hfe*^{-/-} mice (on a C57BL/6J background)^[16], were fed a high calorie diet (HCD) (SF03-020, Speciality Feeds, Glen Forrest, WA, Australia) (*n* = 34) for a period of 10 wk. The mice were then randomly assigned (*n* = 7-9 per group) to receive either HCD alone, or HCD and 1 mg/g curcumin (Curcumin C³ complex, Sabinsa Corporation, Sydney, NSW, Australia), HCD and 1.5 mg/g vitamin E or HCD and a combination of 1 mg/g curcumin and 1.5 mg/g vitamin E for a further 10 wk (Specialty Feeds). The fat composition of the HCD by weight was 150 mg/g saturated fat, 20 mg/g polyunsaturated and 60 mg/g monounsaturated fat, derived from a predominant mixture of cocoa butter (50 mg/g), partially hydrogenated vegetable oil (131 mg/g) and canola oil (50 mg/g) with added cholesterol (1.9 mg/g). Animals were allowed *ad libitum* access to diets and drinking water. After 20 wk of dietary treatment, animals were sacrificed under anaesthesia following cardiac puncture for blood collection. The livers were removed and portions were snap-frozen in liquid nitrogen and stored at -80 °C prior to analysis. Separate portions were collected for histology or dried for the determination of hepatic iron concentration (HIC). Epididymal fat pads were weighed and stored at -80 °C.

Histopathological analysis

Liver samples were fixed in 40 g/L neutral-buffered formalin, embedded in paraffin, sectioned and stained with haematoxylin-eosin (H and E). Histological parameters were staged and graded according to accepted criteria by a specialist liver pathologist in a blinded fashion^[17]. Steatosis was graded according to the percentage of steatotic hepatocytes (grade 0, < 5% affected; grade 1, 5%-33% affected; grade 2, 34%-66% affected; and grade 3, > 66% affected). Lobular inflammatory activity was scored based on the number of inflammatory foci per 200x field (0, none; 1, < 2 seen; 2, 2-4 seen; and 3, > 4 seen) and ballooning was scored based on the degree of hepatocyte ballooning (0, none; 1, few; and 2, many). Activity was also scored using NAFLD activity score (NAS) established by the NASH Clinical Research Network (CRN), which is an unweighted sum of scores for steatosis (0-3), lobular inflammation (0-3) and ballooning (0-2)^[17].

Fibrosis stage was assessed following Picro Sirius Red staining for collagen according to the criteria established by Brunt *et al.*^[18].

Serum biochemistry and hepatic iron concentration

Serum concentrations of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured on a Cobas Integra 400 Chemistry Automated Analyser as per manufacturer's instructions (Roche Diagnostics, Castle Hill, NSW, Australia). HIC was measured as previously described^[6,19].

Hepatic antioxidant enzyme assay

Total cellular glutathione peroxidase (GPx), reduced

(GSH) and oxidized glutathione (GSSG), catalase and mitochondrial manganese superoxide dismutase (MnSOD) activities were measured on homogenized liver tissue using commercial assay kits as per manufacturer's instructions (Cayman Chemical, Ann Arbor, MI, United States).

RNA extraction and quantitative real-time polymerase chain reaction

Total RNA was extracted using TRIzol reagent (Invitrogen, Mount Waverley, Victoria, Australia), subjected to deoxyribonuclease I digestion and transcribed into cDNA using Superscript III according to the manufacturer's instructions (Invitrogen). Quantitative gene expression was performed by real-time polymerase chain reaction (RT-PCR) (ViiATM 7, Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, United States) using Quantifast SYBR green as per manufacturer's conditions (Qiagen, Chadstone Centre, VIC, Australia). The expression of individual genes were normalised to the geometric mean of three house-keeper genes: Basic transcription factor 3 (*Btf-3*), Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and β 2-microglobulin (*β 2-m*). Mouse primer sequences for genes investigated are shown in Supplementary Table 1.

Western blot and densitometry analysis

Protein extracts (30 μ g) from liver were electrophoresed in sodium dodecyl sulphate-10% polyacrylamide gel for 30 min at 100 V and blotted onto polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA, United States) as previously described^[6]. Membranes were immunostained with primary antibodies for ACOX (Abcam, Cambridge, MA, United States) and mitoNEET (Proteintech, Chicago, IL, United States). Signals were detected using standard chemiluminescence (Supersignal West Femto, ThermoFisher Scientific, Scoresby, Victoria, Australia) on an Image Station 4000MM Pro and quantified using Carestream Molecular Imaging software (Carestream Australia, East Melbourne, Victoria, Australia).

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 22 (IBM, Armonk, NY, United States). Non-parametric tests were used to evaluate differences between groups, and data are depicted using box and whisker plots showing median, minimum and maximum values. A Kruskal-Wallis test with Dunn's correction for multiple comparisons was performed to compare intervention groups. A cut-off *P* value of 0.05 was used to determine significance. Statistical review of the manuscript was performed by a biomedical statistician.

RESULTS

Serum transaminase concentrations are decreased by curcumin and vitamin E combination therapy

Total body weight and liver weight were not altered by dietary treatment in *Hfe*^{-/-} mice (Figure 1A). Epididymal

Table 1 Percent steatosis, lobular and non-alcoholic fatty liver disease activity score were reduced due to combination therapy

	HCD (<i>n</i> = 9)	HCD + Cu (<i>n</i> = 9)	HCD + VE (<i>n</i> = 7)	HCD + Cu + VE (<i>n</i> = 9)
Histology diagnosis	NASH (9)	Steatosis (2)/NASH (7)	Steatosis (2)/NASH (5)	Steatosis (4)/NASH (5)
Steatosis grade	3 (3-3)	3 (2-3)	3 (2-3)	3 (2-3)
% steatosis	95 (80-100)	80 (40-100)	95 (70-100)	75 (40-95) ^a
Lobular score	2 (1-2)	2 (1-3)	1 (0-2)	1 (0-3)
Ballooning	2 (1-2)	2 (1-2)	1 (1-2)	1 (0-2)
NAS score	6 (5-7)	6 (4-8)	6 (4-6)	5 (3-8)
Fibrosis stage	1a (0-1a)	0 (0-1a)	0 (0-1a)	0 (0-1a)

Values are expressed as the median (range). ^a*P* < 0.05 vs HCD alone, Kruskal-Wallis ANOVA. HCD: High calorie diet; Cu: Curcumin; VE: Vitamin E; NAS: Non-alcoholic fatty liver disease activity score.

fat pad weight was significantly reduced in the combination treatment group compared to curcumin alone (*P* < 0.02, Figure 1A).

Serum ALT concentrations were significantly reduced by combination treatment compared to HCD alone (*P* = 0.02, Figure 1B). AST concentrations were not significantly different across groups (*P* = 0.12). There was no significant effect of dietary treatment on HIC (*P* < 0.30, Figure 1B).

Attenuation of severe steatosis and lobular inflammation by curcumin and vitamin E combination therapy

Hfe^{-/-} HCD-fed mice developed moderate to severe steatosis with hepatocyte ballooning and inflammatory cell infiltration present in 100% of mice meeting the criteria for steatohepatitis (Table 1 and Figure 2A). Combination treatment with curcumin and vitamin E reduced the presence of NASH to only 55% of mice. Combination treatment also significantly reduced percent steatosis compared with HCD alone (*P* = 0.03). Lobular inflammatory score and NAS score were also reduced in combination treated *Hfe*^{-/-} mice (Table 1 and Figure 2A). Treatment with either curcumin or vitamin E alone had minimal effects on hepatic histology (Figure 2A).

Centrilobular (stage 1) fibrosis (as detected by Sirius red staining) was observed in 7/9 *Hfe*^{-/-} mice fed the HCD and only in 3/9 combination treated mice, as evidenced by reduced staining (data not shown). However, hepatic α1(I)-procollagen (*Col1a1*) mRNA, transforming growth factor β1 (*Tgfb1*) and tissue inhibitor of metalloproteinase 1 (*Timp1*) gene expression were not significantly altered by dietary treatment (*P* = 0.05, *P* = 0.40 and *P* = 0.26 respectively, Figure 2B). Gene expression of monocyte chemoattractant protein-1 (*Mcp-1*) and two acute phase reactants, orosomucoid 1 (*Orm-1*) and serum amyloid A1 (*Saa1*) were not significantly altered by combination treatment when compared to HCD or monotherapy (*P* = 0.08, *P* = 0.07 and *P* = 0.17 respectively, Figure 2C).

Enhanced gene expression of fatty acid oxidation pathways with curcumin and vitamin E combination therapy

Hepatic adiponectin receptor 2 (*AdipoR2*) mRNA expression was significantly increased in vitamin E and combination treatment groups compared to HCD alone

(*P* = 0.02 and *P* < 0.01, respectively).

Downstream of *AdipoR2*, peroxisome proliferator-activated receptor α (*Ppara*) mRNA expression was significantly increased in combination treated *Hfe*^{-/-} mice compared to HCD alone and curcumin alone (*P* < 0.012 and *P* = 0.01, respectively, Figure 3A). Carnitine palmitoyl transferase 1A (*Cpt1a*) expression was also increased due to dietary treatments where vitamin E and combination treatment significantly increased *Cpt1a* expression in *Hfe*^{-/-} mice compared to HCD alone (*P* = 0.05 and *P* < 0.01, respectively, Figure 3A). In addition, combination therapy increased *Cpt1a* expression above curcumin treatment alone (*P* < 0.01, Figure 3A). The expression of acyl-coenzymeA oxidase 1 (ACOX1) protein, the first enzyme in the β-oxidation pathway, was significantly different following dietary intervention. Combination treatment up-regulated ACOX1 expression compared with HCD alone (*P* < 0.01, Figure 3B). These results suggest an increase in lipid metabolism by β-oxidation which has been altered by long-term HCD feeding.

Effects on hepatic antioxidant enzyme activities and mitochondrial function

Neither glutathione peroxidase activity (GPx) nor hepatic mitochondrial manganese superoxide dismutase (MnSOD) activity were significantly altered by dietary treatments (*P* = 0.4 and *P* = 0.80, respectively, Figure 4A). However both curcumin and combination treatment increased catalase when compared to HCD alone (*P* = 0.01 and *P* < 0.01, respectively, Figure 4A).

We have previously shown mitochondrial dysfunction in *Hfe*^{-/-} mice fed a HCD^[6], therefore we then investigated if combination treatment could correct this defect. Indeed, nuclear transcription factor 1 (*Nrf-1*) mRNA expression was significantly altered by dietary treatments in *Hfe*^{-/-} mice (Figure 4B), where vitamin E and combination treatment significantly increased expression compared to HCD alone (*P* = 0.01 and *P* < 0.01, respectively). Likewise, transcription factor B2 mitochondrial (*Tfb2m*) mRNA was significantly increased in *Hfe*^{-/-} mice treated with vitamin E and combination treatment compared to HCD (*P* < 0.01 and *P* < 0.01, respectively, Figure 4B). These results suggest an up-regulation in mitochondrial biogenesis due to vitamin

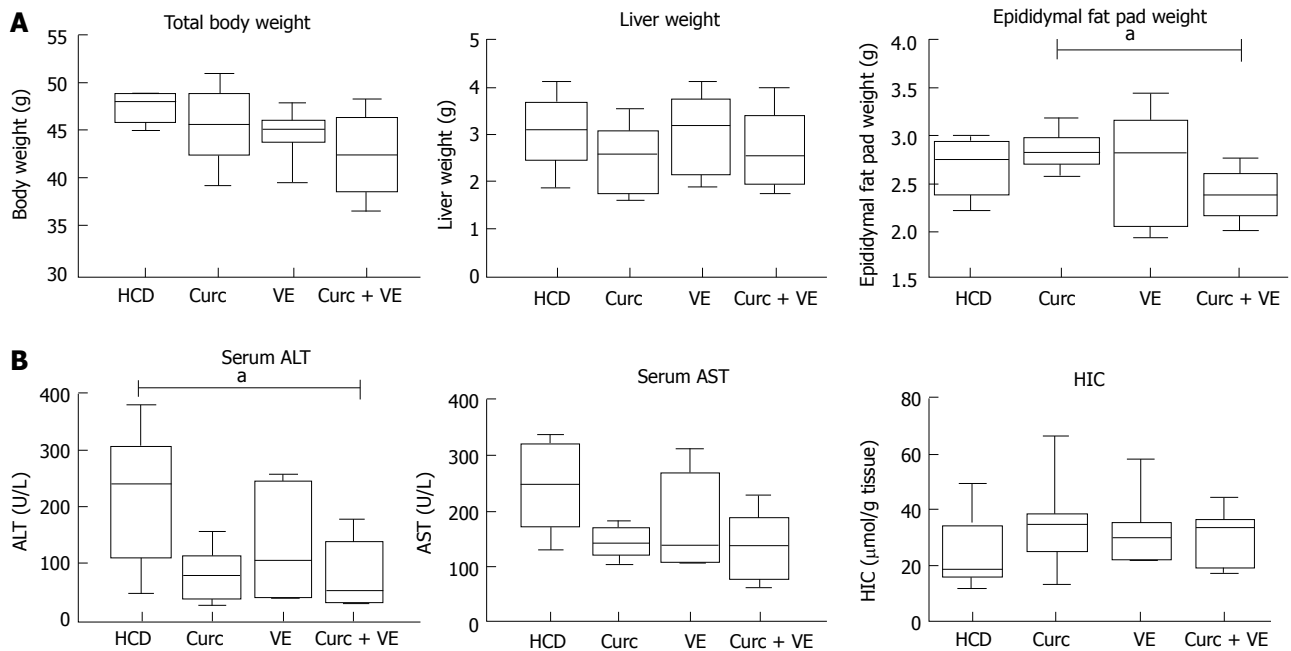


Figure 1 Epididymal fat pad weights and serum transaminase concentrations are decreased by curcumin and vitamin E combination therapy. *Hfe*^{-/-} mice were fed HCD for 10 wk then HCD + Cu, HCD + VE or HCD + Cu + VE for a further 10 wk (*n* = 7-9). A: Body, liver and epididymal fat pad weights were determined. B: Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) concentrations were determined on final cardiac bleeds. Following sacrifice, a dried liver segment was digested to determine hepatic iron concentration (HIC). Data are depicted using box and whisker plots showing median, minimum and maximum values. Statistical significance was tested using Kruskal-Wallis test with Dunn's correction for multiple comparisons, ^a*P* ≤ 0.05. HCD: High calorie diet; Cu: Curcumin; VE: Vitamin E.

E and combination treatment. Interestingly, mitoNEET, a protein that is a driving force behind mitochondrial biogenesis^[20], was not significantly different in combination treated animals compared with HCD alone (Figure 4C).

DISCUSSION

In this study we demonstrated that the combination of curcumin and vitamin E therapy attenuated steatosis and lobular inflammation in *Hfe*^{-/-} mice even with ongoing feeding of a HCD. *Hfe*^{-/-} mice fed a HCD for 20 wk developed steatosis and 100% had histological features consistent with steatohepatitis and the majority of animals showed centrilobular fibrosis. Monotherapy with either vitamin E or curcumin had no effect on percent steatosis, but combination therapy reduced percent hepatic steatosis as well as lobular inflammation, ballooning degeneration and fibrosis. This implies that vitamin E and curcumin exert a synergistic effect over that provided by each individual therapy.

Our previous studies have shown that *Hfe*^{-/-} mice fed a HCD develop steatosis and the pathophysiology of this injury involves altered β -oxidation, impaired fatty acid uptake and mitochondrial dysfunction^[6]. To assess the mechanisms through which curcumin and vitamin E may exert their beneficial effect we examined fatty acid uptake, β -oxidation and mitochondrial function in this study.

Curcumin has been reported to increase the metabolism of lipids by β -oxidation^[21,22] and this appears to

be a mechanism by which combination therapy results in reduced hepatic steatosis in this study. This was evidenced by increased *AdipoR2*, *Ppar- α* and *Cpt1a* mRNA expression in the combination fed *Hfe*^{-/-} mice; facilitating increased mitochondrial uptake of free fatty acids. *AdipoR2* has been shown to activate *Ppar- α* and fatty acid oxidation genes^[23], and these results imply that combination therapy induced *AdipoR2* expression, resulting in the up-regulation of fatty acid oxidation pathways^[24]. However, it is worth noting that the change in *AdipoR2* and *Cpt1* expression with vitamin E monotherapy alone was similar to that achieved by combination therapy. Increased ACOX1 expression, facilitating increased β -oxidation, was also observed in combination treated animals, providing further evidence of increased fatty acid β -oxidation.

We examined defence mechanisms against oxidative stress as another potential reason for the benefits of combination therapy since both curcumin and vitamin E are potent anti-oxidants. Curcumin and combination treatment in *Hfe*^{-/-} mice raised catalase activity illustrating that an up-regulation in β -oxidation was counteracted by an up-regulation of an enzyme that removes excess reactive oxygen species. These observations are consistent with other models of hepatic injury where the anti-oxidant effects of curcumin and vitamin E are mediated by altering catalase and MnSOD activity^[25,26].

Vitamin E and combination treatment increased gene expression of *Nrf-1* and *Tfb2m* which are consistent with up-regulated mitochondrial biogenesis. It has been

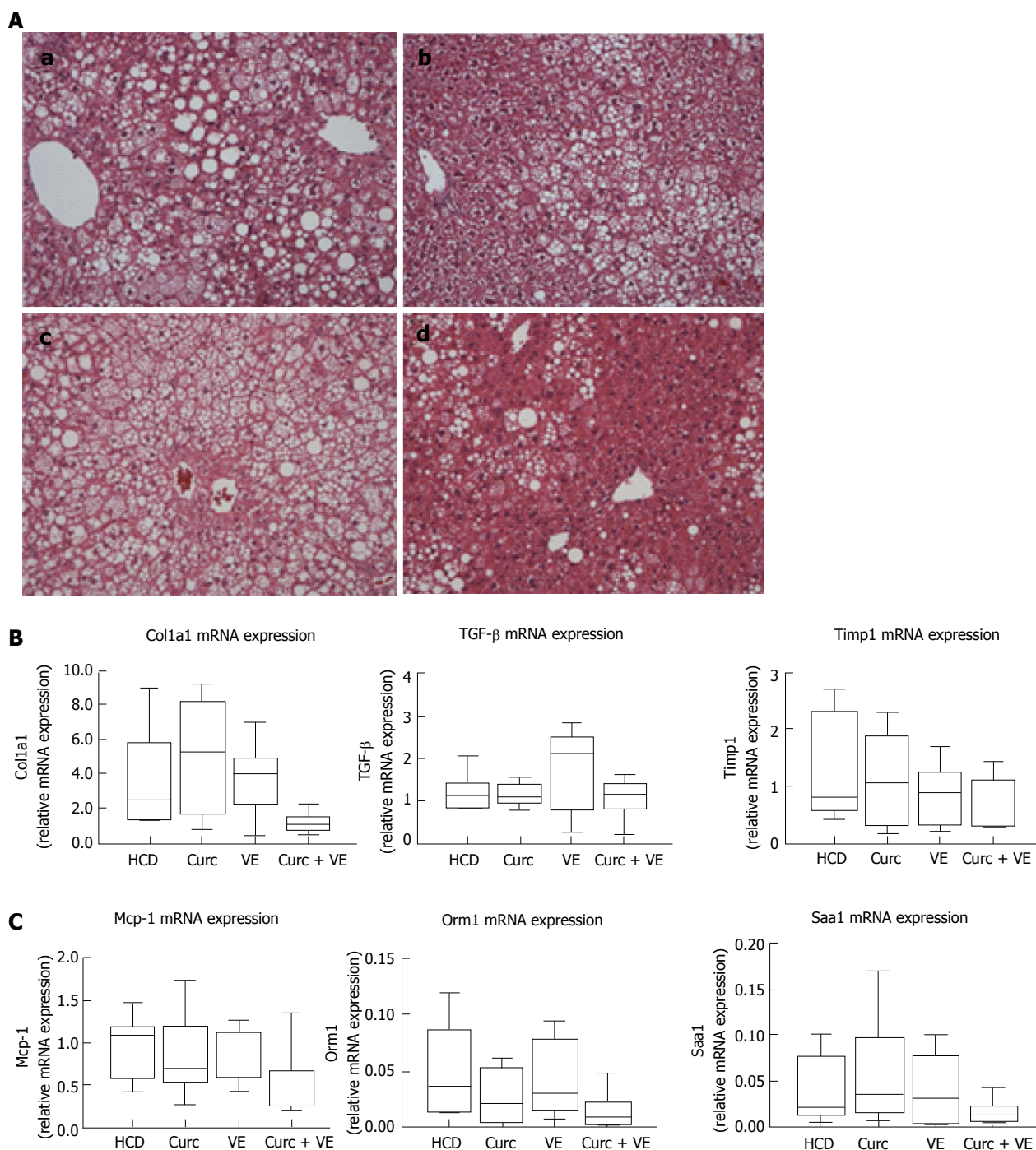


Figure 2 Steatosis is attenuated by curcumin and vitamin E combination therapy while the expression of fibrotic and inflammatory genes are not significantly altered. A: Representative liver paraffin-embedded sections stained with hematoxylin and eosin (H and E) (original magnification $\times 20$). a: *Hfe*^{-/-} mice ($n = 7-9$ per group) fed HCD (20 wk); b: HCD (10 wk) then HCD + 1% curcumin (10 wk); c: HCD (10 wk) then HCD + 1.5% vitamin E (10 wk); d: HCD (10 wk) then HCD + 1% curcumin + 1.5% vitamin E (10 wk). qRT-PCR was used to determine expression levels of hepatic (B) fibrogenic genes: $\alpha 1(I)$ -procollagen (Col1a1); Transforming growth factor β (TGF- β); Tissue inhibitor of metalloproteinase 1 (Timp1); C: Hepatic inflammatory genes: monocyte chemoattractant protein-1 (Mcp-1); orosmuoid 1 (Orm1) and serum amyloid A 1 (Saa1). Data are depicted using box and whisker plots showing median, minimum and maximum values. Statistical significance was tested using Kruskal-Wallis test with Dunn's correction for multiple comparisons. HCD: High calorie diet; Cu: Curcumin; VE: Vitamin E.

suggested that curcumin may be similar to resveratrol in this respect through activating PPAR γ coactivator 1- α (PGC-1 α)^[21,22], a regulator of mitochondrial biogenesis^[27]. Indeed, curcumin increases PGC-1 α and *Nrf-1* gene expression *in vitro*, protecting against mitochondrial impairment induced by high free fatty acids^[28]. A recent study in a rat model of NASH demonstrated attenuation

of liver injury by curcumin which the authors suggested was *Nrf-1* mediated^[29]. Combination treatment increased *Nrf-1* and TfB2m expression in *Hfe*^{-/-} mice but failed to increase the expression of mitoNEET. These results are similar to MnSOD activity where combination treatment failed to restore its activity above a HCD-induced suppression. Down-regulation of mitoNEET causes

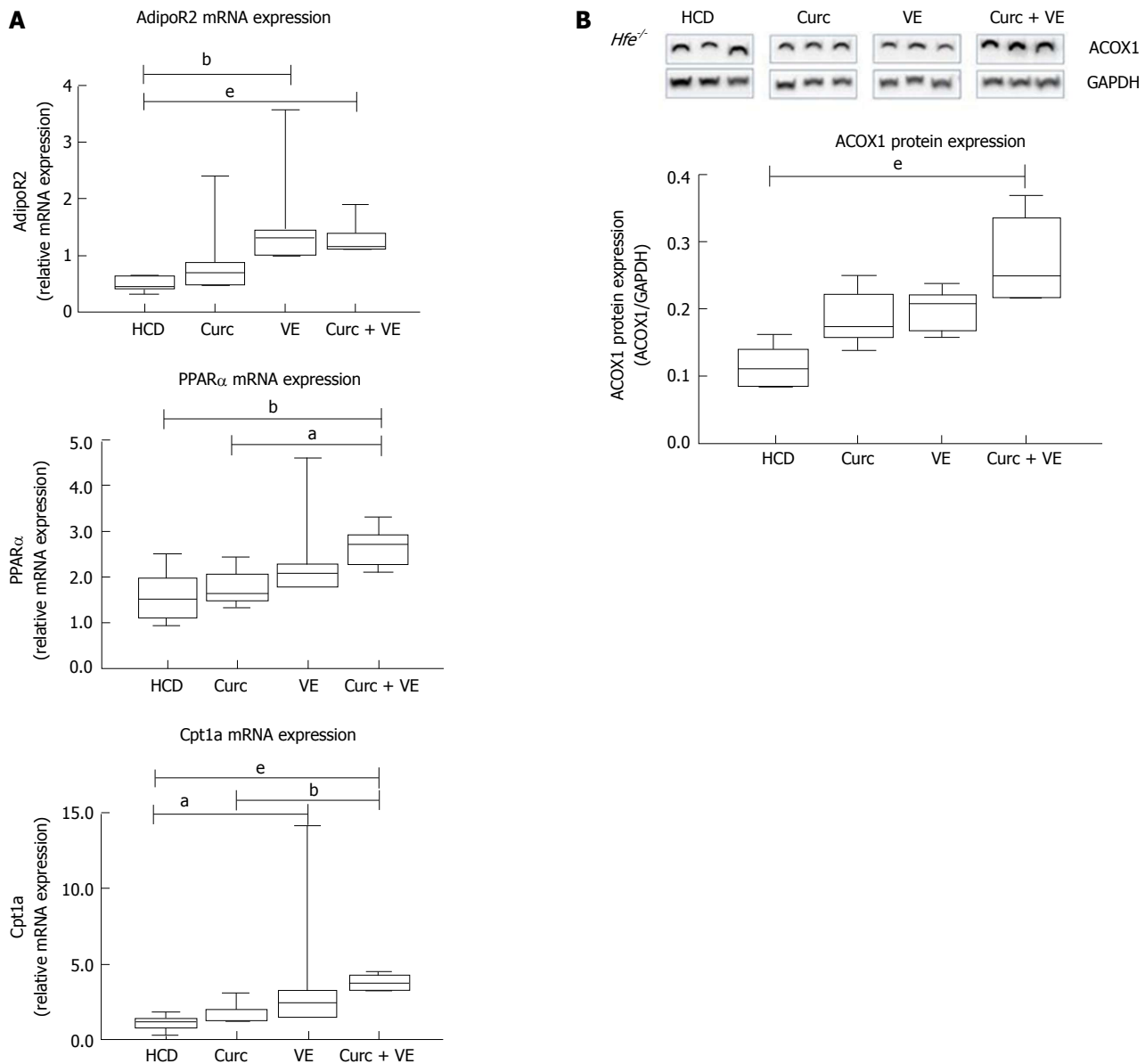


Figure 3 Altered lipid signalling following high calorie diet feeding is abrogated by curcumin and vitamin E combination therapy. qRT-PCR was used to determine expression levels of hepatic fatty acid oxidation genes in *Hfe*^{-/-} mice fed HCD, HCD + Cu, HCD + VE or HCD + Cu + VE. A: Adiponectin receptor 2 (AdipoR2) mRNA; peroxisome proliferator-activated receptor α (PPAR α) and carnitine palmitoyl transferase 1A (Cpt1a) mRNA; B: Western blotting and densitometry analysis was performed to determine levels of acyl-coenzymeA oxidase 1 (ACOX) and glyceraldehyde 3-phosphate dehydrogenase (Gapdh). Data are depicted using box and whisker plots showing median, minimum and maximum values. Statistical significance was tested using Kruskal-Wallis test with Dunn's correction for multiple comparisons, ^a $P \leq 0.05$, ^b $P \leq 0.01$, ^c $P \leq 0.001$. HCD: High calorie diet; Cu: Curcumin; VE: Vitamin E.

an increase in iron content into the mitochondria and decreases the mitochondria's capacity to carry out electron transport and oxidative phosphorylation^[20], potentially increasing oxidative stress and further damaging mitochondria. Indeed, mitochondrial dysfunction is associated with insulin resistance and the development of type 2 diabetes where diminished oxidative capacity is thought to be involved in disease pathogenesis^[30]. Taken together, these findings of our study suggest that combination treatment manages to enhance the capacity for fatty acid disposal by β -oxidation without increasing oxidative stress.

This study is limited by the use of an animal model which cannot fully replicate the pathophysiology of

human NASH. The dietary model incorporated high levels of curcumin which may not be easily replicated in humans. Further studies examining dosing of curcumin and vitamin E in humans are still required. While these studies have suggested potential mechanistic pathways involved in efficacy of combination treatment, we have not fully elucidated mechanisms responsible for the synergistic effects of combination therapy over monotherapy.

The high prevalence of obesity and the metabolic syndrome suggests that many patients with liver disease of varying etiologies will have co-existent non-alcoholic fatty liver disease. Indeed contemporary clinical practice in hepatology is often characterised by

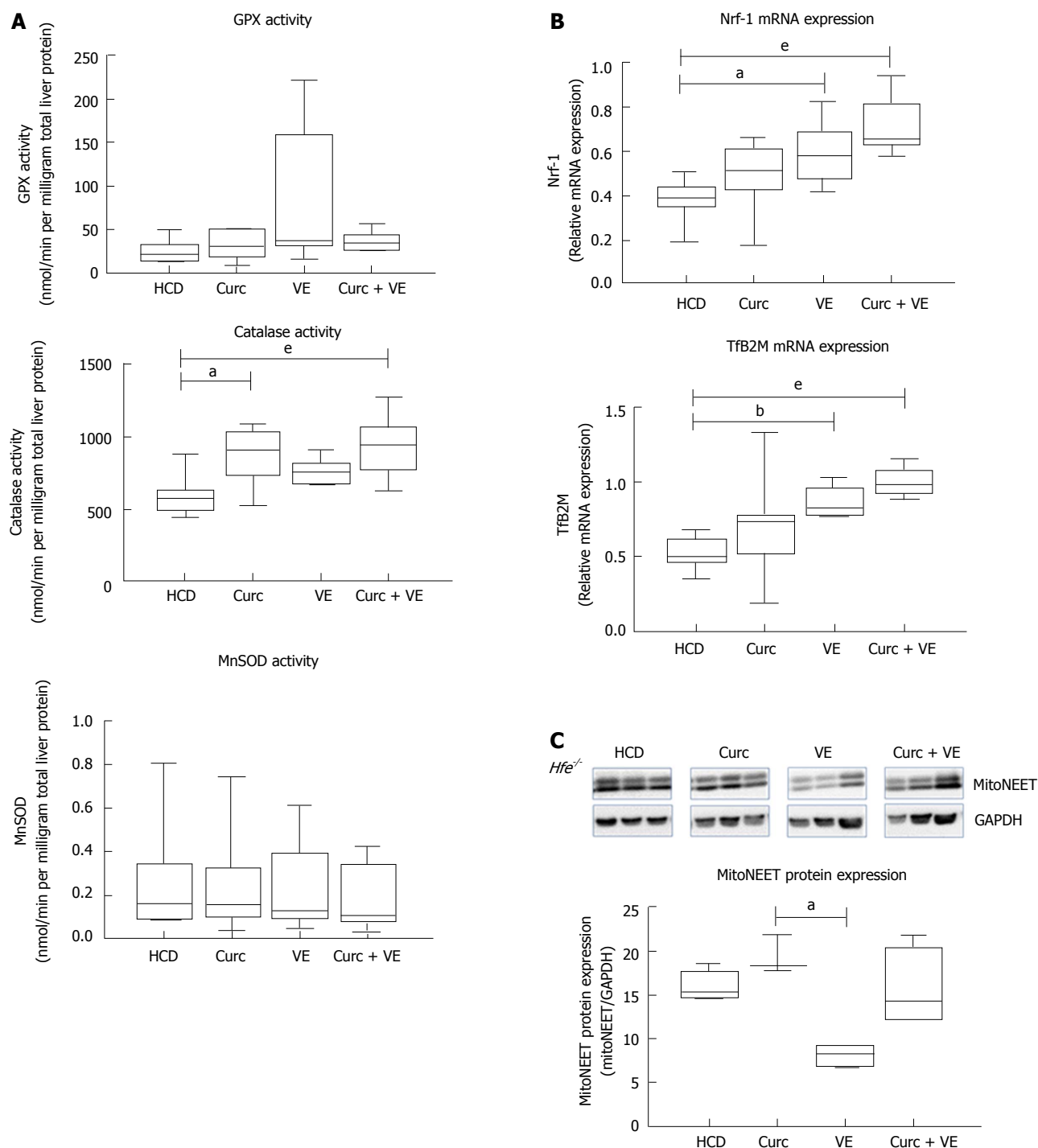


Figure 4 Curcumin and vitamin E treatments increase hepatic antioxidant enzyme activities and increase mitochondrial biogenesis. Hepatic tissue from *Hfe*^{-/-} mice fed HCD for 10 wk then HCD + Cu, HCD + VE or HCD + Cu + VE for a further 10 wk ($n = 7-9$) was analysed for (A) glutathione peroxidase (GPx) activity, catalase activity and mitochondrial superoxide dismutase (MnSOD) activity. qRT-PCR was used to determine expression levels of hepatic (B) nuclear transcription factor 1 (Nrf-1) and transcription factor B2 mitochondrial (Tfb2m). C: Western blotting and densitometry analysis was performed to determine levels of hepatic mitoNEET and glyceraldehyde 3-phosphate dehydrogenase (Gapdh). Data are depicted using box and whisker plots showing median, minimum and maximum values. Statistical significance was tested using Kruskal-Wallis test with Dunn's correction for multiple comparisons, ^a $P \leq 0.05$, ^b $P \leq 0.01$, ^c $P \leq 0.001$. HCD: High calorie diet; Cu: Curcumin; VE: Vitamin E.

the need to address multiple co-toxins in one patient. The results of the present study illustrate of the concept of co-toxic liver disease since our model incorporates increased HIC in combination with steatosis and was associated with necroinflammation and early hepatic fibrosis. Curcumin and vitamin E therapy resulted in

attenuation of steatosis through increased fatty acid β -oxidation, increased catalase activity and upregulated mitochondrial biogenesis. Because of the beneficial effect of combination therapy, we believe vitamin E and curcumin should be investigated in other animal models of NASH, and could be moved rapidly into human

studies if a beneficial effect is demonstrated, and if appropriate dosing strategies can be developed.

ACKNOWLEDGMENTS

Associate Professor Graeme Macdonald from the Gastroenterology and Hepatology unit of the Princess Alexandra Hospital for his knowledge and continual input into the project. Dr. Sarah McLeay for her review of the statistical methods in the manuscript.

COMMENTS

Background

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease in Western countries and encompasses a range of hepatic pathologies from simple steatosis to the more aggressive non-alcoholic steatohepatitis (NASH). Homozygosity for the C282Y mutation in the *HFE* gene is the most common cause of hereditary hemochromatosis (HH). Steatosis is common in patients with HH and is associated with increased hepatic fibrosis; conversely, heterozygosity for the C282Y mutation in *HFE* is common in patients with NAFLD. Even mild increases in hepatic iron concentration (HIC) may play an important role in the transition from simple steatosis to NASH. Curcumin and vitamin E have been used with therapeutic benefit in experimental models of NASH, therefore this study investigated the role of curcumin and vitamin E in ameliorating injury in a model of iron overload and fatty liver.

Research frontiers

Therapies for NASH and fatty liver disease are lacking and due to the growing prevalence of fatty liver disease are increasing important for treatment of this growing number of patients.

Innovations and breakthrough

This study utilised combination treatment with curcumin and vitamin E which differed from previous studies which only examined monotherapy. In addition, this study examined the efficacy of treatment in established fatty liver disease, not solely at the initiation of injury.

Applications

As both curcumin and vitamin E have been used clinically the authors suggest that combination treatment could be easily moved into human clinical trials.

Terminology

Non-alcoholic fatty liver disease: Encompasses a range of hepatic pathologies from simple steatosis to the more aggressive non-alcoholic steatohepatitis; Haemochromatosis: An iron-overload disease which results in hepatic iron accumulation.

Peer-review

Manuscript's content is interesting, overall well written and timely.

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P- Reviewer: Trovato GM **S- Editor:** Gong ZM **L- Editor:** A
E- Editor: Wu HL



Observational Study

Endoscopic therapy for biliary strictures complicating living donor liver transplantation: Factors predicting better outcome

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Author contributions: Ahamed H and Panicker S helped in subject recruitment and data entry of the study; Rao HB and Venu RP were involved with data analysis, interpretation and drafted the manuscript; Sudhindran S revised the article critically for important intellectual content.

Institutional review board statement: The study was reviewed and approved by the Amrita Institute of Medical sciences Research Review Board (Kochi, Kerala, India).

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

Conflict-of-interest statement: There are no conflicts of interest to report.

Data sharing statement: No additional data are available.

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Manuscript source: Invited manuscript

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Received: January 28, 2017
Peer-review started: February 14, 2017
First decision: March 7, 2017
Revised: March 23, 2017
Accepted: April 18, 2017
Article in press: April 19, 2017
Published online: May 15, 2017

Abstract

AIM

To identify factors predicting outcome of endoscopic therapy in bile duct strictures (BDS) post living donor liver transplantation (LDLT).

METHODS

Patients referred with BDS post LDLT, were retrospectively studied. Patient demographics, symptoms (Pruritus, Jaundice, cholangitis), intra-op variables (cold ischemia time, blood transfusions, number of ducts used, etc.), peri-op complications [hepatic artery thrombosis (HAT), bile leak, infections], stricture morphology (length, donor and recipient duct diameters) and relevant laboratory data both pre- and post-endothorapy were studied. Favourable response to endothorapy was defined as symptomatic relief with > 80% reduction in total bilirubin/serum gamma glutamyl transferase. Statistical analysis was performed using SPSS 20.0.

RESULTS

Forty-one patients were included (age: 8-63 years). All had right lobe LDLT with duct-to-duct anastomosis. Twenty patients (48.7%) had favourable response to endothorapy. Patients with single duct anastomosis, aggressive stent therapy (multiple endoscopic retrograde

cholangiography, upsizing of stents, dilatation and longer duration of stents) and an initial favourable response to endotherapy were independent predictors of good outcome ($P < 0.05$). Older donor age, HAT, multiple ductal anastomosis and persistent bile leak (> 4 wk post LT) were found to be significant predictors of poor response on multivariate analysis ($P < 0.05$).

CONCLUSION

Endoscopic therapy with aggressive stent therapy especially in patients with single duct-to-duct anastomosis was associated with a better outcome. Multiple ductal anastomosis, older donor age, shorter duration of stent therapy, early bile leak and HAT were predictors of poor outcome with endotherapy in these patients.

Key words: Biliary strictures; Living donor liver transplantation; Endotherapy; Bile leaks

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Core tip: Living donor liver transplantation (LDLT) is the definitive treatment for end stage liver disease. Biliary complications complicating LDLT is a major source of morbidity and mortality. Anastomotic strictures seen with LDLT are notorious for gross phenotypical variations which have led to variable results with endotherapy as a treatment option in these patients. In this study, we have looked into the factors that can predict the response with endotherapy (endoscopic retrograde cholangiography, sphincterotomy and stent placement) thereby allowing for better prognostication and selection of patients in order to optimize patient care.

Harshavardhan RB, Ahamed H, Panicker S, Sudhindran S, Venu RP. Endoscopic therapy for biliary strictures complicating living donor liver transplantation: Factors predicting better outcome. *World J Gastrointest Pathophysiol* 2017; 8(2): 77-86 Available from: URL: <http://www.wjgnet.com/2150-5330/full/v8/i2/77.htm> DOI: <http://dx.doi.org/10.4291/wjgp.v8.i2.77>

INTRODUCTION

Living donor liver transplantation (LDLT) as a treatment for end stage liver disease is an approach that is becoming increasingly common in Asian centres. Biliary complications, especially bile duct strictures (BDS), remain a significant complication of LDLT^[1-4], although there seems to be a decline in incidence over the last 2 decades^[5]. Unlike deceased donor liver transplantation (DDLT), the incidence of BDS in patients undergoing LDLT is higher and ranges between 18%-32%^[6,7]. Most of these patients present with symptoms of bile duct obstruction such as jaundice, pruritus and/or cholangitis. Endotherapy with endoscopic retrograde cholangiography (ERC), sphincterotomy and placement of endoprosthesis have been shown to be technically feasible and safe

for BDS in post LDLT^[8,9]. However, therapeutic efficacy for BDS post LDLT has been variable^[9-11]. Therefore, additional studies are needed looking into the factors that can predict a favourable therapeutic response to endotherapy.

The higher incidence of BDS post LDLT have been attributed to difficult surgical techniques of anastomosis owing to smaller diameter of the ducts along with multiple ductal anastomoses. The endotherapy protocol (type of stents, sphincterotomy, stricture dilatation, number of ERC with stent exchange and duration of stenting) has not been standardized in earlier reports, possibly accounting for the varying results of endotherapy in these studies^[9-15]. The stricture morphology, type and number of stents used, duration of stent therapy may also have a direct bearing on therapeutic outcome. In this study various risk factors like anatomical contortions of the stricture, type and number of ductal anastomosis, duration, diameter as well as number of stents placed and their impact on the clinical as well as biochemical outcomes are carefully analysed.

MATERIALS AND METHODS

Patient population and data collection

This was a single centre, observational study where all patients referred to the Gastroenterology service with a diagnosis of BDS post LDLT from January 2012 till November 2015 were evaluated. Recipient and donor demographics (age and gender), transplant setting (emergency or elective), aetiology of liver disease, pre-operative Model for End-stage Liver Disease score, intra-operative variables like cold ischemia time, number of blood transfusions, number of bile ducts anastomosed, ductoplasty, conduit used (duct or jejunal loop) and post-operative complications like Hepatic artery thrombosis (HAT), bile leaks; were recorded in pre-designed performas. Bile leaks were considered persistent if the leak did not resolve in four weeks.

Endotherapy

All patients who were medically fit to undergo therapeutic ERC were included in the study. All ERCs were performed by an experienced endoscopist under general anaesthesia and the choice of anaesthetic was left to the discretion of a dedicated anaesthetic doctor who was present throughout the duration for all procedures. A standard adult ERC scope (Olympus TJF Q180V) with an outer diameter of 13.7 mm and channel diameter of 4.2 mm was used. All procedures were performed in the endoscopy suite under fluoroscopic guidance (Fexavision, Shimadzu). Technical success was defined as selective cannulation of CBD with an adequate cholangiogram obtained. Definition of biliary stricture: Patients who had at least two of the three following features were included in the study: (1) Symptoms of cholestasis like Jaundice, pruritus and/or cholangitis in

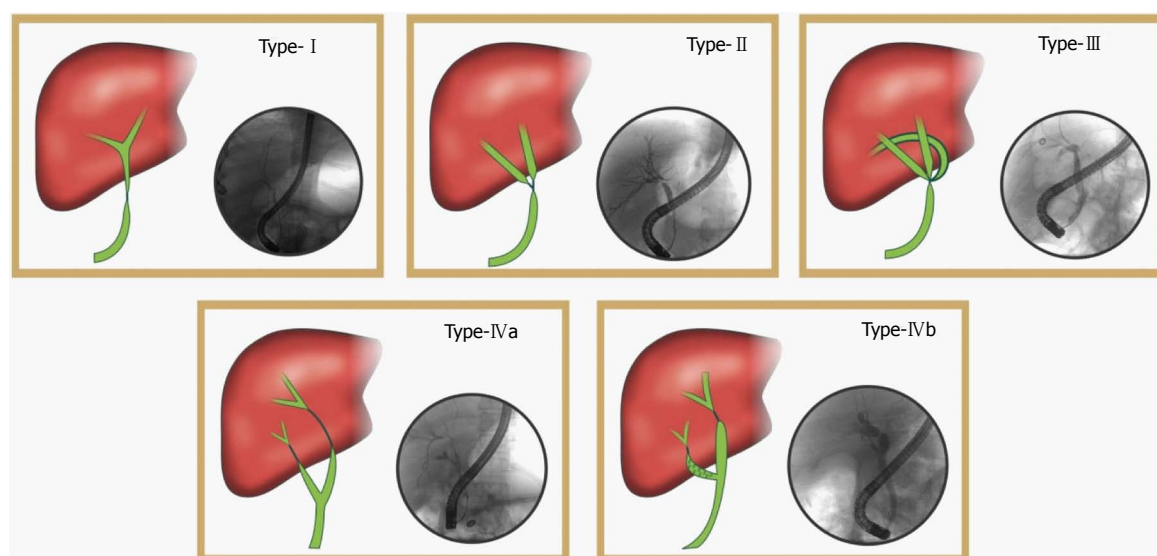


Figure 1 Classification of anastomotic bile duct strictures post living donor liver transplantation.

conjunction with elevated serum bilirubin and/or serum gamma glutamyl transferase (GGT) to more than twice the upper limit of normal; (2) Magnetic resonance cholangiopancreatography showing a narrowing with proximal duct dilatation; and (3) ERC cholangiogram showing significant narrowing at the anastomotic site with/without ductal dilatation.

The cholangiograms were then reviewed for stricture morphology (length, diameter, type of anastomoses). The anastomoses were classified as type 1 when single donor duct was anastomosed to a single recipient duct. Type 2 is when two donor ducts are connected to a single recipient duct. Type 3 is when three donor ducts were anastomosed to a single recipient duct. Type 4 is when two recipient ducts (cystic duct and common hepatic duct or right and left hepatic ducts) are anastomosed with the donor duct (Figure 1). The number of ducts anastomosed including the use of cystic duct for anastomoses were also studied.

Therapeutic protocol and follow up: Institutional protocol for endoscopic management of benign biliary strictures includes Endoscopic sphincterotomy (5-7 mm), which was performed in all patients and stricture dilation using 4-7 mm balloon dilators were performed. A polyethylene stent of 5-7 Fr diameter and 12-15 cm long was then deployed across the stricture. The stent placement was repeated and the stent graduated to a higher diameter upto 10 Fr, if possible. The procedure was repeated every 3 mo for stent replacement for a minimum period of 1 year. Patients, who did not undergo this optimal stent exchange therapy for 1 year due to non-compliance, were studied for recurrence of symptoms and relevant laboratory studies at subsequent clinic visits.

Patients in whom ERC was unsuccessful (due to tight stricture or complex stricture), a guide wire was

advanced into the duodenum across the stricture *via* a percutaneous trans hepatic puncture of the intrahepatic duct (PTBD). ERC, dilatation and stent placement was then carried out using a rendezvous approach. All patients were followed up for immediate and delayed complications.

Surgical repair was undertaken only when both endoscopic and transhepatic approaches failed to cross the stricture, the so called "defiant strictures". Unlike BDS in deceased donors, those occurring in LDLT are surgically formidable to correct, since the length of donor duct (proximal to anastomosis) tends to be very short. BDS following LDLT therefore tend to extend intrahepatically and defining an appropriate donor duct for anastomosis can be a very formidable task surgically particularly in type 4 strictures.

Outcome definition

Outcome was classified into favourable and unfavourable to endotherapy. Patients with favourable response had both biochemical and clinical improvement. Clinical improvement is characterised by relief from symptoms of bile duct obstruction (pruritus, jaundice and cholangitis) and biochemical improvement is considered when there was 80% reduction in total bilirubin (BR)/GGT. Unfavourable response was considered to be partial if either clinical or biochemical improvement was present and Non responsive if neither clinical nor biochemical improvement was seen.

Statistical analysis

Statistical analysis was carried out using IBM SPSS software version 20.0. Comparison of means in the three groups were done using ANOVA test for parametric or Kruskal Wallis for non-parametric variables. Response was also classified as complete responders and partial/No response where independent 2-sample *t* test and

Table 1 Baseline characteristics of the patient population stratified according to response to endotherapy

Parameters	Total (n = 41)	Complete response (n = 20)	Partial response (n = 14)	No response (n = 7)
Recipient mean age (year $\pm$ SD)	40.02 $\pm$ 15.45	37.5 $\pm$ 17.03	41.0 $\pm$ 14.3	45.29 $\pm$ 13.15
Donor mean age (year $\pm$ SD)	39.9 $\pm$ 8.9	38.5 $\pm$ 6.85	39.86 $\pm$ 11.2	44.0 $\pm$ 9.03
Recipient gender - male, n (%)	36 (87.8)	16 (80)	14 (100)	6 (85.7)
Donor gender - male, n (%)	9 (22)	4 (20)	4 (28.5)	1 (14.2)
Transplant setting, n (%)				
CLD	32 (78)	14 (70)	12 (85.7)	6 (85.7)
ALF	9 (22)	6 (30)	2 (14.3)	1 (14.3)
Etiology of CLD (n = 32)				
Alcohol	12 (37.5)	4 (33.3)	6 (50)	2 (16.7)
Cryptogenic	13 (40.6)	6 (46.1)	5 (38.4)	2 (15.5)
Others (Wilson's, hepatitis B, hepatitis C, autoimmune)	7 (21.8)	3 (42.8)	2 (28.6)	2 (28.6)
MELD	23.45 $\pm$ 6.4	25.07 $\pm$ 7.2	22.46 $\pm$ 4.75	21.83 $\pm$ 6.52
Intra-operative variables				
Cold Ischemia time (secs) (mean $\pm$)	51.5 $\pm$ 29.2	53.3 $\pm$ 34.4	54.08 $\pm$ 25.6	43.57 $\pm$ 28.4
Blood transfusions (mean $\pm$)	4.1 $\pm$ 2.8	4.3 $\pm$ 2.3	4.3 $\pm$ 3.5	3.2 $\pm$ 2.2
Post op complications				
Hepatic artery thrombosis, n (%)	5 (12.2)	0 (0)	3 (60)	2 (40)
Bile leak	26 (65)	12 (60)	10 (71.4)	4 (57.1)
Mean interval between LT and leak (d)	18.4	20.85	12.6	5.6
Persistent bile leak > 4 wk, n (%)	14 (34.1)	2 (14.2)	8 (57.1)	4 (28.7)
Clinical presentation, n (%)				
Cholangitis	15 (36.6)	6 (30)	7 (42.1)	2 (13.4)
Pruritus alone	16 (39)	7 (43.75)	4 (25)	5 (31.25)
Jaundice	16 (39)	5 (31.25)	6 (37.5)	5 (31.25)

CLD: Chronic liver disease; ALF: Acute liver failure; MELD: Model for end-stage liver disease.

Mann Whitney *U* tests was used for parametric and non-parametric variables respectively. Categorical variables were analysed using χ^2 test/Fisher's Exact test in the univariate analysis. Multivariate analysis was then carried out using multiple logistic regression analysis. Kaplan Meier curves were computed to look for a survival advantage. A *P* value < 0.05 was taken as significant. The statistical methods of this study were reviewed by Mr Unnikrishnan UG, MSc Statistics, Lecturer, Department of Biostatistics, Amrita Institute of Medical Sciences, Kochi, Kerala, India.

RESULTS

Of the 458 LDLT performed, 47 patients had biliary strictures (10.2%); of which 41 patients had complete data and were included in the study. The mean age of the population was 40.02 $\pm$ 15.45 years with a male preponderance (87.8%). Majority of the patients were transplanted in a non-emergency setting (78%) with alcohol (37.5%) and cryptogenic (40.6%) cirrhosis being the most common etiologies for liver disease. The intra-operative variables did not have any association with therapeutic efficacy in this study. However, patients with post-operative HAT and BDS did not have any response to endotherapy despite re-establishment of flow in 4/5 patients. Majority (85.8%) of patients with persistent bile leak > 4 wk had only partial/no improvement to endotherapy (*P* value < 0.05). Demographic variables and baseline data are shown in Table 1.

Technical success and therapeutic efficacy of endotherapy

ERCP was successful in 31 patients (75.6%) while 10 patients (24.4%) required a combined PTBD with a rendezvous approach. Favourable response to endotherapy was seen in 20 patients (48.7%); 14 patients (34.1%) had partial unfavourable response to endotherapy and 7 patients (17.1%), had no response to endotherapy (Figure 2).

Endotherapy in BDS post LDLT

A total of 117 ERCPs were performed in 41 patients (2.8 procedures per patient) (Table 2). Patients who had complete improvement to endotherapy (favourable response) had a significantly higher number of total ERC (3.01 in patients with favourable response whereas 1.71 in patients with unfavourable response) and the initial favourable response to endotherapy was also higher (85% vs 57.1% respectively) (*P* < 0.05) in these patients. Aggressive stent therapy was performed in 23 patients (56%) and these patients had a significant improvement after endotherapy, 16 patients (69.5%) had complete favourable response while 7 patients (30.5%) had partial/no improvement (*P* value 0.04). Duration of stent therapy showed a direct impact on therapeutic efficacy where patients with a favourable response to endotherapy having the stent in place for a mean duration of 16.4 mo, whereas, patients who had a partial/no response to endotherapy had the stent in place for a mean duration of 6.8 mo (*P* value = 0.03).

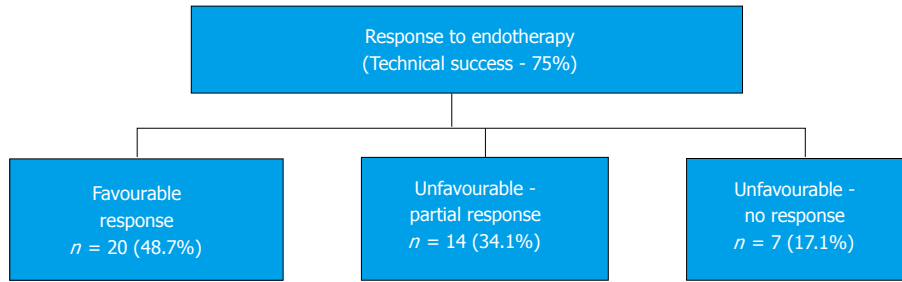


Figure 2 Therapeutic efficacy of endotherapy in patients with bile duct strictures post living donor liver transplantation.

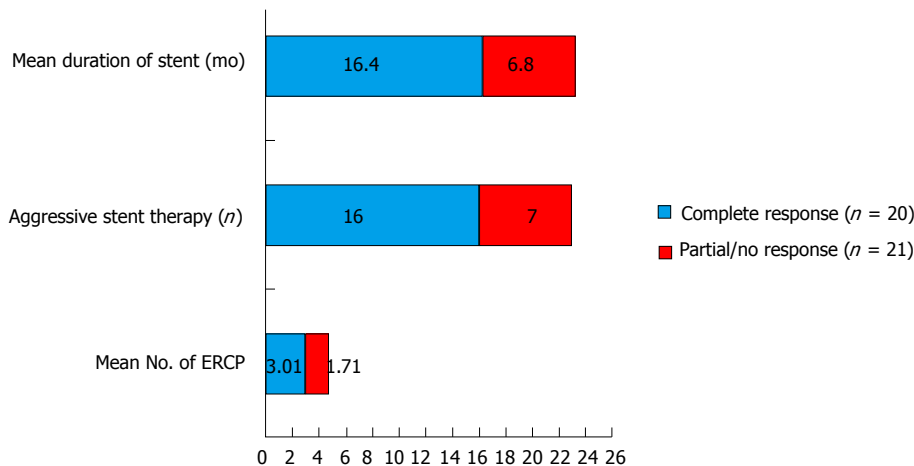


Figure 3 Statistically significant ($P < 0.05$) correlations of good response to endotherapy.

Table 2 Impact of endotherapy protocol and morphology of the stricture on therapeutic efficacy

Endotherapy details	Total (n = 41)	Complete response (n = 20)	Partial/no response (n = 21)
Mean time interval between LT and first ERC (d)			
Mean No. of ERC ^a	368.3	352.6	444.1
Mean Stricture length (cm)	2.85	3.01	1.71
Initial response to ERC, n (%)	0.84	0.85	0.82
Aggressive stent therapy ^{a,b}	29 (70.7)	17 (85)	12 (57.1)
Mean duration of stenting (mo)	23 (39)	16 (69.5)	7 (23.3)
Morphology of the stricture	5.75	16.4	6.8
Mean length of stricture (mm)	84	82	85
Type of anastomosis (Figure 1), n (%)			
Type I	13	10 (76.9)	3 (23.1)
Type II	16	9 (56.25)	7 (43.75)
Type III	5	1 (20)	4 (80)
Type IV	7	0 (14.2)	7 (100)

^a P value < 0.05 ; ^bAggressive stent therapy involves sphincterotomy, serial dilatation of stricture and increase in number/size of stents. LT: Liver transplantation; ERC: Endoscopic retrograde cholangiography.

(Figure 3). A minimum duration of 6.75 mo of stent therapy was found to be predictive of a favourable response to endotherapy (AUROC 0.75).

Stricture morphology: Stricture length and duct diameters did not have a significant correlation to eventual response to endotherapy. The type of anastomosis, however (Figure 1), did influence the response to endotherapy. All patients with type IV (2 recipient ducts) anastomosis had a poor response to endotherapy (Table 2).

Surgical repair was performed only for one patient in this cohort, who remains well following surgery. In one other patient, who developed secondary biliary cirrhosis, retransplantation was required.

Safety and complication

Endotherapy in this patient cohort was safe and only 1 patient had mild acute pancreatitis and 1 patient had cholangitis. A total of 9 patients died (21.9%), including the one with cholangitis. However, a statistically significant survival advantage was observed in patients

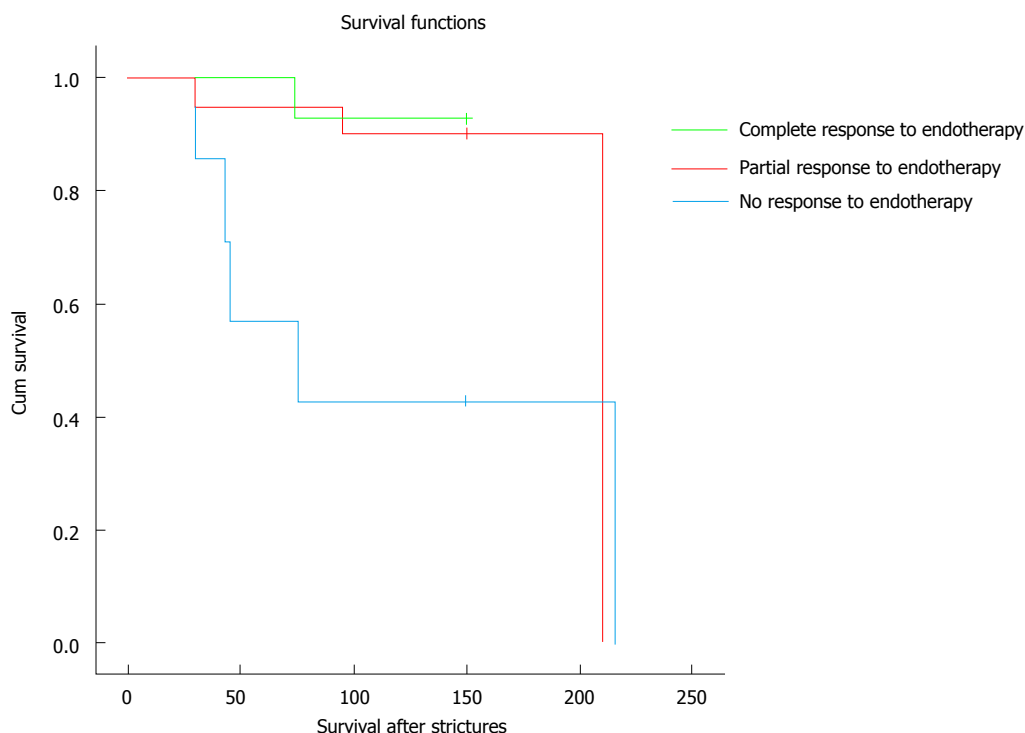


Figure 4 Kaplan meier curves showing a clear survival benefit in patients who had a response to endotherapy.

who had a favourable response to endotherapy as opposed to patients who did not (P value 0.02) (Figure 4).

DISCUSSION

Liver transplantation remains the only definitive treatment for end stage liver disease. Transplantation using living donors, which is commonly practised in most Asian centres including India, is fraught with unique technical challenges and specific complications with as yet, evolving avenues of therapy. Biliary complications (BDS and Bile leaks) in particular, continue to be a stumbling block resulting in considerable morbidity and mortality among these patients^[2,16-18]. The incidence of BDS is higher in LDLT patients (10%-30%) compared to DDLT possibly related to smaller diameter of the ducts and multiple ductal anastomoses^[19,20].

BDS in the post-transplant setting is classified into one of two types - anastomotic (AS) and non-anastomotic (NAS) strictures. NAS, more common with DDLT, with an incidence of 5%-15%^[17,18] are longer, multiple and located proximal to the anastomotic site. NAS are further divided into microangiopathic (with prolonged cold ischemia times), macroangiopathic (in association with HAT) and immunogenic (in association with rejection). AS on the other hand, are seen more frequently in patients who have received living donor livers and tend to be shorter, confined to the anastomotic site and present later in the course of follow up^[5]. Multiple factors contribute to the development of AS including surgical technique, number of

ducts anastomosed and ischemia^[21]. The complexity of the microvasculature of the bile ducts makes them particularly vulnerable to ischemia. Management of biliary strictures with endotherapy, *i.e.*, ERC with endobiliary stenting, is the treatment of choice especially for patients with AS^[22]. They are notoriously difficult to treat with a recurrence rate between 9%-37%^[23]. However, contrary to this, a report from the Adult-to-Adult Living Donor Liver Transplantation Cohort Study consortium (A2ALL)^[24] suggested a comparable efficacy of endoscopic treatment in anastomotic strictures (in both DDLT as well as LDLT setting) based on which a recommendation to reserve endoscopic therapy as a first line treatment for all patients with anastomotic strictures could be made^[22]. Unfortunately, some of these patients may not have a favourable response to endotherapy resulting in significant morbidity and even mortality. These patients with the so-called "defiant" biliary strictures may end up with re-surgery or repeat transplantation which will add to the financial burden of the patients^[22].

Several investigators have reported a favourable response following endotherapy in the range of 53%-88%^[3,19,25,26]. In our patient population, a favourable response was seen in only 48.7% of patients, while partial relief (clinical or biochemical) was noted in 34.1% of patients. Seven/41 patients (17.1%) in our study had no response to endotherapy. Moreover patients who did not respond to endotherapy were at a survival disadvantage with high mortality rate. This critical observation provided us with the impetus to study the factors that might predict outcome with endotherapy, especially

since there is a considerable variation of results and lack of uniform treatment protocols.

Donor age was found to have a significant bearing on response to endotherapy. The age related variations in duct calibre and fibrotic response to ischemia/inflammation of the ducts may be possible pathogenic factors for the development of complex BDS. HAT in the post-operative period was a significant predictor of poor response to endotherapy despite re-establishment of flow in most of the patients (4/5 patients, 80%). This suggests that the ischemic stress even though "short-lived" was sufficient to significantly compromise the viability of the anastomotic site leading to complex and defiant strictures.

Bile leaks have previously been shown to be associated with an increased incidence of BDS^[27-29]. Inflammatory reactions due to the extravasated bile leading to fibrosis has been proposed as the pathogenetic mechanism in these patients. Alternatively, bile leak may be a surrogate to indicate ischemia and necro inflammatory reaction which eventually results in BDS^[30]. Our study looked into not just the immediate perioperative bile leak but also the duration of leak, of more than 4 wk. Patients who had persistent bile leak more than four weeks had a poor outcome following endotherapy which may in part, be related to a dose response relationship between persistent inflammation caused by the extravasated bile. As a corollary to this, an early and aggressive treatment protocol with endoscopic sphincterotomy and stent placement may positively impact endoscopic outcome for BDS in these patients.

LDLT strictures are notoriously complex possibly resulting from multiple ductal anastomoses, mismatch of duct sizes and altered anatomy^[31]. Only 75% technical success rate was achieved with ERC in our patient population with 10 patients requiring PTBD followed by rendezvous approach for stent placement. In all these patients the strictures assumed complex anatomical contortions.

Multiple ductal anastomoses are seldom considered as a risk factor for BDS post LDLT^[32]. In a study by Chan *et al*^[33], they found that a single hepatic duct is ideal for a duct to duct anastomosis. If two ducts are present, a duct to duct anastomosis can still be performed provided the ducts are less than 3 mm apart. If the ducts are more than 3 mm apart, they suggested separate Hepaticojejunostomies using a Roux-en-Y loop^[33]. Another study by Asonuma *et al*^[34] explored the feasibility of the use of the recipient cystic duct for biliary reconstruction in right liver donor transplantation when two bile duct orifices were present. Our study also revealed that employing multiple recipient ducts for anastomosis can cause defiant and complex strictures resistant to endotherapy. Moreover when recipient cystic duct was used for anastomosis, the outcome for endotherapy was especially poor. The stricture length did not correlate with response to endotherapy suggesting a complex anatomical spatial alteration as the likely cause

for poor response to endotherapy than longitudinal extent of the stricture.

There is no uniform consensus on the ERC protocol for the treatment of post LDLT BDS. Several reports have suggested that balloon dilatation with endobiliary stenting is superior to dilatation alone^[17,35-37]. However, in a meta-analysis published recently, balloon dilatation with stenting did not afford a significant advantage to dilatation alone^[38]. Current practices for management of BDS post LDLT suggests a 3 mo interval for repeat procedures with stricture dilatation and stent exchanges, which has been shown to minimize stent occlusion and prevent cholangitis or stone formation^[17,22,39]. Our results also indicate that an aggressive endotherapy protocol which included stent exchanges every 3 mo, stent up-sizing to a maximum of 10 Fr, serial dilatation of the strictures and prolonged duration of stent therapy were associated with a favourable response to endotherapy. Similar to our experience Verdonk *et al*^[30] had a 75% success rate for post LDLT strictures with a median of 3 endotherapy sessions per patient. They also showed that higher number of endotherapy sessions with increasing number of stents had a good outcome. A predictive cut-off of 6.75 mo for the duration of stent therapy was found to have reasonable validity for eventual favourable response to endotherapy. Most studies in this area have found an average stent duration between 6 mo to 1 year with 3-4 endotherapy sessions to be adequate in preventing recurrences with minimal complications and morbidity^[7,40,41].

Reported complications for endotherapy in BDS post transplantation include post-ERC pancreatitis, cholangitis, bleeding, pain and stent migration. In this study, ERC followed by endotherapy was found to be safe with only one patient developing mild acute pancreatitis and one developing cholangitis. Premature removal of stents without replacement or up-sizing of stent was associated with recurrent bile duct stricture and cholestatic jaundice.

The treatment of "defiant" BDS remains an elusive area of study where innovative endoscopic approaches like the use of digital single operator cholangioscopy (DSOC)^[42], covered self-expanding metallic stent (c-SEMS)^[43], use of multiple plastic stents and novel dilatation balloons^[22] are steadily gaining ground over traditional eventual surgical options which include hepaticojejunostomy and retransplantation^[22,44]. In this study, 2 patients with "defiant" BDS underwent surgery [Hepaticojejunostomy (1) and Retransplantation (1)] and one patient had a c-SEMS placed. The use of c-SEMS has recently been studied in a meta-analysis^[38] where it was shown to have superior efficacy rates as compared to multiple plastic stenting. However, the same analysis showed high complication rates, especially stent migration^[38]. In our study, 2 patients with difficult BDS underwent DSOC with the help of which we were able to traverse the stricture successfully only in 1 patient. Our approach to "defiant" BDS currently centres around the

use of multiple plastic stents with only selected patients receiving c-SEMS. Surgical correction is reserved for patients who have particularly crippling symptoms along with frequent recurrence of strictures despite maximal stent therapy for over a period of 15 mo.

In summary, BDS remains a major complication of LDLT. Early identification of risk factors such as bile leaks and HAT in conjunction with adopting early treatment measures may improve therapeutic efficacy of endotherapy in these patients. Aggressive endotherapy protocols seem to have a favourable outcome after endotherapy thereby reducing the associated morbidity and mortality of BDS post LDLT. The use of c-SEMS, DSOC in the management of “defiant” BDS is an area that requires further study.

COMMENTS

Background

Bile duct strictures (BDS) are a major cause of morbidity and mortality in patients who have undergone a living donor liver transplantation. Endotherapy for BDS in these patients includes endoscopic retrograde cholangiography, sphincterotomy and stent placement. Results of endotherapy are variable and factors predicting outcome are not well elucidated.

Research frontiers

Factors that can predict endotherapy outcome and result in optimal patient selection. Endotherapy protocols/guidelines which can help roadmap this heterogeneous group of patients towards better healthcare.

Innovations and breakthroughs

Unique outcome definition: The outcome of endotherapy was considered favourable only when there was symptomatic and biochemical improvement. Partial responders were considered unfavourable owing to significant morbidity in this group. This sub group of patients is particularly tricky as they find themselves in a therapeutic “no man’s land” so to speak, where established treatment has provided some improvement, but with residual yet significant morbidity. Aggressive endotherapy protocols with repeated procedures, serial dilatation and stent upsizing for a minimum period exceeding 6 mo can yield good outcomes in majority of patients. Early endotherapy in patients with persistent bile leak (> 4 wk) may prevent the development of complex BDS. Use of two or more recipient ducts, especially cystic ducts are particularly poor responders to endotherapy.

Applications

This study may aid in the establishment of uniform endotherapy protocols which can have a wide applicability beyond the confines of single centre experiences. Bile duct anastomosis using multiple recipient ducts may be avoided, if possible; in favour of innovative surgical practices that may impact overall outcome in these patients. The outcome definition which identifies partial responders may suggest a sub group that needs a more detailed assessment which can lend clarity on optimal management of these patients.

Terminology

Type of anastomosis has been classified in terms of number of anastomosis into 4 types which have a significant bearing on overall outcome. Outcome definition was defined as favourable (symptomatic and biochemical improvement) and unfavourable (partial/non responders). Partial responders are those with either symptomatic and biochemical improvement and non-responders were labelled as those with neither.

Peer-review

The manuscript focuses on the endoscopic treatment of biliary strictures in living donor liver transplantation. It is interesting and well written.

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P- Reviewer: De Carlis R, Kim SM, Wejman J **S- Editor:** Ji FF
L- Editor: A **E- Editor:** Wu HL



Differential diagnosis in ulcerative colitis in an adolescent: Chronic granulomatous disease needs extra attention

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Author contributions: All authors contributed to this manuscript.

Institutional review board statement: This case report was exempt from the Institutional Review Board standards at Gazi University in Ankara.

Informed consent statement: Informed consent of the subject is taken.

Conflict-of-interest statement: None to declare.

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Manuscript source: Invited manuscript

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Received: November 22, 2016

Peer-review started: November 24, 2016

First decision: December 15, 2016

Revised: March 11, 2017

Accepted: April 6, 2017

Article in press: April 10, 2017

Published online: May 15, 2017

Abstract

Chronic granulomatous disease (CGD) is a primary immune deficiency that is commonly diagnosed under the age of 5 years (95%) and is rarely seen in adulthood. CGD may manifest as inflammatory bowel disease (IBD) in childhood. Without proper diagnosis, these patients may be monitored for years as IBD; some may even be regarded as steroid-resistant ulcerative colitis (UC) and end up having a colectomy. In this case report, we described a patient who had been followed-up for years as UC and subsequently underwent colectomy, but was finally diagnosed in adulthood as primary immune deficiency.

Key words: Ulcerative colitis; Chronic granulomatous disease; Inflammatory bowel disease; Immunodeficiency; Childhood

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Core tip: In the context of current knowledge, monogenetic diseases are at the basis of early-onset inflammatory bowel disease (IBD). Genetic and immunological studies on this subject have gained momentum in recent years. Chronic granulomatous disease (CGD) is an immune deficiency and may manifest itself as IBD. In an adolescent patient, CGD may present with clinical

signs of IBD without evidence of immune deficiency. It should be kept in mind that CGD in adolescents may extend to adulthood.

Kotlarz D, Egritas Gurkan O, Haskologlu ZS, Ekinici O, Aksu Unlusoy A, Gürcan Kaya N, Puchalka J, Klein C, Dalgic B. Differential diagnosis in ulcerative colitis in an adolescent: Chronic granulomatous disease needs extra attention. *World J Gastrointest Pathophysiol* 2017; 8(2): 87-92 Available from: URL: <http://www.wjgnet.com/2150-5330/full/v8/i2/87.htm> DOI: <http://dx.doi.org/10.4291/wjgp.v8.i2.87>

INTRODUCTION

Chronic granulomatous disease (CGD) is a primary immunodeficiency seen in approximately 1 in 200-250000 individuals^[1]. CGD is caused by abnormalities in the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex resulting in defective production of reactive oxygen species by phagocytic cells. The NADPH oxidase enzyme complex consists of at least five subunits: gp91phox, p22phox, p47phox, p67phox, and p40phox. Approximately, 66% of all CGD cases result from mutations within the X-linked [*CYBB* gene (encoding gp91phox)], followed by the autosomal recessive forms of CGD (%30) with defects in the (*NCF1*) gene coding for p47phox. Only 5% of the cases are due to mutations in *CYBA*, *NCF-2* or *NCF-4* which encode for p22phox, p67phox and p40phox respectively^[2,3].

As a result of the failure to activate the respiratory burst, patients with CGD present with severe recurrent intracellular bacterial and fungal infections such as pneumonia, lymphadenitis, cutaneous and hepatic abscesses, osteomyelitis and septicemia^[3]. These severe infections are caused predominantly by *Staphylococcus aureus* Aspergillus species, *Candida*, enteric gram negative bacteria, *Serratia marcescens*, *Burkholderia cepacia* complex, or *Mycobacterium tuberculosis*. CGD patients may also present with diffuse granulomas that can become large enough to cause obstructive or painful symptoms in the esophagus, stomach, biliary, intestinal, urogenital, or pulmonary systems^[1-3].

Colitis is an important gastrointestinal manifestation of CGD, typically seen more commonly, but not exclusively in the X-linked form of the disease^[4]. The clinical presentation of colitis is similar to that of inflammatory bowel disease (IBD), either ulcerative colitis (UC) or Crohn's disease, and includes diarrhea, abdominal pain and rectal bleeding. A diagnosis of CGD is usually made by gastroenterologists who have previously labeled patients as therapy-resistant IBD^[1-3].

CASE REPORT

A 15-year-old male patient was admitted to a medical center with persistent mucoid, bloody diarrhea. A

diagnosis of UC had been made and the patient was followed for six months after discharge. In the interim, treatment comprised pulsed steroids, 5-aminosalicylic acid, and azathioprine. The patient was referred to our center due to persisted symptoms. Medical history revealed absence of recurrent bacterial and fungal infections or frequent antibiotic use. His parents were non-consanguineous.

On physical examination body weight was 51 kg (25th percentile), and the height was 156 cm (10th percentile). Systemic examination was normal. Perianal modifier was not observed. Laboratory results showed hemoglobin of 11.4 g/dL, white blood cell count of 8900/mm³, neutrophils count of 7900/mm³, lymphocyte count of 2200/mm³ and platelet count of 448000/mm³. Stool microscopy showed the presence of occult blood and leukocytes. Total protein was 6.2 g/dL, albumin was 3.8 g/dL, erythrocyte sedimentation rate was 68 mm/h, C-reactive protein was 38 mg/dL, and PANCA was positive. IgA: 68 mg/dL, IgG: 1100 mg/dL, IgM: 79.7 mg/dL, IgE: 38.5 mg/dL, IgA: 217 mg/dL, total C3: 128 mg/dL, total C4: 26.3 mg/dL. Liver and kidney function tests were normal. Colonoscopy was compatible with the diagnosis of pancolitis, but the terminal ileum was normal. Upper gastrointestinal endoscopy revealed pangastritis. Histopathologic evaluation showed cryptitis, crypt abscesses, and pseudopolyps of the entire colonic mucosa; granulomas were not detected.

The subsequent medical treatment and follow-up of the patient are shown in Figure 1. Due to the refractory course of the patient, familial Mediterranean fever (MEVF mutation) and Behcet's disease (Pathergy test, HLA B5 test) were considered and excluded. The patient had a steroid-resistant course and total colectomy was performed. Light microscopic examination of colectomy specimen revealed crypt distortion, cryptitis, crypt loss and crypt abscesses throughout whole colonic mucosa. Epithelial ulcerations, regenerative changes and pseudopolyps were also noted. Neutrophils were present in lamina propria, dense and mixed type inflammatory cell infiltration was seen. These changes were limited thorough mucosa and submucosa. Granulomas and dysplasia were not encountered. The surgically resected specimen was compatible with UC (Figure 2). Acute phase proteins remained slightly increased and soiling was the major problem in the postoperative period. Anorectal manometry showed increased rectal sensitivity, significantly low resting anal sphincter pressure, normal rectoanal inhibitor reflex, and adequate pressure increase in voluntary sphincter contraction. However, perianal dermatitis and impaired perianal wound healing persisted (Figure 3).

Ileoscopy performed 8 mo after colectomy operation revealed a second lumen layer outside the lumen. Both lumen openings were hyperemic, edematous, and eroded (Figure 4). Ileal biopsies revealed backwash ileitis, without signs of Crohn's disease.

At the age of 18 years, the patient was referred

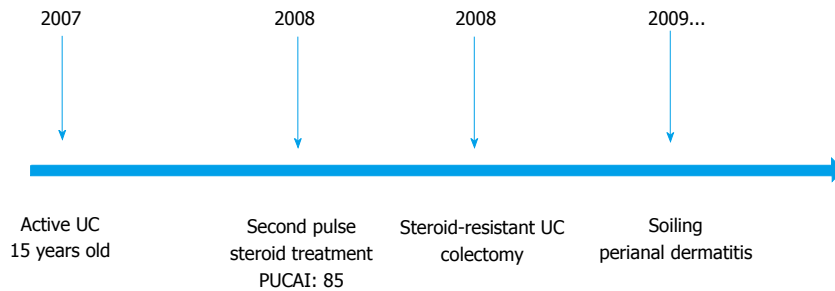


Figure 1 The medical treatment and follow-up of the patient. UC: Ulcerative colitis.

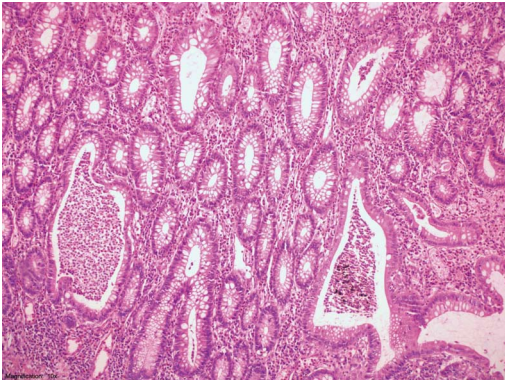


Figure 2 Histopathologic appearance of colonic mucosa.



Figure 3 Perianal dermatitis and impaired perianal wound healing.

to adult gastroenterology section. Pelvic magnetic resonance imaging and fistulography revealed an ileoileal fistula. At the age of 23 years, there was persistence of the perianal lesions and diarrhea despite treatment.

Genetic analysis

To screen for monogenetic forms of IBD, we have performed whole exome sequencing of the patient sample using an exome enrichment kit (SureSelect V4UTR; Agilent) and next-generation sequencing device (Illumina HiSeq 2500). Analysis of rare genetic variants that affected protein sequence or interfered with mRNA transcription revealed a mutation in the *NCF2* gene (c.326A>G, p.Y109Y>C). Bioinformatic analysis of this sequence variant predicted that the amino acid exchange that was deleterious to the protein function.

A diagnosis of CGD was confirmed biologically by a nitroblue tetrazolium test (NBT) and flow cytometry-based dihydrorhodamine (DHR) or 2'-7'-dichlorofluorescein diacetate assays. An NBT slide test with *Escherichia coli* lipopolysaccharide (840 W Sigma-Aldrich) was used to stimulate respiratory burst in phagocytic cells. Normally the percentage of blue-stained neutrophils in an NBT test (Figure 5) is close to 100 - but this percentage is close to 0 in CGD patients. In this patient, the NBT test confirmed a defective respiratory burst in the neutrophils. DHR test (Figure 6) uses flow cytometry to measure the oxidation of dihydrorhodamine 123 to rhodamine 123 in phorbol myristate acetate-stimulated neutrophils, a marker for cellular NADPH oxidase activity. In this test the generation of hydrogen peroxide oxidizes

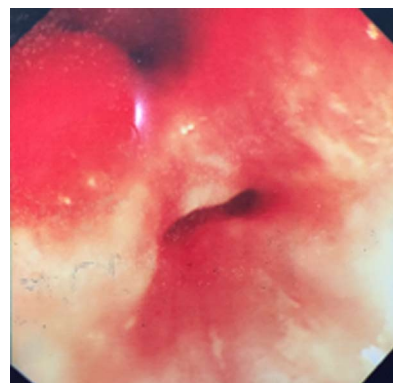


Figure 4 Two lumens visualized during ileoscopy.

the dye, leading to the emission of fluorescence. Mean fluorescence intensity of the activated cells correlates directly with (and thus serves as a reliable surrogate for) superoxide production. In this patient, the diagnosis of CGD was confirmed by using the NBT test and the DHR assay.

Based on the treatment-refractory course and the underlying primary immunodeficiency in the patient, we considered hematopoietic stem cell transplant (HSCT) for cure. The patient had been referred to the Department of Hematology and is currently being prepared for HSCT.

DISCUSSION

CGD is a primary immunodeficiency caused by a defect

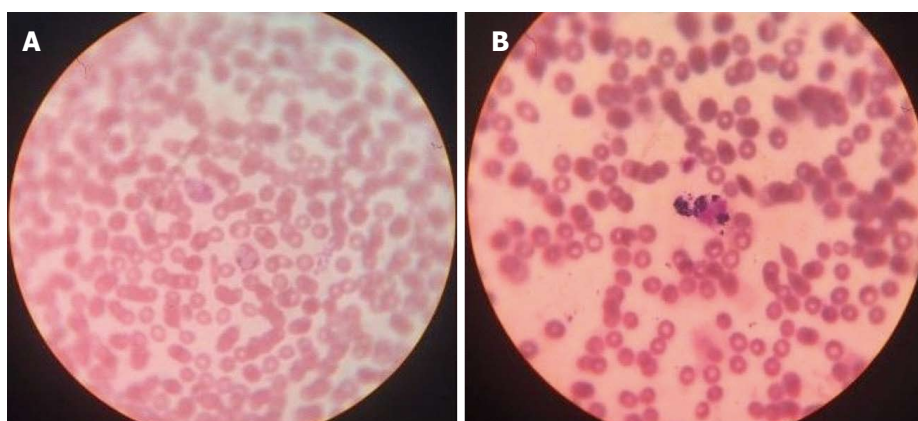


Figure 5 Nitroblue tetrazolium test. A: Neutrophils from a patient with CGD fail to reduce the NBT dye and appear clear; B: Normal (unaffected) cells reduce the NBT dye and stain blue/purple. CGD: Chronic granulomatous disease; NBT: Nitroblue tetrazolium test.

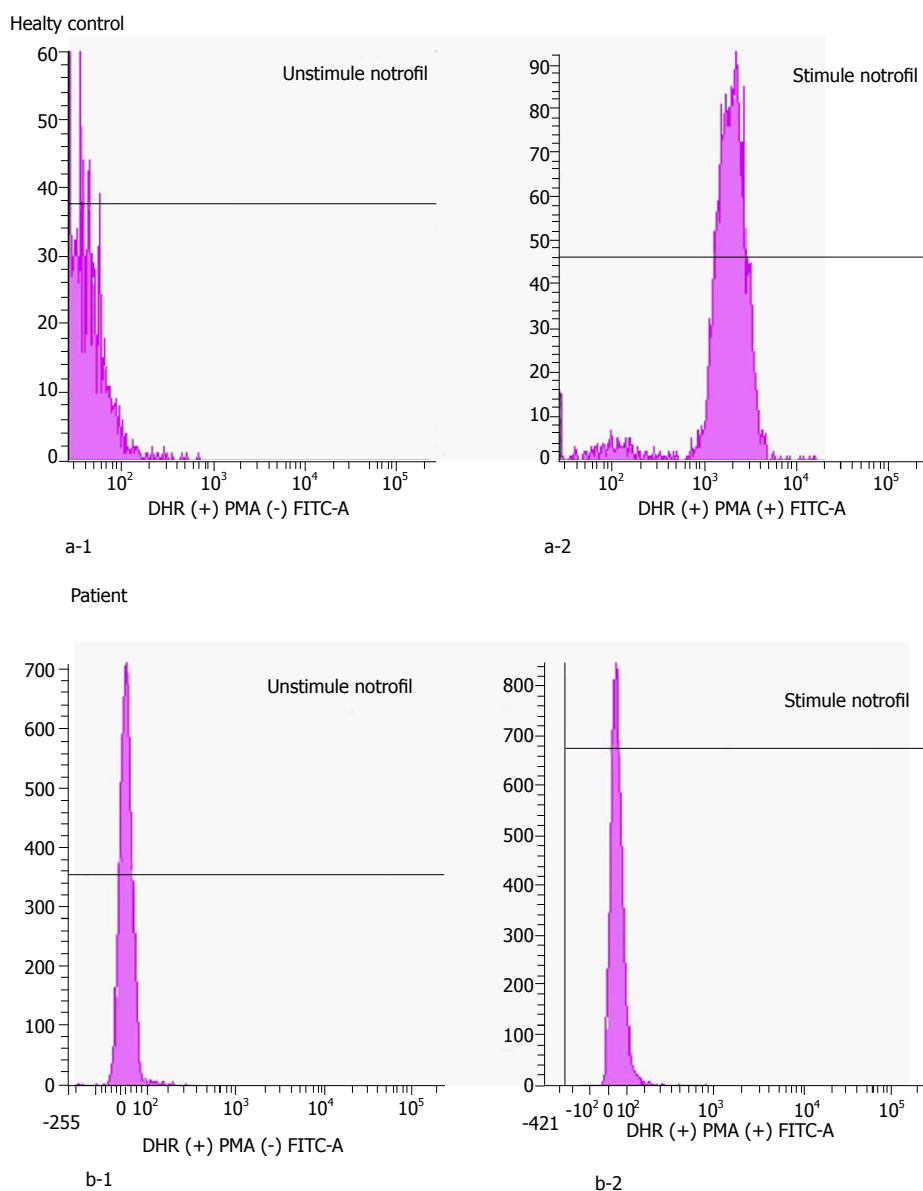


Figure 6 Flow cytometry-based dihydrorhodamine test. DHR: Dihydrorhodamine.

in a superoxide formation. More than 95% of cases are diagnosed before the age of 5 years. However, some

patients may be diagnosed only in adulthood^[1]. During evaluation of very early onset IBD under six years of

age, one can easily consider the presence of underlying immune deficiencies. However, it will be tough to consider this point in an adolescent patient with UC alone, normal physical development and negative history of recurrent infections like our patient.

Gastrointestinal tract involvement in CGD patients causes significant morbidity and mortality. Its phenotype might be similar in presentation-with IBD. Intestinal motility dysfunction, obstruction, ulceration and recurrent infection may occur along the entire tract^[5]. Gastrointestinal involvement was reported in 32% to 48% of patients with CGD^[1,6-8]. A study by van den Berg *et al*^[6] found that 48% of CGD patients had experienced at least one episode of gastrointestinal manifestation. Colitis, perianal abscess and fistula formation may be the first symptoms for gastrointestinal involvement^[9,10]. In our patient, the multiple colonoscopies revealed only large bowel involvement and normal terminal ileum. Therefore, the patient was monitored for years with a diagnosis of UC and eventually underwent colectomy due to the absence of response to medical therapy.

The endoscopic and pathological findings of patients with bowel involvement might be indistinguishable from that of IBD^[10]. Cryptitis, crypt abscesses, eosinophilic infiltrates and granulomas may be detected in affected intestinal segments of CGD patients. Although presence of large pigment laden histiocytes in involved organs of CGD patients is significant, these findings are not specific or sensitive^[11]. In our patient evaluation of colectomy specimens revealed no granulomas, therefore Crohn's disease or Crohn's-alike diseases were not considered. In addition, there were no recurrent bacterial and fungal infections or frequent antibiotic use. Immunologic tests for primary immunodeficiency were not considered due to the late onset of IBD. Eventually, unbiased whole exome sequencing revealed a homozygous missense mutation in NCF2, which was indicative of CGD diagnosis that was confirmed by NBT assay. Genetic studies showed NCF2 mutation for CGD which shows an autosomal recessive trend. Consanguineous marriage was not noted in family history, but coming from same village and the CGD disease with an autosomal recessive trait made us think of a distant relativity between parents.

Very early onset-IBD (VEO-IBD) (below-six years of age) has been shown to be associated with monogenetic etiologies in particular immune deficiencies^[12]. Accordingly immunologic tests and genetic analysis are recommended in VEO-IBD patients. In IBD patients over the age of 6 years, initial search for immunologic parameters is recommended for the following reasons: (1) presence of lesions in the perianal region; (2) consanguineous parents; (3) unresponsive to treatment or steroid dependent; (4) family history of early-onset IBD; and (5) the presence of skin, nail, or hair abnormalities. With the rise in genetic studies, we learned that similar to IBD, immunodeficiencies like CGD may occur in after adolescence. Our experience on this case would make one wonder whether immunologic tests are necessary for every IBD patient, regardless of age.

In literature, basic immunologic tests as the first step in IBD patients were not recommended regardless of the presence of risk factors. However, considering the prolonged follow-up required of this chronic disease, we recommend otherwise.

COMMENTS

Case characteristics

Main symptoms were bloody mucoid diarrhea.

Clinical diagnosis

Main clinical findings were perianal modifiers after colectomy operation.

Differential diagnosis

Crohn's disease should be kept in mind in patients with perianal modifiers.

Laboratory diagnosis

Elevated acute phase reactants and mutation in the NCF2 gene (c.326A>G, p.Y109Y>C) were seen.

Imaging diagnosis

Colonoscopic appearance was compatible with pancolitis.

Pathological diagnosis

Histopathologic evaluation showed cryptitis, crypt abscesses, and pseudopolyps of the entire colonic mucosa; granulomas were not detected.

Treatment

Treatment was consisted of steroids and immunosuppressive drugs.

Experiences and lessons

Chronic granulomatous disease may present itself as ulcerative colitis in adulthood.

Peer-review

This case report was well organized and well investigated. This paper will give us a new information especially in the field of inflammatory bowel disease.

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P- Reviewer: Freeman HJ, Jamali R, Naito Y, Ruffolo C, Sipos F

S- Editor: Ji FF **L- Editor:** A **E- Editor:** Wu HL



Duodenal localization of plasmablastic myeloma

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Author contributions: Licci S made the histological diagnosis, reviewed the literature and conceived and wrote the case report.

Institutional review board statement: This case report was exempt from the institutional review board standards at Santo Spirito Hospital (Rome, Italy).

Informed consent statement: The patient involved in this study gave her written informed consent authorizing use and disclosure of her protected health information.

Conflict-of-interest statement: The author has no conflicts of interest to declare.

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Manuscript source: Invited manuscript

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Received: November 4, 2016

Peer-review started: November 5, 2016

First decision: December 29, 2016

Revised: January 27, 2017

Accepted: March 12, 2017

Article in press: March 13, 2017

Published online: May 15, 2017

Abstract

Gastrointestinal involvement in plasma cell neoplasms,

either as primary localizations (extramedullary plasmacytomas) or as secondary involvement in systemic multiple myeloma, is a well-known event. Accurate histological examination is crucial in defining the diagnosis. In this report, an uncommon case of duodenal localization of myeloma with plasmablastic features is described, with emphasis on the role of clinical data and findings from ancillary immunostaining techniques to avoid misdiagnosis.

Key words: Gastrointestinal tract; Myeloma; Plasma cell neoplasm; Plasmablastic; Duodenum

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Core tip: In cases of gastrointestinal involvement by high-grade plasma cell neoplasia, the presence of large atypical cells infiltrating the lamina propria of the mucosa may lead to an erroneous diagnosis of poorly differentiated carcinoma. Clinical data and findings from ancillary immunostaining techniques are crucial to avoid misdiagnosis.

Licci S. Duodenal localization of plasmablastic myeloma. *World J Gastrointest Pathophysiol* 2017; 8(2): 93-95 Available from: URL: <http://www.wjgnet.com/2150-5330/full/v8/i2/93.htm> DOI: <http://dx.doi.org/10.4291/wjgp.v8.i2.93>

INTRODUCTION

Involvement of the gastrointestinal tract by plasma cell neoplasms is a well-known event, and many cases have been described in the literature, either as primary localizations (extramedullary plasmacytomas)^[1] or as secondary involvement in systemic multiple myeloma^[2]. In this report, the immunomorphological findings of an uncommon case of duodenal localization of myeloma with plasmablastic features are described.

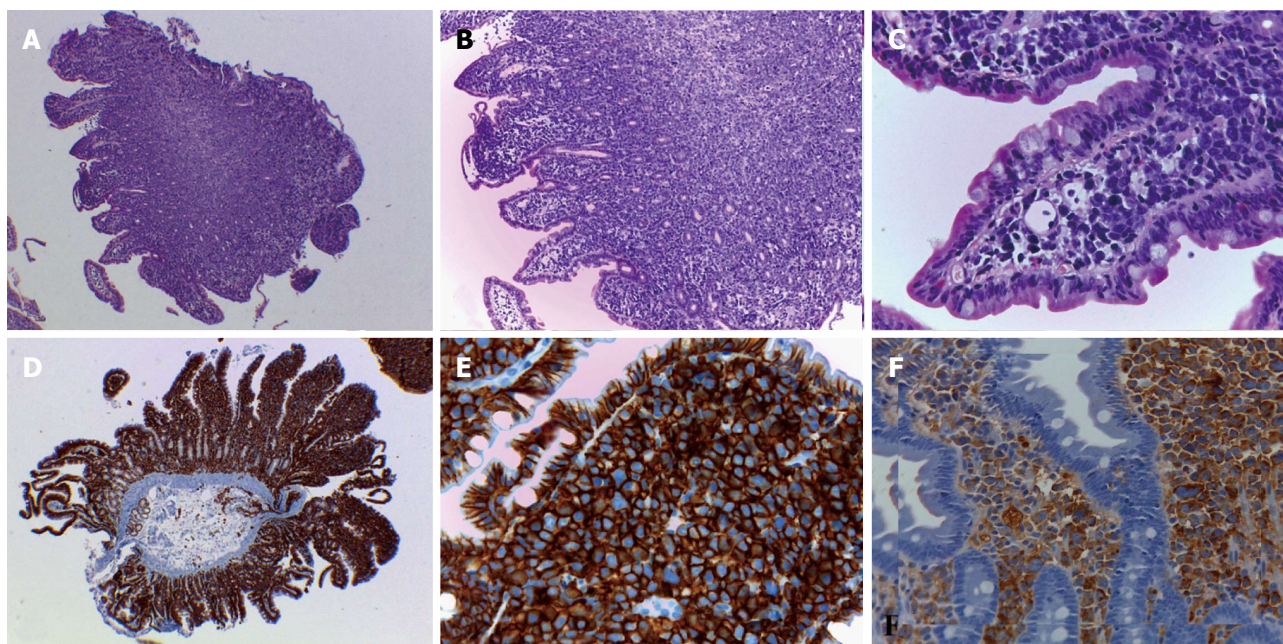


Figure 1 Histological examination of duodenal mucosa biopsy samples. A: Low-power histological examination of duodenal mucosa fragments shows a hypercellular inter-glandular stroma (hematoxylin-eosin, original magnification $\times 4$); B, C: At medium- and high-power; B: Hematoxylin-eosin, original magnification $\times 10$; C: Hematoxylin-eosin, original magnification $\times 40$), an infiltration of the lamina propria by atypical cells is better appreciated, with neoplastic cells displaying medium to large size pleomorphic nuclei, sometimes with prominent nucleoli, and scanty cytoplasm; D-F: CD138 immunostaining shows a strong and diffuse membrane staining, revealing the plasma cell nature of the neoplastic infiltrate (D: Original magnification $\times 4$; E: Original magnification $\times 40$), with prevalent λ chain immunohistochemical expression (F: Original magnification $\times 40$).

CASE REPORT

A 60-year-old woman presented with unintentional weight loss, anemia and thrombocytopenia. Serum protein electrophoresis revealed a monoclonal peak of IgG (30 g/L), and immunofixation identified λ light chains. For better hematological evaluation, a bone marrow trephine biopsy was performed. Marrow spaces showed remarkably increased cellularity (about 90%), mainly represented by immature/atypical CD138⁺ plasma cells with prevalent λ chain immunohistochemical expression (about 80% of the total cellularity). Accordingly, the diagnosis was plasma cell neoplasm, with morphological features consistent with "high-grade" plasma cell myeloma. The residual cellularity was composed of trilineage hematopoietic cells with reactive changes, rare CD34⁺ blast cells and some B (CD20⁺) and T (CD3⁺) reactive small lymphocytes, showing interstitial and micronodular distribution.

In the days following the initial presentation to clinic, the patient re-presented with an acute worsening of anemia and an episode of melena. Suspected gastrointestinal bleeding was investigated by esophagogastroduodenoscopy; the esophageal and gastric mucosa appeared normal, while the duodenal mucosa was characterized by presence of multiple micropolyps, which were biopsied for histology. Microscopic examination revealed a diffuse infiltration in the lamina propria by medium- to large-sized cells with high nuclear-cytoplasmic ratio, atypical pleomorphic nuclei and prominent nucleoli, consistent with malignant neoplasia

(Figure 1A-C). Immunohistochemical study excluded infiltration by poorly differentiated carcinoma (*i.e.*, AE1/3 cytokeratin immunostaining was negative) and revealed a diffuse and strong positivity for the CD138 plasma cell marker (Figure 1D and E). Further immunostaining analyses showed a prevalent λ chain expression (Figure 1F).

Ultimately, the diagnosis of duodenal localization of plasma cell neoplasm showing plasmablastic myeloma features was made on the basis of the cell morphology findings and consistent with the previous bone marrow trephine biopsy diagnosis.

DISCUSSION

In cases of gastrointestinal plasma cell neoplasia, histological examination can represent a diagnostic pitfall, especially when the clinical data are missing; the underlying plasma cell neoplasia can remain unknown and the tumor cells can appear immature and/or atypical. In fact, the presence of neoplastic atypical cells diffusely infiltrating the lamina propria among glandular structures may be easily misdiagnosed as a poorly differentiated carcinoma. Furthermore, even in well differentiated cases with recognizable plasma cells, the issue can be complicated by the presence of numerous Russell bodies - spherical intracytoplasmic eosinophilic immunoglobulin - containing structures, easily detectable by microscopic observation-either in association with neoplastic plasma cell proliferation, in cases of lymphoproliferative disorders displaying a certain degree of plasma cell differentiation, or in non-neoplastic,

inflammatory processes characterized by a conspicuous plasma cell infiltrate, such as the so-called Russell body gastritis^[3]. In such cases, plasma cells can take on the form of the signet-ring cells of poorly differentiated mucinous carcinoma of the gastrointestinal tract. Thus, careful microscopic observation must be integrated with ancillary techniques, mainly immunohistochemical staining analyses, to formulate the right diagnosis. Immunoreaction for cytokeratins is determinant for excluding a carcinoma, and demonstration of plasma cell [CD138⁺ and/or CD38⁺ and/or VS38c (plasma cell p63)⁺] proliferation with κ or λ light-chain restriction enables distinction between a reactive and a neoplastic plasma cell infiltrate.

For the case presented herein, the clinical data represented an important tool for making the right diagnosis, similar to a previously described case of extramedullary plasmablastic myeloma of the small bowel^[4]. The patient's experience of acute worsening of anemia and an episode of melena suggested gastrointestinal bleeding; esophagogastroduodenoscopy disclosed the duodenal mucosa lesions and the histological diagnosis were supported by the clinical-anamnestic data of a previous diagnosis of multiple myeloma. In such cases of secondary gastroenteric involvement by myeloma, therapy options include induction chemotherapy with immunomodulatory agents or proteasome inhibitors and corticosteroids. Stem cell transplantation may improve the remission rates and overall survival.

COMMENTS

Case characteristics

A 60-year-old woman with recent clinical history of multiple myeloma presented for acute worsening of anemia and an episode of melena.

Clinical diagnosis

To address suspected gastrointestinal bleeding, an esophagogastroduodenoscopy was performed and revealed multiple micropolyps of duodenal mucosa, which were biopsied for histology.

Differential diagnosis

Adenomatous duodenal polyps; Primary duodenal adenocarcinoma; Secondary duodenal involvement by myeloma; Other neoplasia.

Laboratory diagnosis

Serum protein electrophoresis monoclonal peak of IgG for the underlying disease; Severe worsening of anemia.

Imaging diagnosis

Bone osteolytic lesions for the underlying disease.

Pathological diagnosis

Secondary duodenal localization of plasmablastic myeloma.

Treatment

Proteasome inhibitors and corticosteroids; Stem cell transplantation.

Related reports

Because of the possible morphological overlap, a secondary gastrointestinal localization of high-grade myeloma can be mistakenly diagnosed as poorly differentiated adenocarcinoma.

Term explanation

The term "plasmablastic" in myeloma indicates a low degree of tumor cell differentiation, related to a more aggressive biological behavior.

Experiences and lessons

Correlation of the histopathological findings with clinical and medical history is the basis of a correct diagnosis for plasmablastic myeloma with duodenal localization.

Peer-review

The strength of this report lies in the importance of the histopathological study, deeply influenced by the knowledge of the clinical and medical history.

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P- Reviewer: Mica L S- Editor: Qi Y L- Editor: A
E- Editor: Wu HL



Late onset pulmonary metastasis more than 10 years after primary sigmoid carcinoma

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Author contributions: Daniels AM was responsible for sample collection, acquisition of patient data, drafting and revising of the manuscript; Vogelaar JFJ planned the study and revised the manuscript.

Institutional review board statement: Approval from the Local Ethical Committee for this study was granted.

Informed consent statement: Informed consent was obtained from the patient.

Conflict-of-interest statement: Both authors have no conflicts of interests to declare.

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Manuscript source: Unsolicited manuscript

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Received: October 25, 2016

Peer-review started: October 27, 2016

First decision: December 1, 2016

Revised: January 3, 2017

Accepted: March 12, 2017

Article in press: March 13, 2017

Published online: May 15, 2017

Abstract

According to current guidelines, follow-up of patients

with colorectal cancer is ended after five years. Also, chest X-ray is not part of standard investigation during follow-up. We describe a case of a 74-year-old patient, more than ten years after a sigmoid resection because of carcinoma of the sigmoid. No recurrence was detected during intensive follow-up. However, ten years after resection of the sigmoid adenocarcinoma, complaints of coughing induced further examination with as result the detection of a solitary metastasis in the left lung of the patient. Within half-a-year after metastasectomy of the lung metastasis, she presented herself with thoracic pain and dyspnea resulting in discovering diffuse metastasis on pulmonary, pleural, costal and muscular level. Five year follow-up of colorectal carcinoma without chest X-ray can be questioned to be efficient. The growing knowledge of tumor biology might in future adjust the duration and frequency of diagnostic follow-up to prevent (late) recurrence in patients with colorectal carcinoma.

Key words: Colorectal cancer; Metastasis; Tumor biology; Tumor dormancy; Follow-up

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Core tip: This case report presents a case of a woman with pulmonary metastases 10 years after a sigmoid resection. No recurrence of the primary intestinal tumor was detected during follow-up. Upcoming knowledge on tumor behavior based on its biology might give new insights in late-onset metastases. Therefore, follow-up protocols and current therapy guidelines might have to be re-evaluated.

Daniels AM, Vogelaar JFJ. Late onset pulmonary metastasis more than 10 years after primary sigmoid carcinoma. *World J Gastrointest Pathophysiol* 2017; 8(2): 96-99 Available from: <http://www.wjgnet.com/2150-5330/full/v8/i2/96.htm> DOI: <http://dx.doi.org/10.4291/wjgp.v8.i2.96>

INTRODUCTION

Colorectal carcinoma (CRC) is the most common type of gastrointestinal cancer. The current screening methods enable early detection, for example adenomatous polyps, to prevent the development of invasive carcinoma^[1]. Etiology of CRC comprises genetic involvement, molecular events, environmental factors and inflammatory conditions^[2]. CRC usually develops over a period of 10-15 years^[3], most often affecting people aged over 50^[4]. After a curative resection, prognosis of CRC is worse in poorly differentiated, mucinous carcinomas with large invasion depth and in the presence of lymph node metastases^[5]. Follow-up of patients with CRC according to the national guidelines consists of echography or computed tomography (CT) of the abdomen, colonoscopy and determination of carcinoembryonic antigen (CEA) until five years after surgical resection^[6].

CRC can spread by lymphatic and hematogenous dissemination, as well as by contiguous and transperitoneal routes. Metastasis, at initially diagnosis present in 20% of all patients, is the main cause of mortality in patients with CRC^[7,8]. When cancer spreads to one single organ, the malignancy presents itself with a specific biological profile and clinical characteristics. The site of the metastatic disease influences the prognosis and the possibilities for treatment. Organs most often affected by CRC are the liver and the lungs^[9]. For pulmonary lesions, the criteria for resectability of the metastatic lesion comprise; control of the primary tumor, possible complete resection and adequate pulmonary reserve to tolerate the planned resection^[10].

CASE REPORT

Ten years ago, a 63-year-old woman was admitted to our hospital with changes in bowel habits. At that time, she had no medical history of abdominal complaints or surgical operations. Her father was familiar with lung carcinoma, her mother and both of her sisters suffered from breast carcinoma.

A colonoscopy reveals a sigmoid colon stricture with unknown length due to the impossibility of endoscopically passing the stricture. Biopsy of the sigmoid shows a poorly differentiated adenocarcinoma. Computed tomography and echography of the liver, spleen and of the pelvic, cardio-thoracic, para-aortic and paracaval region show no lesions suspected for malignancy. An uncomplicated surgical resection of the sigmoid took place. The tumor, with a maximum diameter of five centimeters, including five surrounding lymph nodes and infiltrated fat tissue were removed in totality. No lymph node metastases were detected.

One year after the surgical resection, biopsy of a polyp of the ascending colon showed a tubular adenoma with low grade dysplasia. During further follow up of this patients no malignancies or recurrent lesions were disclosed. The CEA was tested every three months with



Figure 1 Computed tomography of the pulmonary lesion.

a maximum value of 2.2 mcg/L. The intensive follow-up period was completed five years after sigmoid resection.

Ten years after resection of the adenocarcinoma located in the sigmoid without recurrent carcinoma's during follow up, our patient presented herself with persistent coughing. Imaging of the thorax shows a lesion of 1.5 centimeters located in the inferior lobe of the left lung (Figure 1). Biopsy reveals an adenocarcinoma with positive CDX-2 staining, corresponding with intestinal origin of the cells. The pulmonary tumor was removed by video assisted thoracic surgery (VATS), presumably in totality. Colonoscopy shows no metachronous neoplasia. Within half a year, she was admitted to our emergency department with complaints of pain on the left side of her chest resulting in dyspnea, the pain was coherent with breathing movement and pain radiation towards her spine. CT-thorax shows extensive pleural, costal and muscular metastases (Figure 2). To reduce pain, palliative chemotherapy and additional radiotherapy were started.

DISCUSSION

Early detection of relapse or metastatic disease can be effectuated by intensive follow up during five years as recorded in the directive of Oncoline^[6]. This follow-up comprises echography and optional CT-abdomen twice a year during 1-2 years, followed by once a year up to 5 years after the surgical resection. CEA should be determined every 3-6 mo during 3 years, followed by every 6 mo until 5 years after treatment. Colonoscopy is performed three, six and twelve months after surgical resection. Based on the number, localization and size of the lesions, repetition of colonoscopy is indicated after 3 or 5 years^[6].

However, several clinical and experimental observations suggest that metastases can develop even in the absence of a detectable primary tumor. Both large and small tumors have the capability to metastasize^[11,12]. Dissemination of tumor cells can occur in pre-invasive stages of tumor progression resulting in the development of early dormant disseminated tumor cells (DTCs). Early DTCs can remain dormant for a long period with



Figure 2 Computed tomography of the pleural metastasis (indicated with arrows).

manifestation of metastatic growth as result^[13,14]. Metastases may thus be initiated by and evolve from dormant DTCs, rather than established primary tumors. These DTCs can generate metastases with different characteristics from those of the primary tumor. The discriminating characteristics may explain the lack of success treating metastases with therapies based on primary tumor characteristics^[14]. Clinical evidence supports that the vast majority of early DTCs seem to be dormant^[13].

In conclusion, five year follow-up of colorectal carcinoma without chest X-ray can be questioned to be efficient with the upcoming knowledge of early DTC's. Adjustment of the duration and frequency of diagnostic follow-up and adjustment of treatment might be effective for early detection of metastatic disease and prevention of late recurrence in patients with colorectal carcinoma.

ACKNOWLEDGMENTS

The authors wish to thank Simons P, MD, PhD for providing the CT-scan figures.

COMMENTS

Case characteristics

A 63-year-old woman presented with a poorly differentiated adenocarcinoma of the sigmoid as primary tumor. No recurrence of the primary intestinal tumor was detected during follow-up. Ten years after resection of the primary tumor, computed tomography reveals a pulmonary metastasis in the left lung of the patient.

Clinical diagnosis

Persistent cough.

Differential diagnosis

The most common causes of chronic cough are postnasal drip, asthma, and acid reflux from the stomach. Less common causes include infections, medications, and lung diseases (e.g., primary/secondary malignancy).

Laboratory diagnosis

No raised CRP or ESR. Normal leucocyte count. Carcinoembryonic antigen 1.7

mcg/L.

Imaging diagnosis

Computed tomography (CT) shows a lesion of 1.5 centimeters located in the inferior lobe of the left lung.

Pathological diagnosis

Pulmonary metastasis of intestinal origin; a poorly differentiated adenocarcinoma.

Treatment

Metastasectomy by video assisted thoracic surgery.

Related reports

Follow-up of colorectal carcinoma consists of echography, optional CT-abdomen, CEA determination and colonoscopy. Without recurrence of the tumor within five years after primary resection, intensive follow-up is ended. However, several clinical and experimental observations suggest that metastases can develop even in the absence of a detectable primary tumor, based on the principle of dormant disseminated tumor cells.

Term explanation

Late onset pulmonary metastases more than ten years after primary colorectal cancer, without recurrence of the primary tumor, is very rare.

Experiences and lessons

Most recent guidelines recommend follow-up until five years after treatment of colorectal cancer. A pulmonary mass on imaging of the thorax, even more than 10 years after curative resection, is suspicious for metastatic disease. In general, more insights in the behavior of cancer (tumor biology) might change follow-up regimens in future.

Peer-review

The authors presented a case of a woman with pulmonary metastases 10 years after a sigmoid resection. This is clinically important and the paper is well written.

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World J Gastrointest Pathophysiol 2017 August 15; 8(3): 100-149





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World Journal of Gastrointestinal Pathophysiology
Volume 8 Number 3 August 15, 2017

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World Journal of Gastrointestinal Pathophysiology (*World J Gastrointest Pathophysiol*, *WJGP*, online ISSN 2150-5330, DOI: 10.4291), is a peer-reviewed open access academic journal that aims to guide clinical practice and improve diagnostic and therapeutic skills of clinicians.

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INDEXING/ABSTRACTING

World Journal of Gastrointestinal Pathophysiology is now indexed in PubMed, PubMed Central.

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NAME OF JOURNAL
World Journal of Gastrointestinal Pathophysiology

ISSN
ISSN 2150-5330 (online)

LAUNCH DATE
April 15, 2010

Frequency
Quarterly

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7901 Stoneridge Drive, Suite 501, Pleasanton, CA 94588, USA
Telephone: +1-925-2238242
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7901 Stoneridge Drive,
Suite 501, Pleasanton, CA 94588, USA
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PUBLICATION DATE
August 15, 2017

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Fatty liver without a large “belly”: Magnified review of non-alcoholic fatty liver disease in non-obese patients

Mohamad H Yousef, Alhareth Al Juboori, Abdulmajeed A Albarrak, Jamal A Ibdah, Veysel Tahan

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Author contributions: All authors contributed to the acquisition of data, writing, and revision of this manuscript.

Conflict-of-interest statement: All the authors have no conflicts of interests to declare.

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Manuscript source: Invited manuscript

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Received: March 19, 2017

Peer-review started: March 20, 2017

First decision: May 9, 2017

Revised: May 22, 2017

Accepted: June 19, 2017

Article in press: June 20, 2017

Published online: August 15, 2017

Abstract

Nonalcoholic fatty liver disease (NAFLD) is well described as a common cause of chronic liver disease, mostly in the obese population. It refers to a spectrum of chronic liver

disease that starts with simple steatosis than progresses to nonalcoholic steatohepatitis and cirrhosis in patients without significant alcohol consumption. NAFLD in the non-obese population has been increasingly reported and studied recently. The pathogenesis of nonobese NAFLD is poorly understood and is related to genetic predisposition, most notably patatin-like phospholipase domain-containing 33 G allele polymorphism that leads to intrahepatic triglyceride accumulation and insulin resistance. Non-obese NAFLD is associated with components of metabolic syndrome and, especially, visceral obesity which seems to be an important etiological factor in this group. Dietary factors and, specifically, a high fructose diet seem to play a role. Cardiovascular events remain the main cause of mortality and morbidity in NAFLD, including in the non-obese population. There is not enough data regarding treatment in non-obese NAFLD patients, but similar to NAFLD in obese subjects, lifestyle changes that include dietary modification, physical activity, and weight loss remain the mainstay of treatment.

Key words: Nonobese; Nonalcoholic fatty liver disease; Hepatic steatosis; Nonalcoholic steatohepatitis; Genetic

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Core tip: Nonobese nonalcoholic fatty liver disease (NAFLD) is likely a different entity than obese NAFLD, with its unique genetic predisposition. NAFLD in the nonobese population has been increasingly reported and studied recently. It is associated with components of metabolic syndrome. Interestingly, even though non-obese NAFLD patients have normal weight ranges, weight loss remains the mainstay treatment and was found to be beneficial. Diagnosis and treatment are similar to the obese non-alcoholic fatty liver disease. Further research is needed for better understanding of the genetic and environmental factors affecting the course of this specific entity.

Yousef MH, Al Juboori A, Albarrak AA, Ibdah JA, Tahan V. Fatty

liver without a large “belly”: Magnified review of non-alcoholic fatty liver disease in non-obese patients. *World J Gastrointest Pathophysiol* 2017; 8(3): 100-107 Available from: URL: <http://www.wjgnet.com/2150-5330/full/v8/i3/100.htm> DOI: <http://dx.doi.org/10.4291/wjgp.v8.i3.100>

INTRODUCTION

With the growing epidemic of metabolic syndrome, nonalcoholic fatty liver disease (NAFLD), has become the most common cause of chronic liver disease in the Western world^[1,2]. It is characterized by hepatic fat accumulation (steatosis) after excluding significant alcohol consumption; more than 21 drinks a week in men and 14 drinks a week in women^[3,4], or more than 140 g weekly^[2]. Disease spectrum ranges from hepatic steatosis, to nonalcoholic steatohepatitis (NASH), which is characterized by hepatic inflammation and hepatocyte ballooning. When advanced, NASH can progress to advanced fibrosis, cirrhosis, and hepatocellular carcinoma^[5-7]. NAFLD is the most common cause of cryptogenic cirrhosis^[8]. Occasionally, NAFLD can progress to hepatocellular carcinoma skipping cirrhosis interval^[9]. NAFLD was first reported in 1980 by Ludwig *et al.*^[10] who described histological findings in 20 patients, mostly obese, with NAFLD. Obesity has been historically associated with NAFLD, however, not all obese patients develop NAFLD^[11]. NAFLD is also reported in non-obese population^[11-16]. NAFLD is a major cause of hepatic morbidity and mortality^[17]. Here, we review basic and clinical features of non-obese NAFLD.

PREVALENCE

The prevalence of non-obese NAFLD using a cutoff body mass index (BMI) of 25 kg/m² ranges from 4.2% to 27.5%^[11]. Using the national health and nutrition examination survey III data collected from 1988-1994, the prevalence of non-obese hepatic steatosis in patients with BMI less than 25 kg/m², was 21%^[11]. In a community-based study in Hong Kong, the prevalence of NAFLD was 19.3% and 60.5% in non-obese patients and obese patients respectively, using BMI of 25 kg/m²^[16]. This is comparable to the prevalence of obese NAFLD which, in most studies, ranges between 6.3%-30%^[3].

PATHOGENESIS AND RISK FACTORS

Intrahepatic triglyceride content plays an integral role in the pathogenesis of non-obese NAFLD^[18]. Hepatic steatosis is chemically defined as increased intrahepatic fatty acids content that are more than 5% of the liver weight^[19], or histologically as 5% or more intrahepatic steatosis in a liver section^[20-22]. There are multiple factors that increase intrahepatic free fatty acids. Increased release from the adipose tissue, and subsequent increase in the free fatty acid uptake by

skeletal muscle and liver tissues, is noted in NAFLD patients. This is facilitated by alterations in the free fatty acid transport, specifically protein CD34, which regulates tissue free fatty acids uptake from plasma, and is found to have decreased expression in the adipose tissue, and increased expression in hepatic and skeletal muscle tissues in subjects with insulin resistance and intrahepatic steatosis^[20].

Factors that lead to increased *de novo* synthesis, and decreased oxidation of free fatty acids in the liver, contribute to the mechanism for development of hepatic steatosis and progression. *De novo* synthesis and reduced free fatty acid oxidation processes are facilitated by sterol regulatory element binding protein and carbohydrate responsive element binding protein, both of which are stimulated by hyperglycemia and hyperinsulinemia^[20,23,24]. It has been proven that in non-obese NAFLD, NASH incidence is higher when associated with polymorphism in the sterol regulatory element-binding factor-2 (*SREBF-2*) gene^[25].

The excretion of intrahepatic free fatty acids from the liver as very low-density lipoprotein particles, VLDL-TG, is also increased, however the excretion rate plateaus eventually, and won't be able to cope with the increased production of free fatty acids, causing increased intrahepatic triglyceride storage^[20].

In a study to determine the relationship between hepatic steatosis and insulin resistance^[18], Korenblat *et al.*^[18] used an euglycemic hyperinsulinemic clamp procedure in 40 non-diabetic, obese patients. They used magnetic resonance spectroscopy to determine the intrahepatic triglyceride content, which ranged in their population study from 1% to 46%; they found that hepatic insulin sensitivity was inversely correlated with intrahepatic triglyceride content ($r = 0.599$, $P < 0.001$), insulin sensitivity on skeletal muscles and adipose tissue was also inversely correlated with intrahepatic triglyceride content (Skeletal muscle: $r = -0.656$; $P < 0.001$, adipose tissue: $r = -0.590$; $P < 0.001$). Intrahepatic triglyceride content was found to be the best predictor of insulin action on these tissues, independent of body mass index^[18]. This study suggests that hepatic steatosis might not only be an outcome of insulin resistance and metabolic syndrome, but also a possible etiology of NAFLD regardless of BMI^[18].

NAFLD is believed to be affected by the adipose tissue by the induction of inflammatory changes through releasing what is known as adipokines. Adiponectin, leptin, ghrelin, resistin, visfatin, retinol-binding protein 4 (RBP4) among many other adipokines been described^[26]. Insulin resistance, obesity and type 2 diabetes mellitus are all risks for having low levels of adiponectin. Adiponectin secreted 50% less in NASH patients than normal individuals. Also, it can be correlated with NAFLD progression^[26,27]. It is proposed that it has anti-inflammatory effects by induction of tumor necrotic factor-alpha (TNF- α) and interleukin-6 (IL-6) and inhibition of cytokine IL-10^[27]. Leptin is believed to have the opposite correlation with regards

to its association with the amount of adipose tissue and also the effects on NAFLD. From studies that observed the results of leptin injection in animal, it is suggested that leptin increase levels of procollagen-I, transforming growth factor- β and TNF- α . This, in return, leads to both inflammatory escalation and progression of fibrosis. The relationship between leptin and NAFLD is not fully understood^[26,28].

When it comes to the risk for developing non-obese NAFLD, it is the fat distribution rather than the total body fat that matters^[29]. Specifically, visceral fat is more important risk factor than total body fat or waist circumference^[11,30]. Subcutaneous fat acts as a reservoir of metabolically benign fat, however, visceral fat has been associated with insulin resistance, hypertriglyceridemia and low HDL^[30]. Visceral fat is a rich source that can saturate portal vein with free fatty acids and pro-inflammatory cytokines, like TNF- α , IL-6, and CRP, and subsequently causes hepatic fat accumulation and subsequent liver injury^[30,31]. This explains why Asians, who are known to have higher than average total body fat and visceral fat, have high prevalence of metabolic syndrome and NAFLD at lower body mass indices when compared to other races^[32].

High fructose diets and beverages also seem to play an important role in the development of NAFLD, especially in the young and the non-obese^[11,33,34]. Fructose is lipogenic, and proinflammatory, it is rapidly phosphorylated in the cells, causing intracellular ATP depletion, uric acid production and subsequent cellular injury^[33]. In one cross-sectional study, and after adjusting for age, gender, body mass index, and total daily calorie intake, NAFLD was significantly associated with higher intake of soft drinks^[34].

HISTOLOGY

The histology spectrum of non-obese NAFLD doesn't differ much from obese NAFLD^[35]. NAFLD classified into simple steatosis and NASH. The later have necroinflammation changes along the finding of steatosis. More than 5% of steatosis involving of the hepatocyte required making the diagnosis. Although NAFLD patients typically show macrovesicular steatosis, microvesicular steatosis could also be observed in about 10% of the cases. According to the American Association for the Study of Liver Diseases Clinical Single Topic Conference on NASH in 2002, NASH finding were assigned to necessary component, usual but not necessary and may be present but not necessary for diagnosis. Hepatocellular ballooning, lobular inflammation and the earlier described steatosis are necessary for making NASH in the biopsy sample. Hepatocellular ballooning is identified when the hepatocyte is swollen with rarefied cytoplasm. The lobular inflammation is usually mixed with inflammatory cells and mild. Perisinusoidal fibrosis, hepatocellular glycogenated nuclei, lipogranulomas, acidophil bodies, fat cysts are usual finding but not needed to the diagnosis. Mallory-Denk bodies, iron deposition and megamitochondria may be

present, but not necessary for diagnosis^[36,37].

Brunt *et al.*^[38] grading of the steatohepatitis depend in the severity of the inflammatory changes and fat deposition. Grade 1, mild, will have steatosis up to 66 %, occasional ballooning in zone 3, scattered intralobular inflammation with or without mild portal inflammation. Grade 2, moderate, steatosis of any degree, obvious ballooning, intralobular chronic inflammation and mild to moderate portal inflammation. In Grade 3, severe, showing panacinar steatosis, ballooning and disarray obvious, in addition to similar intralobular and portal inflammation to grade 2. The extent of fibrosis what is evaluated in NAFLD staging. Focal or extensive perisinusoidal fibrosis is considered in stage 1, whereas, progression to periportal fibrosis is stage 2, bridging fibrosis is stage 3 and cirrhosis is stage 4^[36,38].

It is suggested by some studies that non-obese NAFLD generally has less severe histological appearance, but this doesn't necessarily mean a better outcome^[15]. In a Chinese prospective cohort study that included 307 NAFLD patients, 23.5% were non-obese and were found to have lower fibrosis stage (1.3 ± 1.5 vs 1.7 ± 1.4 ; $P = 0.004$), and liver stiffness measured by transient elastography (6.3 kg vs 8.6 kg; $P < 0.001$)^[15]. In another study that involved 1090 patients with NAFLD confirmed by liver biopsy, the non-obese NAFLD cohort (11.5%) was found to have significantly lower degree of steatosis, and fibrosis, but more severe lobular inflammation; however, there was not much difference between two groups between hepatocyte ballooning and NASH^[7].

GENETIC PREDISPOSITION

A genetic predisposition to non-obese NAFLD is undoubtedly present and different polymorphisms in genes that regulate lipid metabolism have been identified to play an integral role in development and progression of non-obese NAFLD^[39]. It is a challenging task attempting to interpret associations from genome-wide association studies (GWAS) and elucidating the causal effect of the reported associated NAFLD variants^[40]. We will attempt to focus our review on simplifying the following known NAFLD variants; patatin-like phospholipase domain-containing 3 (PNPLA3), SREBP-2, transmembrane 6 superfamily 2, and cholesterol ester transfer protein, and their role in development and progression in non-obese NAFLD.

Intrahepatic triglyceride accumulation that leads to hepatic insulin resistance, characterizes non-obese NAFLD, further impairs the ability of insulin to regulate hepatic glucose and VLDL production leading to hyperglycemia, hyperinsulinemia, hypertriglyceridemia, and a low HDL cholesterol concentration^[18,41]. NAFLD in a non-obese population is not uncommon and GWAS have identified a single nucleotide polymorphisms (rs738409) in the patatin-like phospholipase domain-containing 3 (PNPLA3) gene with development of NAFLD^[42]. PNPLA3 encodes a 481 amino acid protein expressed in the endoplasmic reticulum and at the surface of lipid droplets

in hepatocytes and adipocytes, and has acyl hydrolase activity, which plays a role in the hydrolysis of three major glycerolipids (*i.e.*, triacylglycerol, diacylglycerol, and monoacylglycerol), leading to hepatic triglyceride accumulation^[43].

A meta-analysis by Sookoian and Pirola, revealed that the G allele of rs738409 was associated with increased aminotransferase and severity of NAFLD in various ethnic groups, data also showed 73% higher liver fat content in populations with the G allele than weight-matched subjects homozygous for the C allele^[44]. A study conducted by Romeo *et al.*^[45] showed that the risk allele (G-allele) of rs738409 was more prevalent in Hispanics compared to Europeans. The frequencies reported include 17% in African Americans, 23% in European descendants, 45% in Japanese and 49% in the Hispanic population, explaining a consistent fraction of the inter-ethnic variability in NAFLD susceptibility^[45]. It has been observed that non-obese NAFLD subjects had higher G allele of rs738409 than obese NAFLD subjects and the identified risk allele is strongly associated with increased hepatic fat contents, hepatic inflammation, and elevated ALT levels^[46]. Of important significance is the association between PNPLA3 G allele and liver fat content being independent of insulin resistance and other metabolic comorbidities like obesity and dyslipidemia in non-obese population^[47].

The *SREBF-2* gene codes for sterol regulatory element-binding protein-2 (SREBP-2), which is the nuclear transcription factor responsible for regulating genes involved in cholesterol synthesis, uptake, and excretion^[25]. Altered cholesterol metabolism results in hepatic cholesterol accumulation and subsequent liver injury^[48]. A study by Musso *et al.*^[25,49] has shown the polymorphism rs133291 C/T in the *SREBF-2* gene can be used to predict 7-year incidence of NAFLD in non-obese, and nondiabetic patients without metabolic syndrome at baseline. Histologically, it is observed that in biopsy proven non-obese NAFLD, NASH incidence is higher when it's associated with *SREBF-2*^[25].

Also noteworthy is the newly NAFLD-associated gene variant on the transmembrane 6 superfamily 2 (*TM6SF2*) gene. A variant located in the *TM6SF2* gene, rs58542926 encoding an amino acid substitution p. Glu167Lys (E167K), is associated with hepatic triglyceride content (HTGC). Research shows that the effect of rs58542926 on HTGC was independent of the effect mediated by rs738409, obesity, or insulin resistance^[50]. The rs58542926 T allele (Lys167) has been observed to be associated with disease severity, suggesting that the variant might have a small effect on the risk of NASH. Unlike the overwhelming evidence of the role of PNPLA3 in the modulation of NAFLD and disease progression in different populations around the world, the initial finding of the effect of rs58542926 have not been reliably able to show such correlations. In a study conducted by Dongiovanni *et al.*^[51], they isolated increased risk of NASH and hepatic fibrosis in the presence of *TM6SF-2* from its proposed cardiovascular protective role represented by

TG protective response to insulin resistance^[51].

Cholesterol ester transfer protein plays a role in transferring TG and cholesterol in between lipoproteins^[52]. A well-known two single nucleotide polymorphisms (rs12447924) and (rs12597002) was postulated as a risk factor for NAFLD. More evidently, it was seen in between lean homozygotes and it was as high as 30%, compared to 10%-15% lean heterozygotes and 3%-5% lean wild^[52].

CLINICAL AND METABOLIC FEATURES OF NON-OBESE NAFLD

Non-obese NAFLD is associated with components of metabolic syndrome. Some studies suggested lower prevalence of metabolic profile components in the non-obese NAFLD when compared to the obese NAFLD^[53], on the other hand, other studies suggested similar or even higher prevalence of metabolic syndrome components in non-obese NAFLD^[12,54]. In one retrospective study to determine the factors that are independently associated with non-obese NAFLD, using the national health and nutrition examination survey III data between 1988 and 1994, NAFLD in both obese and non-obese, was correlated, when compared to control subjects, with age, Hispanic heritage, and with components of metabolic syndrome; visceral obesity, diabetes, hypertension and hyperlipidemia^[53]. When comparing non-obese to obese NAFLD, the cohort was found to be younger, with lower prevalence of hyperlipidemia, hypertension, and diabetes. They also had less degree of elevated liver enzymes, specifically AST, and ALT^[53].

Alam *et al.*^[12] evaluated 229 Indian patients with NAFLD by histology, and found that the non-obese population was metabolically similar to the obese, with no significant difference of levels of total cholesterol, triglycerides, HDL, blood glucose, ALT, AST, GGT, and insulin resistance. Kwon *et al.*^[54] non-NAFLD in 12% of 29994 selected cohort who underwent routine comprehensive health evaluations, he also found that non-obese women had higher adjusted prevalence rate of hypertension, hyperglycemia, hypertriglyceridemia and lower HDL, when compared to the obese woman^[54].

In one longitudinal observational study that aimed to investigate the factors associated with the development and regression of NAFLD in non-obese NAFLD, using a cutoff body mass index (BMI) of 25 kg/m², and ultrasound as the diagnosis modality, among all factors of metabolic syndrome, and after adjusting for age, sex, baseline BMI and components of metabolic syndrome, triglyceride level above 150 mg/dL at baseline was significantly associated with the development and regression of NAFLD in the non-obese, ORs = 1.54 (1.10-2.14) and 0.60 (0.38-0.96)^[14]. Body weight change was also significantly associated with development and regression of NAFLD in both obese ORs = 1.16 (1.09-1.24) and 0.69 (0.63-0.75) and non-obese ORs = 1.23 (1.17-1.30) and 0.74 (0.67-0.81). Other factors that were not significantly associated with progression and

regression of NAFLD in non-obese included fasting blood sugar more than 100 mg/dL, blood pressure more than 130/85 mmHg, HDL less than 40 mg/dL in male and less than 50 mg/dL in females^[14].

Following a cohort of NAFLD patients diagnosed by biopsy for 15.7 years in Sweden revealed that the most common mortality cause was cardiovascular. Worth mentioning that 85% of the patients included in the study were overweight or obese at baseline^[2]. There is not enough data about cardiovascular risk of non-obese NAFLD, but being a disease entity enriched with metabolic syndrome risk factors, cardiovascular events seem to remain the main cause of morbidity and mortality in such patients^[2,55].

DIAGNOSIS

Diagnosing Non-obese NAFLD doesn't differ from obese-NAFLD, with liver biopsy continues to be the gold standard diagnostic modality and should be considered in populations with diagnostic uncertainty^[4,56]. The importance of identifying NASH lays in identifying risk of advanced fibrosis, which could lead to cirrhosis and hepatocellular carcinoma^[19]. Despite liver biopsy being the gold standard, it should be taken under consideration that up to 27% of NASH diagnosis could be missed from reading variability and from inadequate sample due to uneven disease involvement in the liver^[57]. There are many non-invasive tools that can be useful for staging NAFLD patients. These tests also have the advantages of availability, and being cheap in addition to been non-invasive. Using Fibrosis 4 (FIB-4) index for advanced fibrosis have negative predictive value (NPV) as high as 96% with using a liberal cutoff (1.3) and of 93% when using a more stricter cutoff of 2.7^[58]. NAFLD fibrosis score also has comparable results.

Two physical modalities are more useful in getting accurate results. Transient elastography and magnetic resonance elastography. The latter is not widely available and has the disadvantage of been expensive. Transient elastography is particularly useful in non-obese NAFLD patients, given its limited utility on patient with BMI > 30 kg/m². In one large study, it was found to have NPV 99% and PPV 46% with a use 10.3 kPa as a cutoff for cirrhosis^[11,58].

TREATMENT

Different pharmacological modalities have been investigated in the treatment of NAFLD and, so far, there is no evidence of effective therapy, life style modification remains the main stay of therapy^[59]. An important mechanism of cellular injury in NASH is oxidative stress, and vitamin E has been investigated as a treatment option^[60]. Vitamin E is currently recommended as a first line therapy in non-diabetic patients with NASH but not in diabetic patients, NAFLD without liver biopsy, NASH cirrhosis, or cryptogenic cirrhosis^[3].

Obeticholic acid (OCA) showed evidence in im-

proving fibrosis and decrease NAFLD score in NASH population^[61]. In a large randomized, multicenter study obtained by Neuschwander-Tetri *et al*^[61], OCA found to reduce fibrosis, NAFLD score and steatosis histology at the primary end point. Although their results were encouraging, there was no difference between treatment and placebo arms in terms of reversing NASH. Farnesoid X receptor activation by OCA are believed to reduce liver lipogenesis by down-regulating SREBP1c and up-regulating SIRT1 and this mechanism can play important role treating NASH^[61]. However this could increase serum cholesterol by inhibiting cholesterol conversion to bile acid.

Interestingly, even though non-obese NAFLD patients have normal weight ranges, weight loss remains the mainstay treatment and was found to be beneficial. Shen *et al*^[62] reported that weight loss and exercise has the greatest impact in treating NAFLD in patients with PNPLA3 GG genotype polymorphism, however the study was limited by lack of data on histological improvement as liver biopsies were not done. A study done by Jin *et al*^[63] concluded that 10% cholesterol reduction and 5% weight loss cause 20% steatosis improvement in his liver donor cohort, including the non-obese and overweight subgroups.

Visceral fat, as mentioned earlier, is strongly associated with hepatic steatosis, and NASH in non-obese patients, and should be the focus for future intervention trials. In one randomized control trial, 50 morbidly obese patient were assigned to 2 groups, gastric bypass with or without surgical removal of the greater omentum; the combined intervention was found to cause much more significant improvement in insulin sensitivity^[64]. This study can inspire further studies on the non-obese population to see if this can help improve the metabolic profile and reduce the risk of advanced liver disease in this subset of patients.

In one retrospective study that followed 619 NAFLD patients in the United States, Europe, and Thailand for more than 12 years, regardless of the other histological features, fibrosis stage was found to be the most important prognostic factor in NAFLD overall and liver-related mortality, liver transplantation, and liver-related events. The presence of diabetes, smoking, age, and lack of statin treatment, were the other factors found to affect long term survival as well in this study^[65]. Not underestimating the importance of treating NAFLD patient to prevent fibrosis, but this study points to the importance of identifying this subset of patients, NAFLD with evidence of fibrosis, even in early stages, for an aggressive and comprehensive approach to lessen long-term effects.

CONCLUSION

Non-obese NAFLD is likely a different entity than obese NAFLD, with its unique genetic predisposition. It is associated with components of metabolic syndrome. Diagnosis and treatment are similar to the obese

NALFD, with weight loss still being the mainstay of the treatment. Further research is needed for better understanding of the genetic and environmental factors affecting the course of this specific entity.

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P- Reviewer: Enomoto H, Surani S **S- Editor:** Ji FF **L- Editor:** A
E- Editor: Lu YJ



Acute and chronic hepatobiliary manifestations of sickle cell disease: A review

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Author contributions: Shah R and Taborda C contributed significantly to literature review and manuscript preparation; Shah R and Chawla S contributed significantly to conception, interpretation and revision.

Conflict-of-interest statement: This review is contributed by all authors as a voluntary work. Authors have no commercial, financial, political, religious or any other conflicts of interest for contributing to this review.

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Manuscript source: Invited manuscript

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Received: March 21, 2017

Peer-review started: March 23, 2017

First decision: May 19, 2017

Revised: June 2, 2017

Accepted: July 21, 2017

Article in press: July 24, 2017

Published online: August 15, 2017

Abstract

Sickle cell disease (SCD) is a common hemoglobinopathy which can affect multiple organ systems in the body. Within the digestive tract, the hepatobiliary system is most commonly affected in SCD. The manifestations range from benign hyperbilirubinemia to overt liver failure, with the spectrum of acute clinical presentations often referred to as "sickle cell hepatopathy". This is an umbrella term referring to liver dysfunction and hyperbilirubinemia due to intrahepatic sickling process during SCD crisis leading to ischemia, sequestration and cholestasis. In this review, we detail the pathophysiology, clinical presentation and biochemical features of various acute and chronic hepatobiliary manifestations of SCD and present and evaluate existing evidence with regards to management of this disease process. We also discuss recent advances and controversies such as the role of liver transplantation in sickle cell hepatopathy and highlight important questions in this field which would require further research. Our aim with this review is to help increase the understanding, aid in early diagnosis and improve management of this important disease process.

Key words: Sickle cell disease; Hepatopathy; Hepatobiliary; Intrahepatic cholestasis; Hepatic sequestration; Sickle cell hepatic crisis; Sickle cell cholangiopathy; Liver transplant; Iron overload

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Core tip: This review: (1) identifies the pathophysiology, common clinical and biochemical features of a spectrum of hepatobiliary manifestations in sickle cell disease; (2) presents the current evidence of role of liver transplant in

end stage liver disease due to sickle cell hepatopathy; and (3) identifies important areas of future research to explore unanswered questions regarding sickle cell hepatopathy.

Shah R, Taborda C, Chawla S. Acute and chronic hepatobiliary manifestations of sickle cell disease: A review. *World J Gastrointest Pathophysiol* 2017; 8(3): 108-116 Available from: URL: <http://www.wjgnet.com/2150-5330/full/v8/i3/108.htm> DOI: <http://dx.doi.org/10.4291/wjgp.v8.i3.108>

INTRODUCTION

Sickle cell disorder is an umbrella term involving all pathologies where hemoglobin S mutation is present on at least one beta chain. Hemoglobin A, also known as normal adult hemoglobin, comprises two alpha and two beta chains ($\alpha_2\beta_2$), with small amount of HbA2 ($\alpha_2\delta_2$) and HbF ($\alpha_2\gamma_2$). When there is a point mutation on beta chain with a substitution of valine for glutamic acid at the 6th position, it leads to formation of Hemoglobin S ($\alpha_2\beta S_2$). HbS has a sticky patch at the site of valine substitution which allows it to bind to other HbS molecules particularly in the deoxygenated state forming long chain polymers, resulting in distortion of erythrocytes causing sickling and increased hemolysis^[1]. In the oxygenated state, although the sticky patch persists, the complementary receptor site is masked and cannot attach to deoxygenated HbS and polymerize. Hence if kept oxygenated, sickling can be prevented despite high concentration of HbS. Following recurrent sickling, subsequent pleiotropic effects include changes in red cell membrane structure and function, disordered red blood cell (RBC) volume control, increased RBC adherence to vascular endothelium misregulation of vasoactivity, and inflammation finally leading to vaso-occlusion and hemolysis.

If the mutation affects only one β globin chain and the other is normal, the patient is said to have the sickle cell trait, which is a relatively benign carrier state and does not have the classic phenotypic features of sickle cell disease (SCD). When both β chains carry HbS mutation, the patient exhibits phenotypic features of SCD which may include recurrent painful crisis, anemia, infections, stroke, organ failure and premature death due to various complications and end organ damage.

Sickle cell disease (SCD) is widely prevalent in the United States affecting about 100000 Americans^[2]. Among different races, it is most common in African Americans. It is estimated that 1 in 365 African American infants have SCD while 1 in 13 has are born with the Sickle cell trait. The 2010 nationwide Center for Disease Control (CDC) survey of state newborn screening programs which screen for sickle cell trait (SCT) reported that incidence of SCT was 73.1 cases per 1000 black infants screened, 3.0 cases per 1000 white infants screened and 2.2 cases per 1000 Asian, Native

Hawaiian or other Pacific Islander infants screened^[3].

Given the high prevalence and the chronic nature of the disease, SCD is a very resource intensive disease, resulting in significant healthcare expenditure for both the society and the individual. A recent study done on Medicaid patients suggested an average cost of approximately \$2500 per patient per month in total SCD direct and indirect care^[4]. Globally SCD affects 300000 infants every year, most prevalent in areas which are endemic for malaria such as Middle-East, Africa and south Asia. It is also estimated that in many African countries, 10%-40% population carries sickle cell trait resulting in about a 2% prevalence of SCD in these countries^[5].

HEPATOBIILIARY MANIFESTATIONS OF SCD

SCD can involve multiple organ systems including the gastrointestinal tract. These gastrointestinal manifestations usually occur due to small vascular infarcts and microvascular occlusion and ischemia presenting as abdominal crisis with severe pain, acute pancreatitis, peptic ulcer disease and rarely ischemic bowel^[6,7]. The hepatobiliary system is one of the most common intra-abdominal organs involved in SCD and hepatic involvement is observed in 10%-40% cases of sickle cell crisis^[8-10].

Clinically, the diagnosis and appropriate management of hepatobiliary manifestations of SCD is challenging as they may present in myriad ways along a spectrum from relatively benign such as gallbladder sludge to as lethal as acute liver failure. The objective of this review is to describe the hepatobiliary manifestations of sickle cell disease with emphasis on their pathophysiology and clinical manifestations. We also organize and discuss existing clinical terminologies used to describe these hepatobiliary manifestations.

CLASSIFICATION

Hepatobiliary involvement in SCD can be divided into acute manifestations (Table 1) occurring during vaso-occlusive crisis and chronic manifestations (Table 2) which persist and may progress outside of the crisis state. It is important to understand that a spectrum of clinical manifestations may be observed for the same underlying pathophysiology depending on severity of vaso-occlusive crisis and the residual physiologic hepatic reserve. Sickle cell hepatopathy is an umbrella term defined as liver dysfunction and hyperbilirubinemia due to intrahepatic sickling process during SCD crisis leading to ischemia, sequestration and cholestasis^[11]. While recurrent acute damage can eventually turn to more chronic liver disease in SCD, slow progressive liver damage can also independently lead to chronic liver disease (CLD) in absence of recurrent acute

Table 1 Acute hepatobiliary manifestations of sickle cell disease

Acute manifestations of SCD	Clinical presentation	Biochemical changes			Management
		Transaminase (AST, ALT) levels	Bilirubin	Alkaline phosphatase	
Acute sickle cell hepatic crisis	Fever, acute onset RUQ pain, jaundice and tender hepatomegaly	Normal to 3 × upper normal	Upto 15 mg/dL, mainly conjugated	Normal to slight elevation	Supportive with treatment of SCD crisis
Acute Hepatic sequestration	Acute onset RUQ pain, hepatomegaly and anemia	Normal	Upto 24 mg/dL, mainly conjugated	Can go upto 650 IU/L	Supportive with blood or exchange transfusion
Acute intrahepatic cholestasis	Fever, RUQ pain rapidly progressing to acute liver failure	Elevated usually > 1000	Elevated in 100 s, mostly conjugated	Normal or elevated > 1000 IU/L	Supportive, exchange transfusion, correction of coagulopathy? Liver transplant

SCD: Sickle cell disease; AST: Aspartate transaminase; ALT: Alanine transaminase; RUQ: Right upper quadrant.

Table 2 Chronic hepatobiliary manifestations of sickle cell disease

Chronic hepatobiliary manifestations of SCD	Clinical presentation	Biochemical changes			Management
		Transaminase (AST, ALT) levels	Bilirubin	Alkaline phosphatase	
Cholelithiasis	RUQ pain, fever, jaundice	Normal or elevated	Normal or elevated	Normal	Cholecystectomy
Choledocholithiasis	RUQ pain, fever, jaundice, cholangitis	Normal or elevated	Elevated	Elevated	ERCP
Iron overload	Asymptomatic elevated LFTs to frank cirrhosis	Normal or elevated	Normal to mild elevation	Normal	Iron chelation
Viral hepatitis	Viral prodrome, fever, hepatomegaly, jaundice	Acute-elevated	Acute-elevated	Acute - normal to slightly elevated;	Based on AASLD guidelines
		Chronic-normal or elevated	Chronic-normal or elevated	Chronic - mostly normal	
Sickle cell cholangiopathy	Obstructive jaundice, itching, cholestatic LFTs	Normal or elevated	Elevated	Elevated	ERCP liver transplant

SCD: Sickle cell disease; AST: Aspartate transaminase; ALT: Alanine transaminase; RUQ: Right upper quadrant; ERCP: Endoscopic retrograde cholangiopancreatography; LFTs: Liver function tests; AASLD: American Association for the Study of Liver Diseases.

manifestation.

ACUTE LIVER INVOLVEMENT IN SICKLE CELL VASO-OCCLUSIVE CRISIS (SICKLE CELL HEPATOPATHY)

The underlying pathophysiology for this disorder is wide spread sickling of erythrocytes during crisis. Intrahepatic sickling of erythrocytes leads to sinusoidal obstruction. Depending upon the degree of sickling and severity of sinusoidal obstruction, sickle cell hepatopathy can manifest in the following forms.

Acute sickle cell hepatic crisis

Acute sickle cell hepatic crisis has been reported in about 10% of patients presenting with vaso-occlusive crisis^[12]. Clinically, this may present similar to acute cholecystitis with acute onset of fever, right upper quadrant abdominal pain and jaundice. Tender hepatomegaly which is commonly observed differentiates this from acute cholecystitis.

Pathophysiology: The underlying mechanism for

this entity is believed to be due to sickled erythrocytes causing sinusoidal obstruction. This obstruction can cause transient liver ischemia and in severe cases can lead to infarction. On histology, sickle cell aggregates are observed in sinusoidal spaces. Depending on severity of the vaso-occlusive crisis, kupffer cell hypertrophy and in most severe cases, severe centrilobular necrosis can also be observed^[13].

Biochemical abnormalities: The biochemical abnormalities observed vary and in most cases do not correlate with the severity of insult or even histological findings^[14]. Serum transaminases - alanine transaminase (ALT), aspartate transaminase (AST) are usually 1-3 times elevated from the normal although levels in the thousands have been reported. The transaminase levels also fall rapidly followed by resolution of crisis unlike viral hepatitis where transaminases are elevated for a prolonged time. Serum bilirubin is elevated with a predominantly conjugated fraction but usually stays < 15 mg/dL^[13]. Biochemical abnormalities resolve within 3-14 d.

Treatment: Treatment is usually supportive with rehydration and oxygenation similar to acute vaso-

occlusive crisis.

Acute hepatic sequestration

This entity is less commonly observed in SCD crisis^[14]. The underlying mechanism is sequestration of large amount of erythrocytes in the spleen, pulmonary vasculature and rarely in the liver. Patients usually present with abrupt onset of severe right upper quadrant (RUQ) pain, rapidly evolving hepatomegaly and acute rapidly worsening anemia. Depending on amount of erythrocyte consumed in reticuloendothelial system, patients can also present with acute symptomatic anemia, shock rapidly progressive towards mortality. There is usually an acute fall in hematocrit and this fall coincides with acute hepatomegaly^[15]. Falling hematocrit is also associated with appropriate rise in reticulocyte count. Smooth but remarkable hepatomegaly is often observed.

Pathophysiology: There is sequestration of large amount of erythrocytes in the spleen, pulmonary vasculature and to a small extent in the liver. The trapped sickled erythrocytes due to Kupffer cell erythrophagocytosis cause massive dilation of sinusoids which exert mass effect and causes compression of biliary tree^[14]. Biopsy shows dilated sinusoids and trapped erythrocytes. Intrahepatic cholestasis and bile plugs are also commonly observed but necrosis is uncommon.

Biochemical abnormalities: Biochemical abnormalities usually include significant hyperbilirubinemia which can go as high as 24 mg/dL. The elevated bilirubin is mainly in conjugated form abiding to obstructive pathophysiology of the disease. Alkaline phosphatase can also be elevated and can rise as high as 650 IU/L. Transaminases are usually within normal limits.

Treatment: Treatment is usually supportive. Simple blood transfusion or exchange transfusion to support tissue oxygenation usually suffices. A consideration in treatment of acute sequestration crisis is that resolution of this condition usually happens in 3-4 d and acute rise in hematocrit can be observed indicating not all the trapped erythrocytes are hemolyzed. Close monitoring of patient's hematocrit is required as rapid rise in resolution phase can increase the hyperviscosity of blood^[16]. An increase in mortality due to heart failure, cerebrovascular accident (CVA) and even acute coronary syndrome (ACS) has been reported due to hyperviscosity^[16]. If rapid rise in hematocrit is observed in the resolution phase, phlebotomy should be considered.

Acute intrahepatic cholestasis

Acute intrahepatic cholestasis is the most severe acute hepatic manifestation of SCD and can be fatal. Fortunately, it is very rare with total of only 17 reported cases so far^[17]. It presents initially as severe acute hepatic crisis with fever, leukocytosis, RUQ abdominal pain, jaundice but can progress rapidly to multi-organ

failure including renal failure and acute liver failure manifesting as encephalopathy (confusion) and bleeding diathesis (coagulopathy).

Pathophysiology: The pathophysiology of this fatal entity is diffuse sickling in the sinusoids leading widespread ischemia. Hypoxia leads to ballooning of hepatocytes and intracanalicular cholestasis. Widespread dilated sinusoids with intrahepatic cholestasis are seen on histology. In more severe cases, widespread anoxic necrosis with areas of acute and chronic inflammation are also seen^[18].

Biochemical abnormalities: Biochemical evidence shows significantly elevated bilirubin levels which are mainly due to rise in conjugated component. Levels as high as 273 mg/dL have been reported^[19]. This extreme hyperbilirubinemia is due to combination of hemolysis causing unconjugated hyperbilirubinemia, and intrahepatic cholestasis and renal impairment contributing to the conjugated component. Transaminase levels above 1000 mg/dL are commonly seen. Alkaline phosphatase can be normal or elevated but levels greater than 1000 IU/mL are rarely observed^[19]. Hepatic dysfunction with derangement of coagulation profile in form of elevated prothrombin time (PT), partial thromboplastin time (PTT), International normalized ratio (INR) as well as hypofibrinogenemia are also observed.

Treatment: Rigorous supportive measures, exchange transfusion and correction of coagulopathy with fresh frozen plasma (FFPs) are proposed treatment measures^[17,19]. This entity carries extremely high mortality. Renal impairment is thought to be due to primary hepatic impairment and few cases might require temporary dialysis. With correction of hepatic abnormality, renal function usually improves.

Overt liver failure without histologic changes

Although exceedingly rare, this entity is fatal and in absence of option for transplant carries extremely high mortality. There are isolated case reports to small case series reported describing acute liver failure in SCD^[20,21]. While clinical presentation of acute liver failure (ALF) is similar to non SCD patients - acute onset liver dysfunction along with encephalopathy and coagulopathy, abdominal pain, tender hepatomegaly, ALF in SCD presents with extremely high Serum Bilirubin and PT^[22], with relatively mild elevation in transaminases. Few cases where liver biopsy was performed showed centrilobular necrosis and very infrequently showed cholestasis.

Role of zinc deficiency

Zinc is a cofactor for ornithine transcarbamylase, an enzyme required in urea cycle^[23]. Zinc deficiency has been suggested as a strong factor when hepatic failure is observed in SCD patients without significant histologic findings on biopsy. In ambulatory setting SCD patients who tend to have high ammonia levels have shown

reduction in Ammonia level on zinc supplementation. It is hypothesized that zinc deficiency predisposes these patients to higher risk of hepatic encephalopathy. Measurement and supplementation of zinc if low is recommended to prevent hepatic encephalopathy (HE)^[24].

CHRONIC HEPATOBILIARY MANIFESTATIONS OF SICKLE CELL DISEASE

Viral hepatitis

Patients with SCD can present with acute or chronic viral hepatitis. Patients with SCD have a higher prevalence of both acute and chronic viral hepatitis due to exposure to risk factors like multiple transfusions *etc.* Prevalence of viral hepatitis in these patients also depends upon factors such as local prevalence of chronic viral hepatitis, transfusion protocols as well as vaccination practices. Some studies have shown that asymptomatic persistent elevation of ALT/AST in the absence of sickle cell crisis, is commonly associated with chronic hepatitis on liver biopsy^[25].

Clinical presentation: Patients with acute viral hepatitis present similar to general population with malaise, jaundice and abdominal pain with tender hepatomegaly. Patients with chronic viral hepatitis are usually asymptomatic with incidentally discovered persistently elevated transaminases or may present with new diagnosis of cirrhosis and are found to have chronic viral hepatitis.

Biochemical abnormalities: Similar to non-sickle cell disease patients, acute viral hepatitis in SCD patients is associated with very elevated transaminase levels (usually 500-1000 IU/mL). In SCD patients however, the total serum bilirubin has been observed to be much higher than non SCD patients and ranges from 8-64 mg/dL with an average of 45 mg/dL^[9].

Chronic Hepatitis can present with variable degree of transaminase elevation based on disease activity. As mentioned previously, most patients are asymptomatic and are diagnosed on workup of persistently elevated transaminases^[25].

Liver biopsy if performed, in these cases reveals balloon cells with cellular derangement and leucocyte infiltration suggestive of viral hepatitis.

Treatment recommendations for viral hepatitis remain similar to AASLD guidelines in general population.

Transfusion iron overload

Hemosiderosis resulting from iron overload from recurrent blood transfusion is not uncommon in SCD and can lead to cirrhosis. It is as a consequence of accumulation of transfused iron, increased gastrointestinal absorption of iron due to intensive erythropoiesis and iron deposition as a result of continuous hemolysis^[12]. Saturation of

transferrin by excess circulating iron results in formation of reactive oxygen species (ROS) such as hydroxyl radicals. Excess iron tends to deposit in the hepatic parenchyma, endocrine organs, and in cardiac myocytes causing end organ damage by ROS-mediated lipid peroxidation^[26].

Clinical presentation: Initially patients with moderate iron overload may present with abnormal liver biochemical tests (mostly hepatocellular) without any other clinical symptoms suggestive of liver disease. However, as the disease advances to cirrhosis, patients present with stigmata of chronic liver disease such as ascites, gastrointestinal bleeding, hepatosplenomegaly and thrombocytopenia. Encephalopathy and coagulopathy signify advanced liver disease. These features along with cardiac and endocrine involvement suggest iron overload as an etiology of liver disease. Physicians should be aware of cardiac and endocrine manifestations of iron overload such as symptoms of heart failure including orthopnea, paroxysmal nocturnal dyspnea or lower extremity swelling as well as endocrine abnormalities including decreased libido, diabetes mellitus, delayed puberty or delayed growth^[26].

Pathophysiology: With multiple blood transfusions, increased deposition of iron occurs within the reticulo-endothelial cells, including Kupffer cells. In a study by Brittenham *et al*^[27], it was reported that patients diagnosed with either thalassemia major or SCD frequently had hepatic iron concentrations above 400 micromoles per gram which is the approximate "toxic threshold" that has been proposed for the development of hepatic fibrosis in patients with hereditary hemochromatosis^[28]. In an autopsy study of 70 patients, Bauer *et al*^[29] reported that micronodular cirrhosis with hemochromatosis, due to blood transfusion, was present in three patients and parenchymal iron accumulation not severe enough to cause fibrosis or inflammation was present in 30 others. Massive iron accumulation can lead to peri-cellular and portal fibrosis which can lead to diffuse fibrosis and ultimately cirrhosis.

Biochemical abnormalities: The gold standard for assessing liver iron stores in the absence of cirrhosis is Hepatic Iron Concentration (HIC), determined by liver biopsy and atomic absorption spectrophotometry^[30]. Normal HIC is between 0.4 and 2.2 mg/g of liver dry weight. Based on data from hereditary hemochromatosis, less than 7 mg/g is not associated with obvious hepatic pathology while 15 mg/g is consistently associated with liver fibrosis^[28].

Serum ferritin can be used as a surrogate marker in sickle cell anemia with repeated blood transfusions to provide an indirect estimate of body iron stores. During vaso-occlusive crises, serum ferritin increases and therefore steady-state levels obtained on multiple occasions outside SCD crisis gives a better estimate of the degree of iron overload^[31]. Analysis of chronically

transfused SCD patients without viral hepatitis from STOP and STOP2 trials, showed that a ferritin level < 1500ng/mL was correlated with low transfusion burden and low measured Hepatic Iron Content (HIC), while a ferritin > 3000 ng/mL was consistently predictive of HIC > 10 mg/g. Thus, it can be inferred that serum ferritin may not be an accurate predictor of liver iron stores in the range of 1500-3000 ng/mL^[32].

Magnetic resonance imaging (MRI) using Ferriscan (biomagnetic liver susceptometry) when available is preferred to estimate iron content of liver in patients receiving multiple blood transfusions. Liver biopsy is reserved for cases where MRI appearance is not consistent with transfusion history or suspicion for iron overload remains high in light of negative MRI study^[33].

Treatment: Iron chelation with intravenous or subcutaneous deferoxamine is the first line therapy. This results in increased urinary and biliary excretion of iron and results in a meaningful decrease in serum ferritin and plasma ALT levels. Recommendation from American Academy of Pediatrics suggest to use chelation to maintain serum ferritin < 1500 ng/mL and HIC < 7 mg/g^[34]. Some experts suggest performing annual liver MRI and initiate chelation when HIC > 3 mg/g or Serum Ferritin is > 1000 ng/mL on 2 or more occasions^[33,35].

Gallstone disease

Cholelithiasis is fairly common in patients with homozygous SCD, with an incidence of 26%-58% in patients aged 10-65 compared to 17% in patients with SC-Hb C disease and SC- β thalassemia^[36-38].

Pathophysiology: Gallstones are commonly made of the black rather than the brown pigment as a result of elevated bilirubin excretion^[12]. Increased unconjugated bilirubin excretion resulting from catabolic breakdown of heme, bilirubin precipitation and the growth of bilirubinate crystals are determinant factors for the formation of gallstones. Up to 50% of gallstones in patients with SCD can be seen on plain films because calcium bilirubinate, which is the main component of these black stones, is radio-opaque^[39].

Clinical presentation: Cholelithiasis: Like the general population, most patients with gallstones are asymptomatic. Intermittent abdominal pain related to fatty food can be elicited in history. Frequently, it goes unnoticed except when patient presents with acute cholecystitis or choledocholithiasis.

Acute cholecystitis: Presentation of acute cholecystitis is similar to the general population. Usual symptoms are abdominal pain, nausea, vomiting, fever and/or jaundice. Oftentimes it is challenging to differentiate from sickle cell hepatic crisis in patients with SCD. Imaging as well as recognition of pattern of acute hepatic crisis in such cases can help to differentiate these two entities.

Choledocholithiasis: Incidence of both asymptomatic and symptomatic choledocholithiasis (CDL) in SCD

can range from 19%-26% which is comparable to the incidence found in patients with cholesterol gallstones^[40]. However, bilirubin stones may frequently be asymptomatic as they only produce low grade obstruction because of their small size and friability. However, if significant obstruction persists they present with right upper quadrant or epigastric pain and jaundice. Presence of fever in this setting may suggest cholangitis and require emergent biliary decompression.

Biochemical abnormalities: Cholecystitis: Patients with acute cholecystitis may present with acute leukocytosis (with increased number of bands). Mild transaminitis can also be observed though serum bilirubin and alkaline phosphatase are usually normal.

Choledocholithiasis: Depending upon degree of obstruction, elevation of bilirubin, alkaline phosphatase with mild transaminitis are observed. These laboratory abnormalities are not very specific in patients with sickle cell disease. Even though serum bilirubin or transaminases are not associated with CDL in SCD, incremental hyperbilirubinemia (with levels higher than 5 mg/dL) is a better predictor of CDL than is bile duct dilation or elevation in either alkaline phosphatase or serum aminotransferase levels. This interestingly differs from cholesterol CDL in which increased levels of alkaline phosphatase and biliary duct dilation are good predictors^[12].

Imaging: Ultrasound is less useful to appropriately make the diagnosis in patients with acute cholecystitis. Tc99m diisopropyl-iminodiacetic acid scan might show prolonged non-visualization of the gallbladder consistent with acute cholecystitis or more commonly, delayed visualization consistent with chronic cholecystitis. On the contrary, hepatobiliary radionuclide scans can safely rule out acute calculous cholecystitis when the gallbladder is visualized. Diagnosis of CLD can be established based on Ultrasound (US), but frequently cross-sectional images such as CT scan and/or MRI abdomen are required.

Treatment: Cholecystectomy is the most common surgical procedure in patients with SCD, comprising about 40% of the procedures on SC patients^[41]. It should be pursued in patients with symptomatic gallstones and when there is difficulty distinguishing it from sickle cell hepatic crisis. However, in asymptomatic patients, it has become a controversial practice. Some authors advocate for early cholecystectomy taking into consideration complications of emergency surgeries, lack of clinical correlation of histologically chronic cholecystitis with clinical symptoms and finally, simplification of medical management by eliminating gallstones as a diagnostic possibility^[12]. In contrast other authors believe that patients might not develop symptomatic biliary tract disease and therefore prophylactic cholecystectomy's risks might outweigh its benefits. The perioperative mortality rate of elective cholecystectomy has been reported to be 1% and the

Table 3 Current evidence of liver transplantation in sickle cell disease

Author	Number of patients	Outcomes
Hurtova <i>et al</i> ^[46]	6	1, 3, 5, and 10-yr survival rates were 83.3%, 66.7%, 44.4%, and 44.4%, respectively
Mekeel <i>et al</i> ^[48]	3	Patient and graft survival was 66%
Baichi <i>et al</i> ^[49]	2	100% mortality in post-transplant period due to multiorgan failure
Emre <i>et al</i> ^[50]	1	Failure of graft in 5 mo due to SCD crisis
Greenberg <i>et al</i> ^[51]	1	Successful but follow up only till day 28
Kindscher <i>et al</i> ^[52]	1	Successful with extrahepatic complications
Lang <i>et al</i> ^[53]	1	Successful at 6 mo
Ross <i>et al</i> ^[54]	1	Successful at 22 mo - death due to PE
van den Hazel <i>et al</i> ^[55]	1	Successful at 5.5 yr
Gilli <i>et al</i> ^[56]	1	Successful at 2 yr
Berry ^[57]	1	Death in post-op period

rate of postoperative complications to be more than 30%^[41,42]. If choledocholithiasis is present, the common bile duct should be cleared of the gallstones to prevent biliary obstruction and cholangitis, which can be fatal. This is usually achieved endoscopically by performing endoscopic retrograde cholangiopancreatography (ERCP) or through surgical common bile duct (CBD) exploration.

Sickle cell cholangiopathy

Sickle cell cholangiopathy is a form of ischemic cholangiopathy which may be encountered in patients with SCD. While hyperbilirubinemia can be multifactorial in these patients, elevated serum bilirubin with abnormal biliary imaging findings should point towards possible evaluation for Sickle cell cholangiopathy.

Pathophysiology: The underlying mechanism of sickle cell cholangiopathy is ischemic injury to the biliary tree due to recurrent sickle cell crisis affecting end arteries of the biliary tree ultimately causing hypoxic injury^[43,44]. While initially this can lead to dilation of biliary ductal system, recurrent insult can result in strictures in extrahepatic and intrahepatic biliary ducts. Biopsy is often not necessary and if obtained, mostly shows cholestasis. Occasionally findings of ischemia such as ischemic bile duct necrosis, biliary fibrosis can be observed

Biochemical abnormalities: Most patients with have elevated bilirubin mainly direct bilirubinemia, elevated alkaline phosphatase and variable elevations of transaminases.

Clinical feature: In early stages, most patients present with cholestatic jaundice. In a study done on 224 SCD patients with cholestatic jaundice receiving total of 242 ERCP, prevalence of dilated biliary ducts was 24.6%^[45]. Common causes of biliary obstruction such as stones, mass have to be excluded prior to attributing these changes to cholangiopathy. As the disease progresses to development of biliary strictures, patients might present with symptoms of obstructive jaundice such as pruritus, dark urine, clay colored stool and jaundice. These patients can also develop ascending cholangitis. Chronic

liver failure/cirrhosis may also occur in advanced stages of disease.

Treatment: Patient who are asymptomatic but are found to be having dilated biliary ducts, should be closely followed up since they are at a high risk of having bile duct stones^[45]. Endoscopic therapy is the mainstay for patients with choledocholithiasis or biliary strictures. The role of liver transplantation for patients with recurrent cholangitis or cirrhosis in patients with sickle cell cholangiopathy remains controversial.

LIVER TRANSPLANTATION IN SCD

Data regarding liver transplant in Sickle cell hepatopathy is limited. Although it has been proposed on a case by case basis, only a few case series with a total of 18 cases where Orthotopic Liver Transplant (OLT) was performed have been reported (Table 3). With more recent advances in transplant management as well as advanced understanding in the disease process of sickle cell hepatopathy, this field appears to have fair potential to be a viable treatment option in SCH as is suggested by the most recent case series reported by Hurtova *et al*^[46]. In this cohort, the liver transplant was performed with selective inclusion criteria as well as strict post-transplant adherence to exchange transfusion protocol at least for first 6 mo to keep HbS < 30% and Hb between 8-10 g/dL. Patients with significant cardiovascular and respiratory co-morbidities were excluded from the trial. The 3 year survival rate close to 67% and 10 year survival rate close to 44% were observed in this study suggesting that although liver transplant does not affect the disease course in SCD, it has potential to improve at least short term and survival rate in this patient population. A common observation among all these liver transplant patients was that efforts to maintain HbS < 25%-30% were associated with improved post-transplant survival^[47]. It should be kept in mind that OLT is not a benign treatment and even post-transplant liver grafts are at increased risk of vascular thrombosis and graft failure as well as risk of infection due to multiple exchange transfusions. Moreover, sickle cell hepatopathy, hepatitis C and

transfusion related iron overload can also develop in the transplanted liver.

CONCLUSION

Sickle cell hepatopathy is a spectrum of disease manifestations with varying levels of severity due acute or chronic changes within the hepatobiliary system in patients with sickle cell hemoglobinopathy. With better understanding of disease pathophysiology, advances in treatment options and improvement in the care of SCD patients, the overall survival of patients with SCD has improved significantly. This paper highlights the pathophysiology of the hepatobiliary manifestations of sickle cell disease, discusses clinical presentation and biochemical features to help identify and manage the appropriate manifestations along this disease spectrum.

This review also raises certain important un-answered questions which need to be further studied. Data to identify risk factors for developing acute hepatopathy is lacking. Treatment for most acute hepatopathy manifestations still remains mainly supportive and the role of hydroxyurea and other anti-sickling agents in preventing the hepatobiliary manifestations has not been defined. The role of liver transplantation, though offered at some centers, still remains controversial and the need for prophylactic cholecystectomy is still questionable. Finally, about 10% SCD patients are found to have cirrhosis on autopsy which cannot be explained by any other etiology and it is yet unclear as to what increases this risk to progression towards cirrhosis.

Research of these unanswered questions can potentially lead to better management of these patients and alter the natural history of disease possibly reducing the morbidity and mortality associated with end stage liver disease in SCD.

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P- Reviewer: Al-Haggag M, Kopljar M **S- Editor:** Kong JX
L- Editor: A **E- Editor:** Lu YJ



Prospective Study

Oral spore-based probiotic supplementation was associated with reduced incidence of post-prandial dietary endotoxin, triglycerides, and disease risk biomarkers

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Author contributions: McFarlin BK designed the study, collected data, interrupted findings, and prepared manuscript; Henning AL, Bowman EM, Gary MA and Carbajal KM collected data, interrupted findings, and prepared manuscript.

Institutional review board statement: The study was reviewed and approved by the UNT Institutional Review Board for Human Subjects Research.

Informed consent statement: Subjects provided written and oral consent to participate using an IRB-approved informed consent form specific to the study in question.

Conflict-of-interest statement: The present study was funded in part by a competitive research grant from Microbiome Labs, LLC (Glenview, IL) to the University of North Texas. The UNT team did not receive direct funding associated with the completion of the present study. The funding agency was not involved in the data collection, analysis, interpretation, and manuscript preparation. Double blind procedures and confidentially were used to conduct the present study in a sound and unbiased manner. As such, the authors report no conflict of interest associated with completing the present study.

Data sharing statement: None.

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Manuscript source: Invited manuscript

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Received: January 26, 2017

Peer-review started: February 8, 2017

First decision: April 17, 2017

Revised: July 4, 2017

Accepted: July 14, 2017

Article in press: July 17, 2017

Published online: August 15, 2017

Abstract

AIM

To determine if 30-d of oral spore-based probiotic supplementation could reduce dietary endotoxemia.

METHODS

Apparently healthy men and women ($n = 75$) were screened for post-prandial dietary endotoxemia. Subjects whose serum endotoxin concentration increased by at least 5-fold from pre-meal levels at 5-h post-prandial were considered "responders" and were randomized to receive either placebo (rice flour) or a commercial spore-based probiotic supplement [*Bacillus indicus* (HU36), *Bacillus subtilis* (HU58), *Bacillus coagulans*, and *Bacillus licheniformis*, and *Bacillus clausii*] for 30-d. The dietary endotoxemia test was repeated at the conclusion of the supplementation period. Dietary endotoxin (LAL) and triglycerides (enzymatic) were measured using

an automated chemistry analyzer. Serum disease risk biomarkers were measured using bead-based multiplex assays (Luminex and Milliplex) as secondary, exploratory measures.

RESULTS

Data were statistically analyzed using repeated measures ANOVA and a $P < 0.05$. We found that spore-based probiotic supplementation was associated with a 42% reduction in endotoxin (12.9 ± 3.5 vs 6.1 ± 2.6 , $P = 0.011$) and 24% reduction in triglyceride (212 ± 28 vs 138 ± 12 , $P = 0.004$) in the post-prandial period. Placebo subjects presented with a 36% increase in endotoxin (10.3 ± 3.4 vs 15.4 ± 4.1 , $P = 0.011$) and 5% decrease in triglycerides (191 ± 24 vs 186 ± 28 , $P = 0.004$) over the same post-prandial period. We also found that spore-based probiotic supplementation was associated with significant post-prandial reductions in IL-12p70 (24.3 ± 2.2 vs 21.5 ± 1.7 , $P = 0.017$) and IL-1 β (1.9 ± 0.2 vs 1.6 ± 0.1 , $P = 0.020$). Compared to placebo post supplementation, probiotic subject had less ghrelin (6.8 ± 0.4 vs 8.3 ± 1.1 , $P = 0.017$) compared to placebo subjects.

CONCLUSION

The key findings of the present study is that oral spore-based probiotic supplementation reduced symptoms indicative of "leaky gut syndrome".

Key words: Metabolic endotoxemia; Chronic disease; Leaky gut syndrome; Probiotics; Multiplex; Cardiovascular disease; Inflammatory cytokines; High-fat meal challenge

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Core tip: Dietary or metabolic endotoxemia is a condition that affects approximately 1/3 of individuals living in Western society. It is characterized by increased serum endotoxin concentration during the first five hours of the post-prandial period following consumption of a meal with a high-fat, high-calorie content. The key findings of the present study, were that 30-d of oral spore-based probiotic supplementation reduced the incidence of dietary endotoxemia, which may be indicative of reduced gut permeability.

McFarlin BK, Henning AL, Bowman EM, Gary MA, Carbajal KM. Oral spore-based probiotic supplementation was associated with reduced incidence of post-prandial dietary endotoxin, triglycerides, and disease risk biomarkers. *World J Gastrointest Pathophysiol* 2017; 8(3): 117-126 Available from: URL: <http://www.wjgnet.com/2150-5330/full/v8/i3/117.htm> DOI: <http://dx.doi.org/10.4291/wjgp.v8.i3.117>

INTRODUCTION

Incidence of gastrointestinal (GI) distress and permeability has increased in prominence in modern society due in large part to the excessive consumption of

highly processed, calorie dense, commercially available foods^[1]. These same dietary choices coupled with low physical activity are believed to be the primary causes underlying the current obesity epidemic^[2]. Recent efforts have focused on the use of over-the-counter probiotics (typically *Lactobacillus* and *Bifidobacterium*) to address symptoms associated with GI abnormalities^[3-5]. The lay literature has generally identified a goal of improved "GI health", but unfortunately this is so broadly defined that it is nearly impossible to identify a single research focus^[6]. Further complicating matters is that probiotic supplementation does not yield consistent results^[7,8]. We have speculated that if an individual doesn't have a pre-existing GI abnormality then they would not be a "responder" to probiotic supplementation. Complicating oral probiotic supplementation efforts is the fact that few traditional probiotic supplements (*i.e.*, *Lactobacillus* and *Bifidobacterium*) delivery fully viable bacteria to the small intestine^[9,10]. Recently it has been speculated gram positive, spore-forming probiotic strains may be a good alternative because the endospores that encapsulate the strains are highly resistant to stomach acid, potentially resulting in the delivery of more viable probiotics to the small intestine^[11,12]. Thus, it appears that two major limitations of the existing probiotic literature lie with an inability to identify "responder" subjects prior to enrollment and issues associated with viable probiotic delivery to the small intestine.

Dietary or metabolic endotoxemia occurs when one's dietary consumption causes disruption in either GI permeability, the microbiota profile, or both^[1,2,4,13-15]. Dietary endotoxemia transiently increases systemic inflammation, which chronically may increase one's risk of a variety of diseases^[2]. Our laboratory and others have demonstrated that consumption of a single, high-fat, high-calorie meal was associated with an increase in serum endotoxin, triglycerides, metabolic biomarkers, inflammatory cytokines, endothelial microparticles, and monocyte adhesion molecules^[16-22]. The post-prandial time course varies for each biomarker, but generally the transient changes occur during the first five hours of the post-prandial period. Given the direct link between nutrition, microbiota, GI permeability, and disease risk, our laboratory and others have speculated that these changes represent an appropriate treatment target for a probiotic intervention^[23,24]. To address known issues with sufficient probiotic delivery, we utilized a "spore-based" probiotic in the present study. According to the literature the biggest advantages of a "spore-based" probiotic is that it is composed of endospores which are highly resistant to acidic pH, are stable at room temperature, and deliver a much greater quantity of high viability bacteria to the small intestine than traditional probiotic supplements^[11,12]. To our knowledge, the present study is the first attempt to clinically leverage the benefits of spore-based probiotics to improve health outcomes. The primary purpose of the present study was to determine if 30-d of spore-based probiotic supplementation reduced post-prandial endotoxemia and triglycerides.

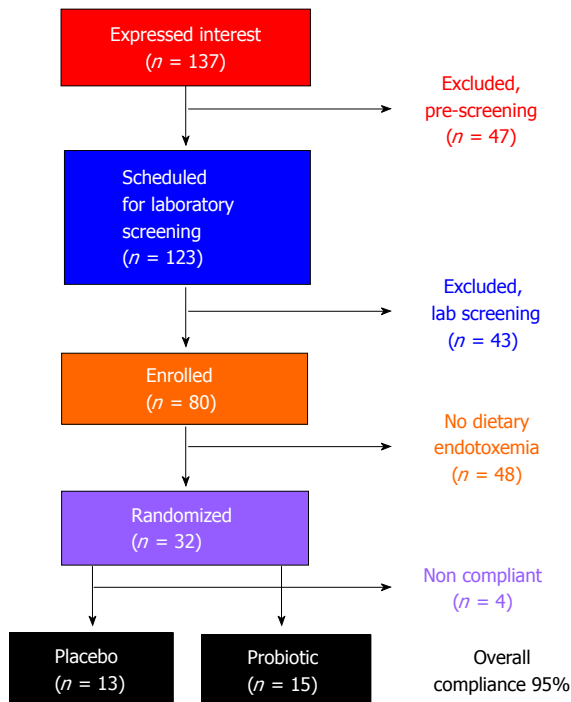


Figure 1 Represents the consort diagram for the study that indicates the number of participants that matriculated through the study. Subjects were carefully screened for exclusion/inclusion criteria and if qualified were enrolled in the study. Consistent with our preliminary data 2 out of every 6 subjects presented a dietary/metabolic endotoxin response following consumption of the high-fat meal. A total of 26 individuals were identified to have the “responder” phenotype and were randomized to participate in either the probiotic or placebo condition.

The study enrollment was unique in that we developed an additional level of screening to only enroll subjects who had dietary endotoxemia (*i.e.*, responders). Our secondary purpose was to determine if other metabolic biomarkers and cytokines, known to change after consuming a high-fat meal, would also be modified by 30-d of spore-based probiotic supplementation.

MATERIALS AND METHODS

Determination of appropriate sample size

All the procedures described in the present study were reviewed and approved by the University of North Texas Institutional Review Board (IRB) for Human Subject’s Research. Subjects provided their written and verbal consent to participate before being enrolled in the study. The present study was completed following a completion of a preliminary proof of concept study in the laboratory (data not shown). From this data, we identified that only 2 of 6 subjects (“responders”) had a measurable dietary endotoxemia response (*i.e.*, at least a 5-fold increase from pre-meal values at 5-h post-prandial). “Responder” subjects experienced a 30% reduction in serum endotoxin (effect size = 0.40) at 5-h post-prandial following a 30-d probiotic intervention (same probiotic used in the present study). Based on these criteria, we identified that we needed to enroll a minimum of $n = 10$ “responders” in placebo and

Table 1 Subject characteristics

Characteristic	Placebo ($n = 13$)	Probiotic ($n = 15$)
Age (yr)	21.8 ± 0.7	21.2 ± 0.5
Height (cm)	167.9 ± 3.2	170.8 ± 2.7
Body mass (kg)	74.2 ± 6.6	71.2 ± 3.1
Body mass index (kg/m ²)	25.9 ± 1.5	24.3 ± 0.9
Body fat (%)	27.8 ± 4.1	25.2 ± 3.0
Fat mass (kg)	21.0 ± 4.3	17.3 ± 2.4
Lean mass (kg)	50.1 ± 3.8	50.0 ± 3.7
Bone mineral mass (kg)	2.9 ± 0.2	2.9 ± 0.1
Resting energy expenditure (kcal/d)	2243 ± 304	2071 ± 108

Values represent group mean ± SEM. No significant differences existed between groups with respect to subject characteristics.

spore-based probiotic groups ($n = 20$ total) in order to achieve at least 80% statistical power. Eighty subjects were screened for a dietary endotoxin response, and 25 “responders” were enrolled (Table 1) and matriculated through the study treatments (Figure 1).

Additional subject screening

Prior to testing for the post-prandial endotoxemia response, subjects also completed a series of other tests to exclude for other pre-existing conditions. Screening included measurement of body composition (whole body DEXA scan; GE Lunar Prodigy, United States), medical history assessment, and resting metabolic rate (RMR; MGC Diagnostics Ultima; St. Paul, MN, United States). Subjects who were currently taking or had taken in the previous 6-mo medications for the treatment of metabolic disease, antibiotics, probiotic supplements, anti-inflammatory medications, and/or daily consumed at least 3 serving of yogurt were excluded from further participation. Within the medical history, we also excluded subjects who were currently being treated for metabolic disease (*i.e.*, diabetes mellitus), currently being treated for cardiovascular disease, and/or were obese (by BMI and/or percent body fat from DEXA). Individuals who met the initial screening criteria were scheduled to consume the experimental meal challenge on a separate day. The experimental meal challenge was used to identify subjects with a dietary endotoxin response that we considered “responders”. Individuals classified as “responders” were enrolled in the supplementation phase of the study.

Identification of “responders”

Experimental meal challenge: Subjects reported to the laboratory between 0600 and 1000 following an overnight fast (> 8-h) and abstention from exercise (> 24-h). Following collection of a pre-meal blood sample, subjects were provided a high-fat meal (85% of the daily fat RDA and 65% of the daily calorie needs based on RMR). Thin crust cheese pizza from a local vendor was used as the high-fat meal source (Table 2). Blood samples were measured for endotoxin concentration after the meal and only those subjects

Table 2 Meal composition

Component	Placebo (n = 13)	Probiotic (n = 15)
Total calories (kcal)	1630.4 ± 134.4	1644.7 ± 94.5
Total caloric needs (% of RMR)	72%	79%
Servings (#)	6.3 ± 0.5	6.4 ± 0.4
Fat (g)	88.8 ± 7.3	89.6 ± 5.1
Fat (kcal)	799.3 ± 6.6	806.4 ± 46.3
Saturated fat (g)	31.7 ± 2.6	32.0 ± 1.8
Trans fat (g)	0	0
Protein (g)	69.8 ± 5.8	70.4 ± 4.0
Carbohydrate (g)	145.9 ± 12.0	147.2 ± 8.5
Carbohydrate (kcal)	583.6 ± 48.1	588.8 ± 33.8
Cholesterol (mg)	152.3 ± 12.5	153.6 ± 8.8
Sodium (mg)	2911.9 ± 240.0	2937.4 ± 168.8

Values represent group mean ± SEM. No significant differences existed between groups with respect to meal composition.

whose endotoxin level increased by > 5-fold at 5-h post-prandial were classified as “responders” and enrolled in the supplementation phase of the study. This same experimental meal challenge was completed at the end of the supplementation period to assess the effectiveness of spore-based probiotic supplementation at modifying the serum endotoxin response.

Supplementation conditions: “Responder” subjects were randomized to either a placebo (rice flour) or spore-based probiotic (Megasporebiotic; Physicians Exclusive, LLC; Glenview, IL, United States) condition. The spore-based probiotic included 4 billion spores from gram-positive, spore-forming strains [*Bacillus indicus* (HU36), *Bacillus subtilis* (HU58), *Bacillus coagulans*, and *Bacillus licheniformis*, *Bacillus clausii*]. Subjects were instructed to consume 2 capsules each day for a total of 30-d. Subjects were asked to promptly report any missed doses. Based on subject reporting, efficacy of intake was > 95% for the study period. All group assignments were completed using double-blind procedures. Subjects were instructed to maintain their habitual dietary and lifestyle habits during the study.

Blood sample collection: Venous blood samples were collected prior to the high-fat meal (PRE), 3-h, and 5-h post meal from a peripheral arm vein into an evacuated serum tube. Serum tubes were held at room temperature for 30-min to allow for clotting. Serum was separated by centrifugation and frozen at -80 °C until additional analysis.

Dietary endotoxin measurement

Serum was analyzed for endotoxin concentration using a commercially available kinetic limulus amoebocyte lysate (LAL) assay (Lonza; Allendale, NJ, United States). Briefly, serum samples were diluted 1:100 in endotoxin-free water and heated at 70 °C for 15-min to remove contaminating proteases. Treated samples were then analyzed in triplicate using an automated chemistry

analyzer (Chem Well T; Palm City, FL, United States) to determine endotoxin concentration against an *E. coli* endotoxin standard.

Serum triglyceride measurement

Serum was analyzed in triplicate for triglyceride concentration using an endpoint enzymatic assay (Pointe Scientific; Canton, MI, United States) on an automated chemistry analyzer (ChemWell T).

Exploratory disease risk biomarkers: Previously frozen serum samples were analyzed as previously described^[25-27]. Briefly, ghrelin, insulin, leptin, MCP-1, GM-CSF, interleukin (IL)-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12(p70), IL-13, and tumor necrosis factor alpha (TNF-α) were measured in duplicate using a commercially available bead-based multiplex assay (Milliplex; MilliporeSigma; St. Louis, MO, United States) and an automated analyzer (Luminex MagPix; Austin, TX, United States). Raw data files were used to calculate unknowns from standards using Milliplex Analyst software (MilliporeSigma).

Statistical analysis

Prior to formal statistical testing data were assessed for normality. Non-normal data was log-transformed to stabilize this assumption prior to formal testing. Data were analyzed using a condition (placebo or probiotic) × experiment time (baseline and 30-d post) × meal time (pre, 3, and 5-h post) analysis of variance (ANOVA) with repeated measurements on the 2nd and 3rd factors. *P*-values were adjusted using the Huynh-Feldt method to account for the repeated measures design. Significance was set at *P* < 0.05. Location of significant effects was determined using separate *t*-tests with a Bonferroni correction for multiple comparisons.

In order to visualize the responses collectively, we log transformed all the responses to normalize the various biomarkers to a similar scale. We then created three radar plots (one for each sampling time point). Each plot contained the log transformed variable response at baseline and 30-d post and a third line for the fold-change from pre-meal response). Heat maps were generated for variables that showed similarity to endotoxin responses using a three-color approach: Red (large increase from pre-meal), yellow (intermediate response), and green (large decrease from pre-meal) (Figure 2). We have used a similar approach to data visualization in past manuscripts and this is an effective and accepted method^[19,28].

RESULTS

Endotoxin and triglycerides

We found significant three-way interaction effects for both serum endotoxin (*P* = 0.011; Figure 3A) and triglycerides (*P* = 0.004; Figure 3B). In each instance, there was no difference between the post-prandial response between the two treatment groups (*i.e.*,

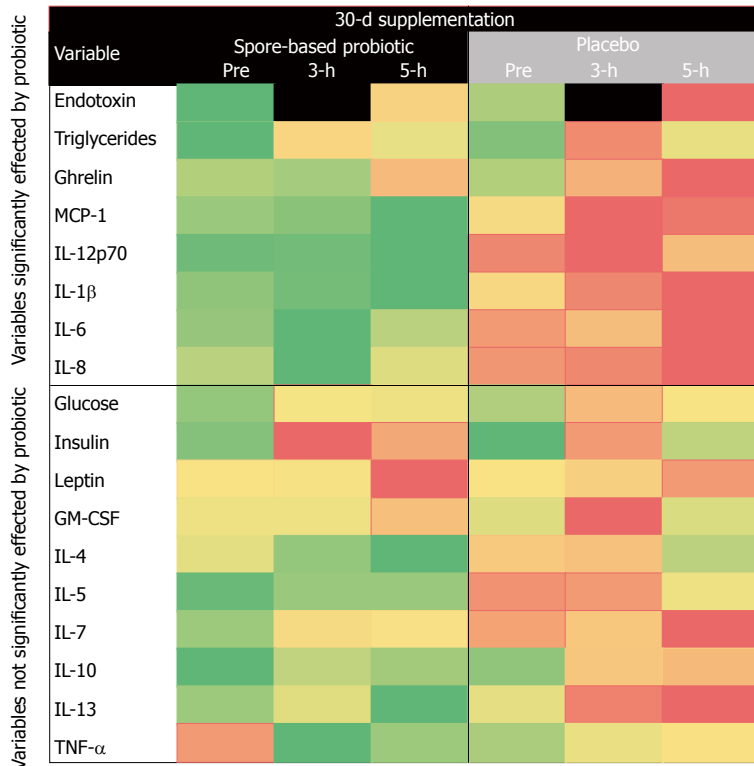


Figure 2 The change score for each variable compared to baseline was calculated and plotted as a heat map for probiotic vs placebo. Venous blood samples were collected prior to 3, and 5-h after consumption of a high-fat, high-calorie meal. Serum samples were analyzed using various accepted methods. Variables were divided into those that demonstrated a significant (upper panel) and those that did not (lower panel) have a significant probiotic effect. Responses were coded a lower (green to yellow) or higher (yellow to red) compared to baseline. An unchanged (yellow) response was also identified. TNF: Tumor necrosis factor; IL: Interleukin.

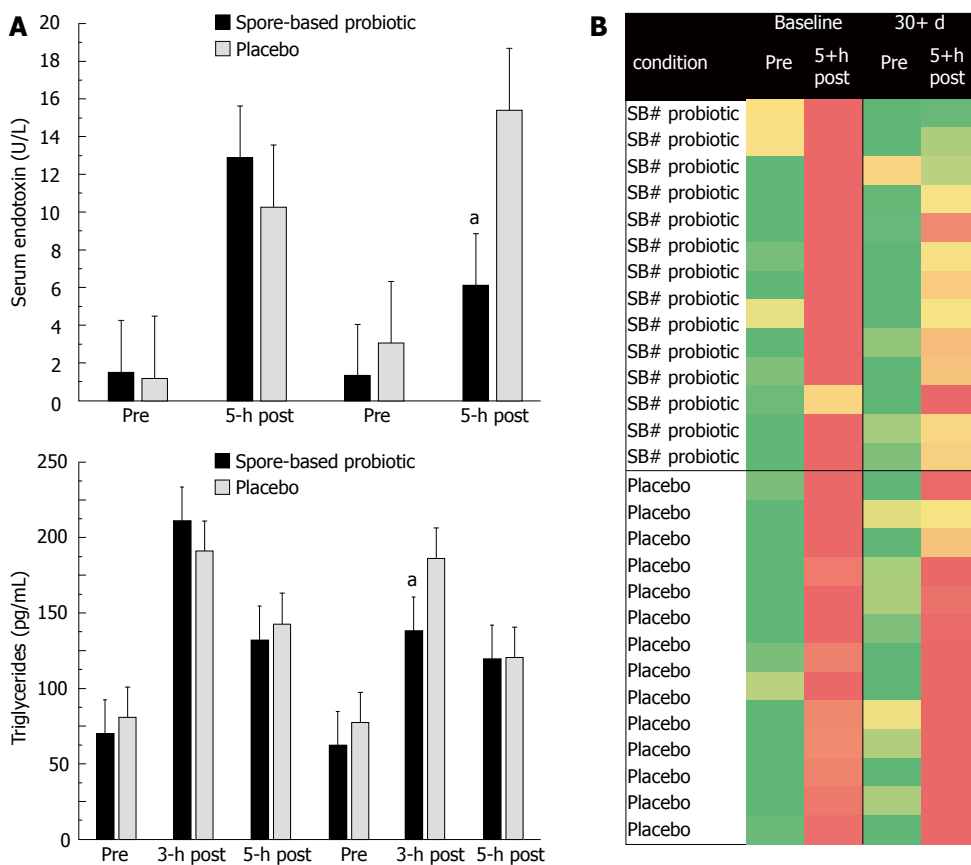


Figure 3 Serum endotoxin (A) and triglyceride (B) response to consumption of a commercially available high-fat, high-calorie pizza meal. ^a $P < 0.05$ indicates significantly less than placebo, less than pre-meal, less than and same time point at baseline. Venous blood samples were collected following an overnight fast and abstinence from exercise. Serum samples were analyzed using an automated chemistry analyzer. Subjects consumed an oral probiotic supplement for 30-d and the experimental meal challenge was completed at baseline and following the 30-d supplementation period. Probiotic responses were compared to placebo. Panel C demonstrates individual variability in the dietary endotoxin response prior to and after the 30-d supplementation period. Red indicates a large dietary endotoxin response (> 5 fold increase from pre-meal) and green indicates a small dietary endotoxin response (< 1 fold increase from pre-meal).

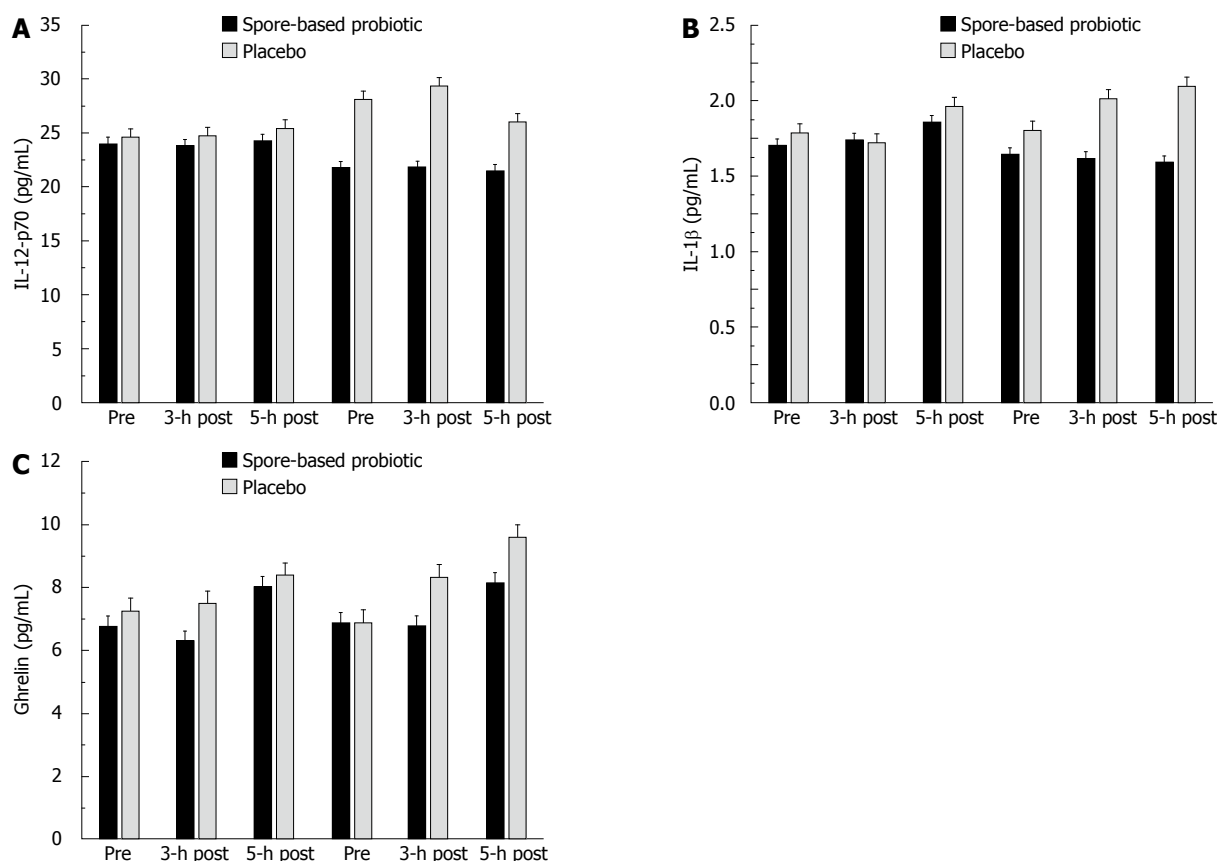


Figure 4 Serum IL-12p70 (A), IL-1 β (B), and ghrelin (C) response to consumption of a commercially available high-fat, high-calorie pizza meal. Venous blood samples were collected following an overnight fast and abstention from exercise. Serum samples were analyzed using an automated chemistry analyzer. Subjects consumed an oral probiotic supplement for 30-d and the experimental meal challenge was completed at baseline and following the 30-d supplementation period. Probiotic responses were compared to placebo. IL: Interleukin.

placebo vs spore-based probiotic) at baseline; however, the significant differences were apparent at post-supplementation. Specifically, spore-based probiotic supplementation was associated with a 42% reduction in serum endotoxin at 5-h post-prandial compared to a 36% increase in placebo at the same time point. Spore-based probiotic supplementation was associated with a 24% reduction in serum triglycerides at 3-h post-prandial compared to a 5% reduction in placebo at the same time point.

Exploratory biomarkers

We found significant trial $\times$ condition interactions for IL-12p70 ($P = 0.017$; Figure 4A), IL-1 β ($P = 0.020$; Figure 4B), and ghrelin ($P = 0.017$; Figure 4C). We also found potentially interesting trends for IL-6 ($P = 0.154$; Figure 5A), IL-8 ($P = 0.284$; Figure 5B), and MCP-1 ($P = 0.141$; Figure 5C). These effects were consistent with the pattern observed for serum endotoxin in that spore-based probiotic intervention was associated with a reduction in a given biomarker at post-supplementation compared to pre-supplementation and placebo.

DISCUSSION

An a priori review of the existing literature^[5,29], lead

our team to speculate that there may be an ideal subject phenotype that was “responsive” to spore-based probiotic treatment. Thus, we designed and implemented a screening protocol for the present study to identify individuals who presented with post-prandial endotoxemia at baseline, which may be a hallmark sign of intestinal permeability and “leaky gut” syndrome^[14,15,22,23]. We believe our approach to subject selection increased the efficacy and applicability of our key findings. Within our “responder” population (who likely had a non-protective microbiome), we were able to demonstrate that 30-d of oral supplementation with a viable, spore-based probiotic was associated with a significant reduction in post-prandial endotoxin and triglycerides. Further, we found that several of our exploratory biomarkers were either significantly reduced (IL-12p70, IL-1 β , and ghrelin) or trended toward reduction (IL-6, IL-8, and MCP-1) with spore-based probiotic supplementation. It is reasonable to speculate that the spore-based probiotic supplement may have exerted its effect by altering the gut microbial profile, altering intestinal permeability, or a combination of the two effects. The present study was designed to assess systemic changes rather than focus on intestinal measures that are invasive or impossible to make accurately in human subjects. The reductions observed

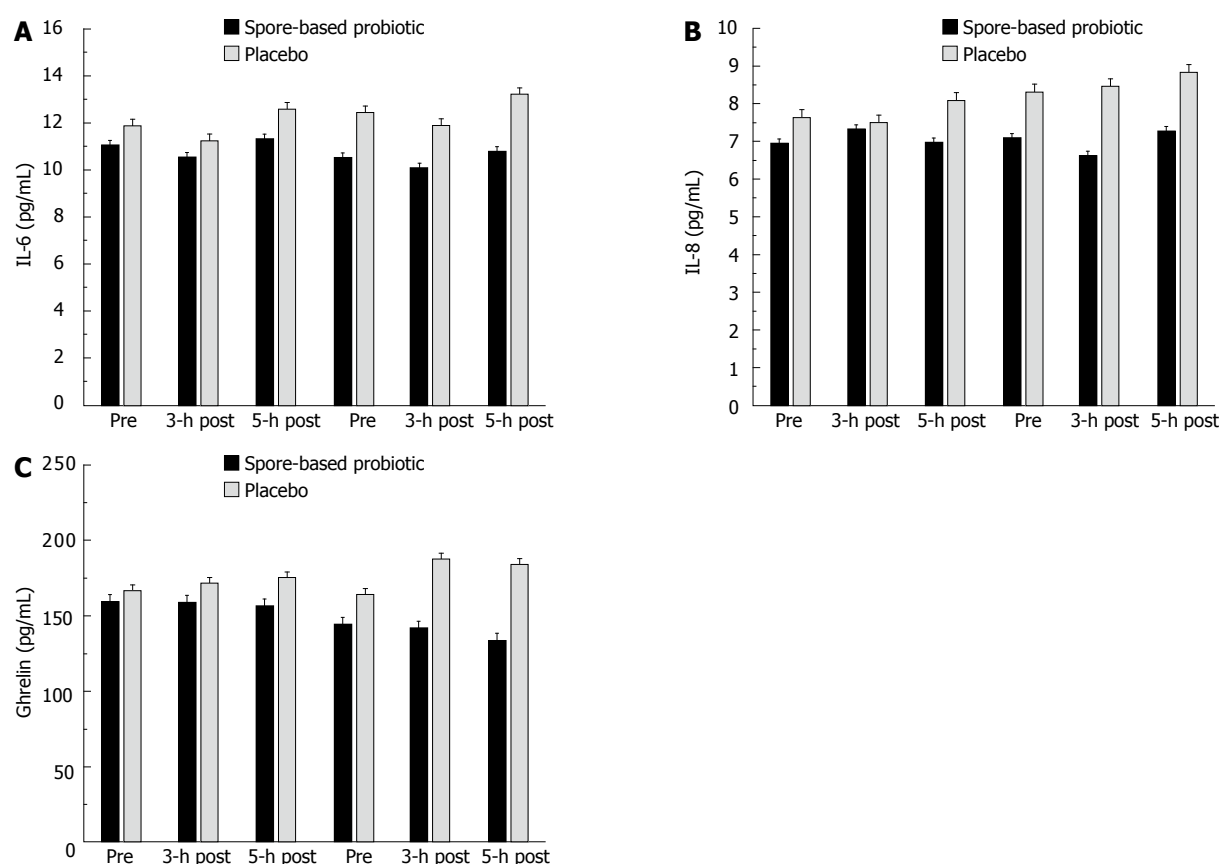


Figure 5 Serum IL-6 (A), IL-8 (B), and MCP-1 (C) response to consumption of a commercially available high-fat, high-calorie pizza meal. Venous blood samples were collected following an overnight fast and abstention from exercise. Serum samples were analyzed using an automated chemistry analyzer. Subjects consumed an oral probiotic supplement for 30-d and the experimental meal challenge was completed at baseline and following the 30-d supplementation period. Probiotic responses were compared to placebo. While effects did not reach statistical significance, trends are consistent with other variables that did significant change (Figures 2 and 3). IL: Interleukin.

in the present study with spore-based probiotic supplementation were consistent with a transient reduction in chronic disease risk. It is also important to note that the reported changes were observed while the college-aged subjects continued to lead their habitual life with no directed modification. They continued to be exposed to many of the stressors that are known to negatively affect gut permeability in college-aged individuals (*i.e.*, consumption of microwaved and other processed food, fast foods, soft drinks with their excess of sugars, including artificial sugars, colorings and flavorings, energy drinks, alcohol consumption, lack of sleep, exam anxiety, *etc.*).

Previous authors consistently speculate that the onset and progression of chronic disease results from the accumulation of transient changes in one's health that result from lifestyle choices^[16,18,19,22,28,30-32]. Unfortunately, the current literature has yet to define the quantity of transient change that must be accumulated to cause disease onset. Instead, previous studies have attempted to use lifestyle modifications (*i.e.*, nutrition, physical activity, *etc.*) to minimize negative changes in health. One such problem, especially in western cultures, is the wide accessibility to high-fat, high-calorie meals, creating an environment where excessive, low-quality nutritional

habits are the norm. In these diets elevated post-prandial endotoxin and triglyceride are consistently reported as problematic changes. Our observed baseline responses mirror previous reports^[22,31-33]. Recently a review article touted the potential of probiotic supplementation to prevent metabolic or dietary endotoxemia^[24], but to our knowledge no published study has yet to demonstrate this outcome. Thus, our finding of a 42% reduction in metabolic endotoxemia is novel and unique. Further interpretation of our finding does reveal a potentially interesting effect, while 30-d of supplementation reduced metabolic endotoxemia by 42%, it did not completely prevent metabolic endotoxemia. It is plausible to speculate that a longer period of supplementation may result in greater reductions in metabolic endotoxemia. Cani *et al.*^[14,15] previously reported in rodents, that the only viable method to "reprogram" the gut microbial response was to initially treat animals with a broad-spectrum antibiotic. For obvious ethical reasons treating human subjects with antibiotics is likely not a viable experimental design consideration, but perhaps the same effect could be achieved with a longer period of probiotic supplementation. In addition to probiotic effects, we also observed an interesting response in placebo subjects. Specifically, the placebo subjects presented

with an even greater metabolic endotoxemia response following a 30-d period. We do not believe that this observation is due to the experimental treatment, but is rather likely due to a diurnal fluctuation in metabolic endotoxemia responses. Thus, placebo subjects trended toward increased metabolic endotoxemia, while probiotic intervention reversed that effect. Since the present 30-d probiotic intervention did not completely prevent metabolic endotoxemia, it is reasonable to speculate that an intervention longer than 30-d may be necessary to completely prevent metabolic endotoxemia.

We have previously demonstrated that the consumption of a high-fat meal causes transient biological changes that were consistent with a transient increase in risk of atherosclerosis^[16,18,21,32]. These changes combined with a post-prandial increase in serum triglycerides creates a milieu that favors foam cell formation and the development of atherosclerotic plaques^[19,31,33,34]. In the present study, the baseline post-prandial meal response presented outcomes that were consistent with published data from our laboratory and others^[16,18,19,22,31-33,35]. Thus, the present study presented an opportunity to assess if a probiotic intervention would change disease risk biomarkers in a similar manner as endotoxin and triglycerides. We found significance across the entire meal combined between conditions, but were unable to tease apart specific time point differences. A *post hoc* sample size analysis revealed that we would have needed to enroll approximately 20 “responder” subjects in each group to delineate specific time point changes for biomarkers. Regardless, we found significant reductions in IL-12p70, IL-1 β , and ghrelin. Previous research has indicated that obese subjects do not have as great of a post-prandial suppression ghrelin than normal weight subjects^[36]. The authors do not explain the nature of the change, but given the observations of the present study, it is reasonable to speculate that obesity status may very well effect the gut microbiome^[36]. It is plausible that in the present study, without changing body weight, we were able to create the microbiome of a normal weight individual thus restoring normal post-prandial ghrelin responses.

Given the pro-inflammatory actions of IL-1 β , the observed reduction with probiotic supplementation was consistent with reductions in post-prandial systemic inflammation. Reduced ghrelin may be indicative of better post-prandial hunger/satiety control with probiotic. IL-12p70 has a variety of metabolic actions, the chief action in the present study is the ability to modulate the release of TNF- α or related inflammatory cytokines following antigenic challenge^[37,38]. In the case of the present study, reduced IL-12p70 with probiotic supplementation may reflect a reduction in systemic inflammatory capacity. In addition to the biomarkers that reached significance, we also found similar numerical trends for IL-6, IL-8, and MCP-1, which are all released by adipose tissues and commonly elevated in obese individuals^[27,31,39]. The biomarkers observed to change in the present study following the

probiotic intervention are involved in the accumulation of systemic inflammation^[38,40-43]. The existing literature has linked elevated systemic inflammation to the pathophysiology of cardiovascular and metabolic diseases, thus even a transient reduction in systemic inflammation biomarkers may be associated with reduced disease risk^[2,24]. The biomarkers measured in the present study are most often measured in the context of long-term weight loss (> 12 wk) interventions. In those weight loss models, it can take up to 16-wk to reduce body weight enough that biomarkers change. It is interesting that we demonstrated similar reductions in inflammatory biomarkers in 1/4 the time, but also in the absence of weight loss. We have presented novel results concerning the ability of probiotic supplementation to elicit transient effects.

In summary, the key findings of the present study demonstrate that 30-d of spore-based probiotic supplementation resulted in a blunting of dietary endotoxin, triglycerides, and potentially systemic inflammation. To our knowledge, the present study is the first to report that a short-term spore-based probiotic intervention altered dietary endotoxemia in human subjects, although the effect has been widely reported in mice^[1,14]. Due to limitations associated with using human subjects, it was not possible to directly measure gut permeability in the present study. Despite this, it is reasonable to speculate that the underlying cause of the observed reductions in post-prandial endotoxemia may be due to changes in the gut microbiome, gut permeability, or a combination of the two. Future research is needed to determine if a longer course of treatment with a spore-based probiotic results in additional health improvements.

ACKNOWLEDGMENTS

The present study was funded in part by a competitive research grant from Microbiome Labs, LLC (Glenview, IL, United States) to the University of North Texas. The UNT team did not receive direct funding associated with the completion of the present study. The funding agency was not involved in the data collection, analysis, interpretation, and manuscript preparation. Double blind procedures and confidentiality were used to conduct the present study in a sound and unbiased manner. As such, the authors report no conflict of interest associated with completing the present study.

COMMENTS

Background

Dietary or metabolic endotoxemia is a condition that affects approximately 1/3 of individuals living in Western society. It is characterized by increased serum endotoxin concentration during the first five hours of the post-prandial period following consumption of a meal with a high-fat, high-calorie content. Long-term repeated dietary endotoxemia may increase the risk of developing a variety of chronic diseases via an inflammatory etiology. Of the available treatments, oral probiotic supplementation has been purported to reduce gastrointestinal (GI) permeability to endotoxin, which in theory should suppress the dietary endotoxin

response.

Research frontiers

GI health is a hot topic and there is great interest in the study of natural substances that have the potential to improve GI health. Probiotics have been studied with inconsistent results, the present study was designed to specifically address previous limitations

Innovations and breakthroughs

For the purposes of this study the authors validated a new method for identifying subjects that may be “responders” to probiotic intervention. This screening method included only enrolling subjects that had at least a 5-fold increase in serum endotoxin at 5-h post prandial. Using this method of subject screening, the authors believe that the present study is the first published account demonstrating a significant reduction in post-prandial endotoxemia. It is also significant that the authors also found reductions in disease risk biomarkers after only 30-d of probiotic supplementation. The approach to subject screening for probiotic studies is a novel approach that the authors hope will become a standard for future studies in the area.

Applications

To the knowledge the present study is the first published report that has conclusively documented that short-term probiotic supplementation can reduce the incidence of leaky gut syndrome. The authors believe that the lessons learned from this study that will be critical to future projects is that: (1) detailed screening is needed to qualify subjects who are certain to respond; and (2) the type of probiotic used should be carefully selected. The present study used a spore-based probiotic that is known to have greater than 90% survivability after exposure to stomach acid. Survivability after exposure to stomach acid is a critical factor in the assessment of commercial probiotics that is often overlooked in the selection and study design.

Terminology

Dietary or metabolic endotoxemia is defined as a rise in blood serum endotoxin concentration during the first five hours after eating a meal. This post-meal period is also known as the post-prandial period. The most common cause of dietary endotoxemia is a disruption of gut barrier function. There is currently no accepted clinical test in humans to measure for gut barrier function. Disrupted gut barrier function cannot be predicted by any combination of baseline measures. Thus, to the knowledge the only means by which to assess gut barrier function was to complete a dietary endotoxemia test used in the present study. The endotoxin measured in the blood comes from bacteria that populate the GI track.

Peer-review

The article is interesting and maybe beneficial to the clinical physician.

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P- Reviewer: Lee HC S- Editor: Ji FF L- Editor: A
E- Editor: Lu YJ



Prospective Study

Assessment of serum angiogenic factors as a diagnostic aid for small bowel angiodysplasia in patients with obscure gastrointestinal bleeding and anaemia

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Author contributions: Holleran G and McNamara D designed research; Holleran G, Hussey M and Smith S Performed research; Holleran G, Smith S and McNamara D analyzed data; Smith S contributed reagents and analytical tools; Holleran G and McNamara D wrote the paper.

Institutional review board statement: Full ethical approval was obtained from the Tallaght Hospital/St James's Hospital Joint research Ethics committee.

Informed consent statement: Written informed consent was obtained from all patients prior to inclusion.

Conflict-of-interest statement: None of the authors had any conflicts of interest to declare.

Data sharing statement: There is no additional data available.

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Manuscript source: Invited manuscript

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Received: December 16, 2016

Peer-review started: December 20, 2016

First decision: March 6, 2017

Revised: March 31, 2017

Accepted: May 18, 2017

Article in press: May 19, 2017

Published online: August 15, 2017

Abstract

AIM

To assess the use of serum levels of angiopoietin-1 (Ang1), Ang2 and tumor necrosis factor- α (TNF α) as predictive factors for small bowel angiodysplasia (SBA).

METHODS

Serum samples were collected from patients undergoing capsule endoscopy for any cause of obscure gastrointestinal bleeding (OGIB) or anaemia. Based on small bowel findings patients were divided into 3 groups: (1) SBA; (2) other bleeding causes; and (3) normal, according to diagnosis. Using ELISA technique we measured serum levels of Ang1, Ang2 and TNF α and compared mean and median levels between the groups based on small bowel diagnosis. Using receiver operator curve analysis we determined whether any of the factors were predictive of SBA.

RESULTS

Serum samples were collected from a total of 120 patients undergoing capsule endoscopy for OGIB or anaemia: 40 with SBA, 40 with other causes of small bowel bleeding, and 40 with normal small bowel findings. Mean and median serum levels were measured and compared between groups; patients with SBA had significantly higher median serum levels of Ang2 (3759 pg/mL) compared to both other groups, with no significant differences in levels of Ang1 or TNF α based on diagnosis. There were no differences in Ang2 levels between the other bleeding causes (2261 pg/mL) and normal (2620

pg/mL) groups. Using Receiver Operator Curve analysis, an Ang2 level of > 2600 pg/mL was found to be predictive of SBA, with an area under the curve of 0.7. Neither Ang1 or TNF α were useful as predictive markers.

CONCLUSION

Elevations in serum Ang2 are specific for SBA and not driven by other causes of bleeding and anaemia. Further work will determine whether Ang2 is useful as a diagnostic or prognostic marker for SBA.

Key words: Angiodysplasia; Small intestinal bleeding; Capsule endoscopy; Angiogenic factors; Angiopoietin-2

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Core tip: Small bowel angiodysplasia (SBA) is an important cause of obscure gastrointestinal bleeding and anaemia but can be difficult to diagnose. This paper assesses the use of novel serum angiogenic factors associated with SBA as potential diagnostic aids. The study has identified a cut-off serum level of Ang2 of 2600 pg/mL which may be useful in predicting patients with the condition. Further studies will be required to determine its use in clinical practice but it may represent a major advancement in the identification of a diagnostic and prognostic marker for angiodysplasia, and also in determining the underlying pathophysiology of the condition.

Holleran G, Hussey M, Smith S, McNamara D. Assessment of serum angiogenic factors as a diagnostic aid for small bowel angiodysplasia in patients with obscure gastrointestinal bleeding and anaemia. *World J Gastrointest Pathophysiol* 2017; 8(3): 127-132 Available from: URL: <http://www.wjgnet.com/2150-5330/full/v8/i3/127.htm> DOI: <http://dx.doi.org/10.4291/wjgp.v8.i3.127>

INTRODUCTION

Small bowel angiodysplasia (SBA) accounts for over 50% of cases of obscure gastrointestinal bleeding (OGIB) and iron deficiency anaemia (IDA)^[1]. It is more common in elderly patients and in those with multiple comorbidities including chronic kidney disease (CKD), cardiovascular disease and chronic obstructive pulmonary disease (COPD)^[2]. The clinical presentation of SBA varies from those who remain asymptomatic or with mild IDA only, to those with acute life threatening overt haemorrhages, with a proportion of patients developing a more chronic and refractory form of SBA, suffering from recurrent bleeding episodes^[3-5].

The majority of SBAs are diagnosed by visualisation of characteristic mucosal vascular lesions by small bowel capsule endoscopy (SBCE) or device assisted enteroscopy (DAE). One of the difficulties in both diagnosing and following up patients with SBA is that almost half of patients do not notice any overt gastrointestinal bleeding, with their condition deteriorating silently until

they become symptomatic of their anaemia^[6,7]. For elderly patients with multiple co-morbidities this can lead to cumulative episodes of cardiac and respiratory decompensation, requiring regular hospital admissions and a resultant poor quality of life. An early and timely diagnosis would allow clinicians the opportunity to arrange more vigilant follow up, and to prevent progression to severe anaemia and subsequent pressure on other organs.

SBCE is the most sensitive diagnostic tool for SBA, however there are limitations to its use. Firstly, although SBAs can bleed significantly, when not bleeding they are generally < 8 mm in size and can be difficult to detect depending on their location, peristaltic activity or intraluminal contents at the time of passage of the capsule, leading to the potential for false negative studies. Secondly, we are as yet unsure as to the significance of the detection of non-bleeding SBAs by SBCE. Not all SBAs will bleed, however it has been shown that once they do bleed, the likelihood of repeated bleeding is significantly increased^[8]. In addition, SBCE is generally used to detect SBAs and guide treatment *via* argon plasma coagulation using DAE. However there are high rates of re-bleeding from previously treated lesions, along with sporadic growth of *de novo* lesions^[9,10]. This means that patients are often dependent on multiple repeat SBCEs on each bleeding episode to direct further treatment, with a resultant economic burden, increase in waiting lists for the procedure and a delay in diagnosis and treatment for patients with SBA. As there are no reliable or useful clinical predictors of SBA activity, it would be helpful if there were some other form of non-invasive marker of disease in order to guide management and predict prognosis for patients.

We have previously published our findings identifying abnormalities in serum angiogenic factors in patients with SBA, specifically the detection of higher levels of angiopoietin-2 (Ang2), and lower levels of angiopoietin-1 (Ang1) and Tumour Necrosis factor α (TNF α) in patients with SBA compared to non-bleeding controls^[11]. If specific for SBA, these factors could potentially be used as a diagnostic aid for diagnosis and management of SBA and reduce our dependence on SBCE.

This study aims to determine whether previously identified abnormalities in serum levels of angiogenic factors are specific for SBA or induced by OGIB or anaemia and assess whether serum levels of these factors could be used to predict a diagnosis of SBA in a cohort of patients with OGIB or IDA prior to SBCE.

MATERIALS AND METHODS

Ethical approval was obtained from our institutions research and ethics committee and any patient over the age of 18 years who was undergoing a SBCE for either IDA or OGIB was invited to participate. IDA was defined as haemoglobin (Hb) of < 11.5 g/dL in females and < 13 g/dL in males along with a serum ferritin of < 14. OGIB was defined as the presence of overt or occult (faecal

Table 1 Levels of serum angiogenic factors, ranges and *P* values compared to the small bowel angiodysplasia group for all 3 groups

	SBA (<i>n</i> = 40)	Abnormal (<i>n</i> = 40)	Normal (<i>n</i> = 40)
Ang1 (pg/mL)			
Median (range)	40976 (974-97511)	44770 (2660-101930)	47639 (17899-95173)
<i>P</i> value vs SBA		0.33	0.04
Ang2 (pg/mL)			
Median (range)	3759 (1915-14731)	2261 (842-14000)	2620 (686-8850)
<i>P</i> value vs SBA		< 0.004	< 0.003
Ratio of Ang1/Ang2			
Median	11.4	20.2	19
<i>P</i> value vs SBA		< 0.006	< 0.001
TNF α (pg/mL)			
Median (range)	5.76 (0.35-40)	9.76 (0.46-58)	10.14 (0.35-38)
<i>P</i> value vs SBA		0.12	0.13

SBA: Small bowel angiodysplasia; TNF: Tumor necrosis factor; Ang1: Angiopoietin-1.

occult blood positivity), and all patients had previously undergone at least one upper and lower endoscopy which had failed to identify a source of their anaemia or bleeding. On the day of their SBCE approximately 10 mL of blood was drawn from participants *via* standard phlebotomy technique. Plasma samples were analysed routinely for Hb level and serum samples were left to clot for 30 min before undergoing centrifugation for 15 min at 1000 rpm. The resultant supernatant was extracted and stored in aliquots at -80 °C for batch analysis.

SBCE was carried out in a routine fashion as previously reported and videos were reported by gastroenterologists trained in SBCE^[12]. Based on the report of their SBCE patients were divided into 3 groups: (1) SBA; (2) any other cause of bleeding; and (3) normal. A diagnosis of SBA was made using the classification published by Saurin *et al* with only definite or P2 lesions being included in the study. Other causes of bleeding included potential malignancies/polyps, any form of enteritis or active bleeding which was not determined to have been from and SBA on subsequent investigations. Recruitment continued until serum samples had been stored on a minimum of 40 patients in each group.

Serum levels of Ang1, Ang2 and TNF α were measured using commercially available solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) kits (R and D systems, Minneapolis, MN, United States). Samples were prepared in duplicate and results were read at 450 nm absorbance. The intra-assay coefficients of variation (CV) were calculated as an average of all of the individual CVs for the sample concentration duplicates analysed by ELISA.

Statistical analysis

Results of all assays and patient demographics were expressed as a mean and/or median and compared between groups using the Student *t*-test, Mann-Whitney *U* test, univariate/multivariate logistic regression analysis or a relative risk (RR) ratio as appropriate. All analyses were performed using SPSS version 22 (SPSS Inc., Chicago, IL, United States). The potential for the use of serum Ang1, Ang2 or a ratio of Ang1/Ang2 as a diagnostic

marker or SBCE screening tool for SBA was explored using receiver operator characteristic (ROC) curve analysis.

RESULTS

Of the group overall (*n* = 120), 58% (*n* = 70) were male, with a mean age of 63 years (18-93). Patients in the normal group (*n* = 40) were significantly younger than both the abnormal (*n* = 40) and angiodysplasia group (*n* = 40) with a mean age of 55 years (18-81) vs 60 years (19-92) and 73 years (53-93) respectively. There was no difference in gender between the groups with 42% (*n* = 17), 67% (*n* = 27), and 65% (*n* = 26) being male in each group respectively. The specific findings in the abnormal group included; non-specific enteritis *n* = 20, active bleeding *n* = 6, polyp/possible malignancy *n* = 5, denuded coeliac mucosa *n* = 2, Meckel's diverticulum *n* = 2, dieulafoy lesions *n* = 2, non-specific mucosal erythema not consistent with SBA *n* = 3.

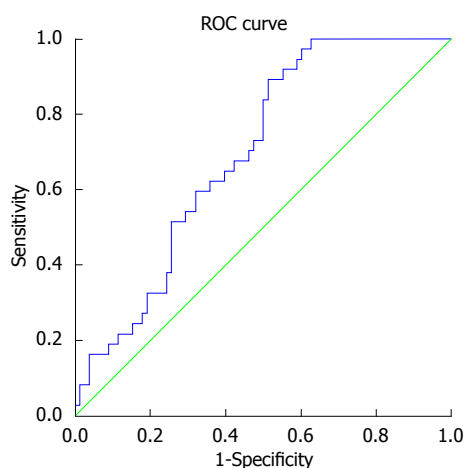
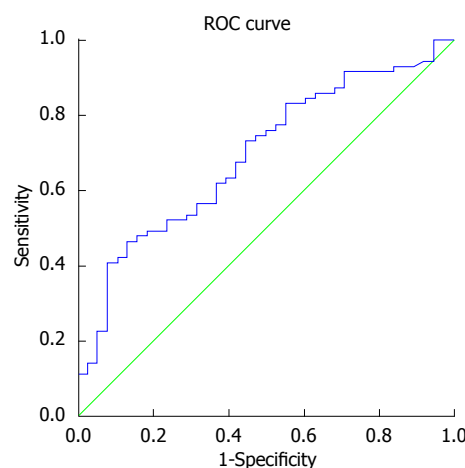
However there were significant differences in Hb levels with patients with normal findings having a higher mean Hb level at the time of SBCE 12.0 g/dL in females and 14.0 g/dL in males) compared to both the abnormal (11.3 g/dL in females and 12.9 g/dL in males) and Angiodysplasia (11.0 g/dL in females and 11.3 g/dL in males) groups *P* values both < 0.02. There was no difference between Hb levels in the abnormal and Angiodysplasia groups, *P* = 0.832.

Ang1 and Ang2 and TNF α levels

The median serum levels, ranges and *P* values compared between the groups is shown in Table 1. Patients with SBA have significantly higher serum levels of Ang2 than patients with other causes of bleeding or anaemia. When Ang2 levels were controlled for anaemia across the groups it was not found to be a confounding factor. Although there was a trend towards lower levels of both Ang1 and TNF α , these were not found to be statistically significant. There were no differences in any of the factors when compared between the other cause of

Table 2 Analysis of sensitivity, specificity and predictive values of a cut-off serum level of Ang2 of 2600 pg/mL

True positive $n = 33$	False positive $n = 40$	Sensitivity = 85%
False negative $n = 7$	False negative $n = 40$	Specificity = 50%
		Positive predictive value = 45%
		Negative predictive value = 85%

**Figure 1** Receiver operator characteristic curve for serum Ang2, area under the curve = 0.695.**Figure 2** Receiver operator characteristic curve for the ratio of serum Ang1/Ang2, area under the curve = 0.692.

bleeding and normal groups. The ratio of Ang1/Ang2 was found to be significantly lower in the SBA group compared to the other groups and again there was no difference in the ratios between the other bleeding cause and the normal groups.

Potential diagnostic tools for SBA

We went on to assess the potential use of serum Ang2 as a diagnostic tool for SBA by using ROC curve analysis as demonstrated in Figure 1. This established a cutoff serum Ang2 level of 2600 pg/mL with an area under the curve (AUC) of 0.695, (standard error 0.048, 95%CI: 0.601-0.789, $P = 0.001$). Table 2 outlines the sensitivity, specificity, and predictive values of this cut-off level. Using the RR model, a serum level of 2600 pg/mL had a RR of SBA of 2.22 ($P = 0.012$, 95%CI: 1.20-4.11). Although the positive predictive value for SBA was only 45%, 53% of the false positive patients did clinically significant findings (including 2 potential malignancies, 2 Meckel's diverticulae and an actively bleeding Dieulafoy's lesion) all of which would warrant urgent further investigation, making the true false positive rate for clinically significant findings only 26%.

ROC curve analysis was also performed using the Ang1/Ang2 ratio which gave an area under the curve of 0.692, (standard error 0.052, 95%CI: 0.51-0.793, $P = 0.001$) (Figure 2). As the AUC was slightly lower than that for Ang2 alone curve (AUC 0.695 vs 0.692 respectively) it was felt that no benefit would be conferred by the need for the measurement of two markers and further analysis was not performed.

DISCUSSION

Due to advances in medical care, particularly cardiovascular and stroke medicine, patients are living longer with a dependency for anticoagulant and antiplatelet therapy, meaning that SBA is increasing in incidence in our aging population. Unfortunately no treatment has yet been shown to prevent re-bleeding from SBAs in the longer term, however there have been suggestions that the earlier diagnosis and intervention of some form of directed treatment prior to respiratory or cardiac decompensation due to anaemia, may improve the longer term outcome for patients.

Although SBCE is the most sensitive diagnostic tool for SBA and is far less invasive than conventional small bowel endoscopy, it is still not widely available in all centres, does carry some risks including false negative studies, and remains a relatively expensive test. Thus the availability of alternative diagnostic and prognostic tools may prove extremely useful in reducing the dependence on SBCE and directing treatment for patients at an earlier stage. As the clinical course of SBA is so unpredictable and patients are often unaware of re-bleeding episodes a non-invasive predictive marker which could be used in addition to and to direct further investigation and treatment would be of great advantage.

Our previously published findings of higher levels of Ang2 and lower levels of Ang1 in serum and tissue levels of patients with SBA offers a credible hypothesis for the pathophysiology of SBA as excess Ang2 has

been shown to lead to the development of enlarged and weakened blood vessels, and Ang1 is required to ensure blood vessel stabilisation and maturity. However, one of the main concerns regarding our initial findings was that our comparison of serum factors in patients with SBA vs non-bleeding controls may have meant that the detected abnormalities in Ang levels may have been driven by OGIB or anaemia overall and not were not specific for SBA. One of the main objectives of this study therefore was to determine the specificity of these findings for SBA compared to OGIB and IDA of varied causes. This study has shown that the elevation of serum levels of Ang2 is specific for SBA and is not a response to bleeding or anaemia of any other cause. In contrast, our results showed that abnormalities in serum levels of Ang1 is not specific for SBA and may be related to other factors such as OGIB or anaemia overall. Interestingly however there was still a significant difference in the ratio of Ang1/Ang2 levels between SBA and non-SBA patients.

The second aim of this study was to determine whether measuring serum levels of these factors could be used as a diagnostic or screening tool to predict patients likely to have SBA and to facilitate earlier detection. The difficulty in establishing a diagnostic tool is defining a cut off level with adequate sensitivity and specificity levels to make them clinically useful. Using ROC curve analysis we determined that an Ang2 level of 2600 pg/mL with a sensitivity level of 85% and a negative predictive value of 85%, was the most clinically appropriate cut-off level. Although an AUC of 0.7 is not particularly accurate for a diagnostic tool, the purpose of this screening tool is to identify patients likely to have SBA. Therefore the most important aspects of this tool were the sensitivity and negative predictive value for a diagnosis of SBA. Although the ratio of Ang1/Ang2 differed significantly from other causes of OGIB, we found no diagnostic benefit to measuring both serum factors, making the test more economically feasible if translated to clinical practice. Further validation of the use of Ang2 at this cut-off level in a larger group of patients with OGIB and IDA are needed to ensure its accuracy as a potential predictive tool, however it offers a potentially cheap and non-invasive marker to aid in the diagnosis of SBA.

Although it is unlikely that the measurement of serum angiogenic factors will replace our need for SBCE, particularly as this is generally useful to guide route of approach for subsequent DAE, they are likely to have a role in assisting diagnosis and in follow up. It was beyond the scope of this study to assess the ability of serum angiogenic factors as biomarkers of disease activity as serum was taken from patients at a single point in time and it is likely that the majority were not actively bleeding at the time. An interesting assessment and a necessity in determining whether serum Ang2 levels could be used as a prognostic marker would be to take serial measurements from patients with known

SBA and establish whether levels correlate with changes in disease course, around the time of active bleeding, or following a definitive treatment intervention.

A recognised weakness of our study is that the patient's with a normal SBCE had lower mean Hb levels than the other groups. All patients at the time of referral were anaemic, however due to significant waiting times for SBCE in our unit a significant proportion of these patients may have had a spontaneous recovery of their anaemia by the time they underwent SBCE. This is likely to be the case in clinical practice as patients will receive some empiric treatment with iron or red cell transfusions and future prospective studies may determine whether a Hb level alone could be used as a predictive or prioritisation tool in milder cases of anaemia, with serum Ang2 reserved for currently anaemic patients only. In addition, our study relied on SBCE being the gold standard for diagnosis of small bowel causes of OGIB, however as mentioned earlier the sensitivity of SBCE is not 100% and there is a possibility that some of the patients in the normal will include those with false negative studies which may have impacted Ang2 levels.

In conclusion, this study has validated our previous findings showing an elevated serum Ang2 in patients with SBA. In addition it has shown that this association is specific for SBA and is not driven by bleeding or anaemia of other causes. We have identified the potential use of serum Ang2 as a diagnostic aid in SBA, and developed a basis for further work to examine its use as an indicator of disease activity. In the future these findings may lead to a more timely diagnosis for patients with SBA and provide a more accessible and non-invasive mode of follow up.

COMMENTS

Background

Small bowel angiodysplasia (SBA) accounts for over 50% of cases of obscure gastrointestinal bleeding and anaemia. Very little is known about the underlying pathophysiology of the condition which significantly limits improvements in diagnostic aids, prognostic markers and focussed treatments. The authors have previously identified an association between SBA and abnormalities in serum levels of angiopoietin-1 (Ang1), angiopoietin-2 (Ang2) and tumour necrosis factor- α (TNF α). Whether these factors were specifically associated with SBA or were driven by overall gastrointestinal bleeding or anaemia, and whether they could be used as diagnostic aids for the condition were not clear from the authors initial study and required further assessment.

Research frontiers

At present diagnosis of SBA is *via* small bowel endoscopy only in the form of capsule or device assisted endoscopy, both of which have very limited access worldwide. In addition there is currently no specific treatment for angiodysplasia, the development of which is limited by a deficient knowledge of the underlying pathophysiology. The overarching aim of our work is to identify angiogenic factors associated with the condition which may be useful both as diagnostic and prognostic markers and as future treatment targets.

Innovations and breakthroughs

This paper has identified Ang2 as a potential serum diagnostic marker for SBA in patients with obscure gastrointestinal bleeding and anaemia. It may be useful in expediting diagnosis and improving outcome.

Applications

Further work will need to be done to validate these findings prior to their use in clinical practice but Ang2 may be useful as a diagnostic aid in patients with obscure gastrointestinal bleeding. It may also be a useful treatment target for anti-angiogenic therapies.

Terminology

Ang1 and Ang2 are both angiogenic factors known to be involved in blood vessel formation. TNF α is an inflammatory cytokine which also has a role in vessel formation.

Peer-review

This preliminary study is an advance in the understanding of the pathophysiology of angiodysplasias of the small intestine and may be a useful clinical tool to be verified in a larger series of patients.

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P- Reviewer: Vega J S- Editor: Gong ZM L- Editor: A
E- Editor: Lu YJ



Prospective Study

Effect of replenishment of vitamin D on survival in patients with decompensated liver cirrhosis: A prospective study

Ashish Kumar Jha, Sharad Kumar Jha, Amarendra Kumar, Vishwa Mohan Dayal, Sanjeev Kumar Jha

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Author contributions: Jha AK drafted the manuscript, oversight of the study, assisted with data analysis and prepared the manuscript; Jha SK recruited the patients, involved with data collection and performed statistical analysis; Dayal VM participated in design and oversight of the study; Kumar A participated in design of the study and oversight of the study, Jha SK was involved with data collection, and assisted with data analysis; all authors read and approved the final manuscript.

Institutional review board statement: The study was reviewed and approved by the Institutional Review Board of Indira Gandhi Institute of Medical Sciences, Sheikhpura, Patna, India.

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

Conflict-of-interest statement: The authors of this manuscript having no conflicts of interest to disclose.

Data sharing statement: There is no additional data available.

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Manuscript source: Invited manuscript

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Received: November 13, 2016

Peer-review started: November 15, 2016

First decision: December 20, 2016

Revised: May 1, 2017

Accepted: June 6, 2017

Article in press: June 8, 2017

Published online: August 15, 2017

Abstract**AIM**

To assess the vitamin D (VD) deficiency as a prognostic factor and effect of replenishment of VD on mortality in decompensated cirrhosis.

METHODS

Patients with decompensated liver cirrhosis were screened for serum VD levels. A total of 101 VD deficient patients (< 20 ng/mL) were randomly enrolled in two groups: Treatment group ($n = 51$) and control group ($n = 50$). Treatment group received VD treatment in the form of intramuscular cholecalciferol 300000 IU as loading dose and 800 IU/d oral as maintenance dose along with 1000 mg oral calcium supplementation. The VD level, clinical parameters and survival of both the groups were compared for 6-mo.

RESULTS

Prevalence of vitamin D deficiency (VDD) in decompensated CLD was 84.31%. The mean (SD) age of the patients in the treatment group (M:F: 40:11) and control group (M:F: 37:13) were 46.2 (± 14.93) years and 43.28 (± 12.53) years, respectively. Baseline mean (CI) VD (ng/mL) in control group and treatment group were 9.15 (8.35-9.94) and 9.65 (8.63-10.7), respectively. Mean (CI) serum VD level (ng/mL) at 6-mo in control group and treatment group were 9.02 (6.88-11.17) and 29 (23-35), respectively. Over the period of time the VD, calcium and phosphorus level was improved in treatment

group compared to control group. There was non-significant trend seen in greater survival (69% *vs* 64%; $P > 0.05$) and longer survival (155 d *vs* 141 d; $P > 0.05$) in treatment group compared to control group. VD level had no significant association with mortality ($P > 0.05$). In multivariate analysis, treatment with VD supplement was found significantly ($P < 0.05$; adjusted hazard ratio: 0.48) associated with survival of the patients over 6-mo.

CONCLUSION

VD deficiency is very common in patients of decompensated CLD. Replenishment of VD may improve survival in patients with decompensated liver cirrhosis.

Key words: Chronic liver diseases; Vitamin D; Vitamin D deficiency; Decompensated liver cirrhosis; Survival

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Core tip: This was a prospective study to assess the vitamin D (VD) deficiency as a prognostic factor for survival in patients with decompensated chronic liver diseases ($n = 101$) and effect of replenishment of VD on all-cause mortality in decompensated liver cirrhosis. Treatment with VD supplement was found associated with survival of the patients over 6-mo. Replenishment of VD along with calcium supplementation may improve survival in patients with decompensated cirrhosis. These findings need to be confirmed in larger multicenter studies.

Jha AK, Jha SK, Kumar A, Dayal VM, Jha SK. Effect of replenishment of vitamin D on survival in patients with decompensated liver cirrhosis: A prospective study. *World J Gastrointest Pathophysiol* 2017; 8(3): 133-141 Available from: URL: <http://www.wjgnet.com/2150-5330/full/v8/i3/133.htm> DOI: <http://dx.doi.org/10.4291/wjgp.v8.i3.133>

INTRODUCTION

Vitamin D (VD) is widely known as a regulator of calcium and bone metabolism. Deficiency of VD, a secosteroid causes rickets in children and osteomalacia in adults. Role of VD is not limited to bone mineral metabolism. It has pleiotropic effects including cellular proliferation and differentiation^[1]. It has important role in activation of lymphocytes and immunomodulation. The role of VD in the activation and regulation of both innate and adaptive immune systems has been described^[2]. There are reports suggesting VD also has anti-inflammatory and anti-fibrotic properties. These functions have been implicated in the pathogenesis and treatment of infections, autoimmune, cardiovascular, and degenerative diseases and several types of cancer^[1-5].

Worldwide, hepatic cirrhosis is a common cause of mortality and morbidity. Liver plays important role

in conversion of inactive VD into active 25-hydroxy VD (25-OH VD)^[6]. Vitamin D deficiency (VDD) is very common in patients of chronic liver diseases (CLD). VDD is well described in cholestatic liver diseases. However, the role of VDD in the natural history of noncholestatic CLD is not well described^[7]. The data regarding the association of VDD with complications and prognosis in patients of CLD are scarce and heterogeneous^[8-10]. There are few reports available regarding the effect of VD supplementation on the natural history of VD deficient people, especially patients with decompensated CLD^[11-14]. Effect of VD supplementation to prevent mortality in VD deficient patients with decompensated CLD is not known. The aims of this study were to assess the VD levels in a cohort of patients with decompensated liver cirrhosis and effect of VD replenishment on all-cause mortality in patient with VD deficient decompensated cirrhosis.

MATERIALS AND METHODS

This study was a single centre prospective study conducted in the Department of Gastroenterology in a tertiary care center of Eastern India over the period of 2-year. All patients provided a written informed consent before enrolment. For patients with altered sensorium, an informed consent was obtained from the next of kin. The study was approved by the institute's ethical review committee.

Inclusion criteria: Decompensated cirrhosis of liver, Child Turcotte Pugh (CTP) score ≥ 10 , age between 18 years to 70 years. The following patients were excluded from the study: Septicemia, infection with HIV, episodes of variceal bleeding within 6 wk, hepatocellular carcinoma or any malignancy, hepatorenal syndrome at the time of enrolment, significant cardiac and respiratory disease, pregnancy, patients being taken up for transplant and refusal to participate in the study.

Detailed clinical history and physical examination of patients was done. All patients were tested for complete blood count, liver function tests, prothrombin time, blood sugar, alpha-fetoprotein, renal function tests, serum electrolytes, serum calcium, serum phosphorus, serum 25-OH VD, urine routine microscopic examination and culture, blood culture for aerobic and anaerobic organism, chest radiographs, endoscopy and color Doppler ultrasonography of abdomen. Patients also underwent diagnostic paracentesis and fluid analysis for protein, albumin, TLC, differential cell counts, and culture sensitivity. All of them were tested for HBsAg, anti-hepatitis B core antigen (HBc) IgG (total), and anti-hepatitis C virus (HCV) by ELISA. Appropriate tests for autoimmune liver disease, Wilson disease, and hemochromatosis (ANA, ASMA, anti-LKM1, serum ceruloplasmin, 24 h urinary copper analysis, and serum ferritin) were performed. The following investigations; anti-hepatitis E virus (HEV) IgM, anti-hepatitis A virus (HAV) IgM, and CT of whole abdomen were done whenever required.

Patients of CLD were diagnosed with the help of characteristic medical history, compatible physical examinations, blood investigations, radiological imaging and endoscopy. The diagnosis of CLD was defined on the basis of at least one of the following: Endoscopic evidence of esophageal varices of at least grade II in size, undisputable evidence on ultrasonography or CT scan of cirrhosis of the liver and/or presence of portosystemic collaterals, and prior liver biopsy showing evidence of liver cirrhosis. Decompensated CLD was defined as CLD complicated with ascites and/or gastrointestinal variceal bleeding^[15].

Patients with decompensated CLD were screened for serum 25-hydroxy VD (25-OH D) levels. Blood for 25-OH VD estimation was collected on admission. The assay was performed within 24 h of sample collection. Quantitative assessment of total 25-OH D was performed by direct competitive chemiluminescence immunoassay using commercially available kit UniceL[®] DxI 800 immunoassay systems (Beckman Coulter, Inc. CA, United States) (detection range 4-150 ng/mL in 250 µL of serum). The classification of the 25-OH VD status were as follows: Severe deficiency: < 10 ng/mL; deficiency: 10-20 ng/mL; insufficiency: 20-29 ng/mL; sufficiency: 30-100 ng/mL; (To convert results into SI units: ng/mL × 2.5 = nmol/L)^[16]. Patient with serum VD level < 20 ng/mL was considered as VD deficient.

Patients with deficient VD level were randomly divided into treatment and control groups. Total number of VD deficient patients enrolled in study was 101. Fifty one patients were included in treatment group who received VD treatment in the form of intramuscular cholecalciferol 300000 IU as loading dose and 800 IU/d oral as maintenance dose along with 1000 mg oral calcium supplementation. Fifty VD deficient patients were not supplemented with VD acts as control group. Monitoring of VD; serum calcium and serum phosphorus was done monthly for 6-mo or till death, whichever occur earlier. Supplementation of VD was stopped if features of hypercalcemia, kidney stone, or serum 25 OH VD level > 80 nmol/L (> 32 ng/mL) observed.

Patients were treated with general supportive measures. Patients with hepatic encephalopathy were treated with anti-hepatic encephalopathy regimen. Renal failure was managed with either intravenous human albumin with or without intravenous terlipressin and dialysis as when indicated. Those without renal failure and encephalopathy were treated with diuretics for ascites. Therapeutic paracentesis was performed as when indicated. Patients were treated with inotropes for hypotension and with assisted ventilation for respiratory failure. Oral anti-viral drugs were used for the treatment of HBV infection. Treatment of alcoholism was done with abstinence and medications to increase abstinence. Those who were discharged in stable condition were followed up on outpatient basis.

Statistical analysis

All statistical analysis were performed using Stata

version 10 (Stata Corp, TX, United States). The primary outcome was survival observed over a period of 6-mo. Continuous variables were presented as mean with 95%CI and categorical variables were reported as frequencies and percentages. Survival analysis was performed using Kaplan-Meier method followed by log-rank test for binary variables and Cox-proportional hazard method for continuous variable. Baseline variables between test and control group was compared using *t*-test for difference of means. A *P*-value of < 0.05 was considered statistically significant.

RESULTS

We evaluated 153 patients of decompensated CLD (CTP Score ≥ 10). Out of 153, 129 (84.31%) patients were deficient in serum VD. Out of 129, 28 patients were excluded from study due to following reason; upper gastrointestinal bleeding within 6 wk (18), hepatorenal syndrome (4), hepatocellular carcinoma (2), age < 18 years (2) and age > 70 years (1). A total of 101 patients were randomly enrolled in two groups: Treatment group (*n* = 51) and control group (*n* = 50). The mean age of the patients in the treatment group and control were 46.2 years (± 14.93) and 43.28 years (± 12.53), respectively. Male:female ratio in the treatment group and control were 40:11 and 37:13, respectively. There was no significant difference in age and sex ratio of two groups. The treatment and control groups were similar with respect to etiology of liver diseases. Most common etiology of CLD was ethanol in both control (*n* = 24; 48%) and treatment (*n* = 19; 38%) groups, followed by HBV (control = 20%; treatment = 24%), cryptogenic CLD (control = 14%; treatment = 20%), and hepatitis C virus (control = 8%; treatment = 8%). Other uncommon etiologies were NASH and Wilson disease.

The baseline parameters in both the treatment and control groups were comparable (*P* > 0.05), except fever and blood TLC which were significantly higher in control group as compared to treatment group (*P* < 0.05) (Table 1). Baseline serum VD (ng/mL) in control group and treatment group were 9.15 (8.35-9.94) and 9.65 (8.63-10.7), respectively. Serum VD (ng/mL) at 6-mo in control group and treatment group were 9.02 (6.88-11.17) and 29 (23-35), respectively. Baseline serum calcium (mg/dL) in control group and treatment group were 7.8 (7.6-8.00) and 7.59 (7.4-7.7), respectively. Serum calcium (mg/dL) at 6-mo in control group and treatment group were 5.5 (4.23-6.6) and 6.7 (5.31-8.08), respectively. Baseline serum phosphorus (mg/dL) in control group and treatment group were 3.8 (3.7-4.06) and 3.68 (3.53-3.83), respectively. Serum phosphorus (mg/dL) at 6-mo in control group and treatment group were 2.68 (2.09-3.27) and 3.31 (2.61-4.10), respectively. Comparisons of level of VD, calcium and phosphorous over a period of 6 mo is summarized in Table 2. As compared to control group the levels of VD, calcium and phosphorous were higher in

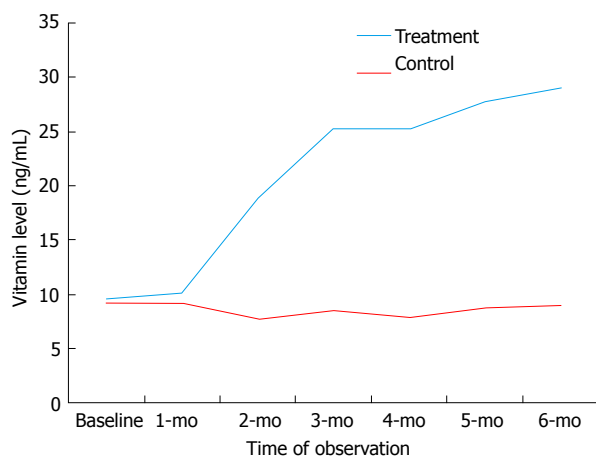
Table 1 Baseline characteristics of patients in treatment (*n* = 51) and control group (*n* = 50) *n* (%)

Parameters	Control groups (<i>n</i> = 50)	Treatment groups (<i>n</i> = 51)	<i>P</i> -value
Age (yr) (mean ± SD)	43.28 ± 12.53	46.21 ± 14.93	> 0.05
Sex (M:F)	37:13	40:11	
Mean (CI) serum bilirubin (mg/dL)	8.95 (6.62-11.29)	6.89 (5.15-8.64)	> 0.05
Mean (CI) PT - INR	1.50 (1.41-1.59)	1.43 (1.37-1.48)	> 0.05
ALT (IU/L)	92 (61-119)	84 (62-106)	> 0.05
AST (IU/L)	157 (98-215)	127 (106-147)	> 0.05
Mean (CI) serum albumin (g/dL)	2.41 (2.32-2.50)	2.38 (2.28-2.49)	> 0.05
Mean (CI) serum creatinine	1.11 (1.04-1.17)	1.18 (1.13-1.24)	> 0.05
Mean (CI) blood TLC (mm ³ /μL)	10902 (9481-12322)	8407 (7238-9576)	< 0.05
Mean (CI) Ascitic fluid TLC (mm ³ /μL)	233.84 (187.41-280.27)	237.92 (193.48-282.32)	> 0.05
Ascites	50 (100)	51 (100)	> 0.05
Jaundice	40 (80)	41 (80.4)	> 0.05
Encephalopathy	21 (42)	27 (52.94)	> 0.05
Fever	19 (38)	10 (20)	< 0.05
Mean (CI) CTP score	10.92 (10.64-11.20)	11.17 (10.83-11.52)	> 0.05
Mean (CI) MELD score	19.02 (17.91-20.12)	18.62 (17.70-19.54)	> 0.05
Etiology of CLD (%)	Ethanol (48), HBV (20), Cryptogenic (14), HCV (8), Ethanol + HBV (6), NASH (2), Wilson's disease (2)	Ethanol (38), HBV (24), Cryptogenic (20), Ethanol + HBV (10), HCV (8), NASH (4)	

PT: Prothrombin time; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; CTP: Child Turcott Pugh.

Table 2 Comparisons of level of vitamin D, calcium and phosphorous over a period of 6 mo

		Baseline	3 rd month	6 th month
Mean (CI) vitamin D (ng/mL)	Control	9.15 (8.35-9.94)	8.54 (6.95-10.13)	9.02 (6.88-11.17)
	Treatment	9.65 (8.63-10.7)	25.35 (21.59-29.10)	29 (23-35)
Mean (CI) calcium (mg/dL)	Control	7.8 (7.6-8.00)	6.2 (5.2-7.2)	5.5 (4.23-6.6)
	Treatment	7.59 (7.4-7.7)	7.7 (6.9-8.6)	6.7 (5.31-8.08)
Mean (CI) phosphorus (mg/dL)	Control	3.8 (3.7-4.06)	3.01 (2.51-3.51)	2.68 (2.09-3.27)
	Treatment	3.68 (3.53-3.83)	3.8 (3.3-4.2)	3.31 (2.61-4.10)

**Figure 1** Comparison of vitamin D level between treatment and control group.

treatment group. Over the period of time the VD levels were better improved in treatment group compared to control group (Figure 1). Although, the level of serum calcium and phosphorus were decreasing over the period of time in both groups but treatment group had less decline compared to control group.

CTP score and model for end-stage liver disease (MELD) score at baseline were similar in treatment and control group (*P* > 0.05). We observed a decline in CTP

score and MELD score in both the groups over the period of 6-mo time. The proportion of survival in treatment group (69%) was higher compared to control group (64%), but the difference was statistically not significant (*P* > 0.05). Also the mean survival duration in treatment group was 155 d (95%CI: 142-167) compared to control group, *i.e.*, 141 d (95%CI: 125-157) (*P* > 0.05). Comparisons of CTP score, MELD score and survival over a period of 6-mo is summarized in Table 3.

In univariate and multivariate analysis, the various parameters including bilirubin, creatinine, ascitic fluid TLC, CTP score, and MELD score were found significantly (*P* < 0.05) associated with 6-mo survival of the patients (Table 4). In univariate analysis, the parameters including hepatic encephalopathy, blood TLC, serum sodium, creatinine, PT- INR were also found significantly (*P* < 0.05) associated with 6-mo survival of the patients.

Serum VD level had no significant association with mortality (*P* > 0.05). In the univariate analysis the unadjusted hazard ratio for the effect of VD supplementation on mortality was 0.92 (95%CI: 0.81-1.03) and it was not found significant (*P* > 0.05). In multivariate analysis, treatment with VD supplement was found significantly (*P* < 0.05) associated with survival of the patients over 6-mo. However, adjusted hazard ratio was < 1 (0.48), implying the significance of this result is low.

Table 3 Comparison of Child Turcott Pugh/Model For End-Stage Liver Disease score and survival (treatment *vs* control group)

Parameters	Treatment group (n = 51)	Control group (n = 50)	P value
CTP score, mean (95%CI)			
At base line	11.17 (10.83-11.52)	10.92 (10.64-11.20)	> 0.05
6 th month	6.09 (4.85-7.33)	5.92 (4.63-7.20)	> 0.05
MELD score, mean (95%CI)			
At base line	18.62 (17.70-19.54)	19.02 (17.91-20.12)	> 0.05
6 th month	9.03 (7.09-10.97)	8.82 (6.81-10.82)	> 0.05
Survival at 6-mo			
Survival	35/51 (69%)	32/50 (64%)	> 0.05
Mean (CI) survival (d)	155 (142-167)	141 (125-157)	> 0.05

CTP: Child Turcott Pugh; MELD: Model for end-stage liver disease.

Table 4 Factors associated with survival

Methods, variables [mean (CI)]	Univariate analysis		Multivariate analysis	
	Crude HR (95%CI)	P value	Adjusted HR (95%CI)	P value
Hepatic encephalopathy	1.53 (1.15-2.01)	< 0.05		
Bilirubin	1.04 (1.01-1.08)	< 0.05	0.90 (0.83-0.98)	< 0.05
AST	1.00 (0.99-1.00)	> 0.05		
Creatinine	228 (21-2445)	< 0.05	57.43 (2.16-1522)	< 0.05
PT-INR	6.63 (2.52-17.5)	< 0.05		
Ascitic fluid TLC	1.004 (1.002-1.006)	< 0.05	1.02 (1.00-1.04)	< 0.05
Blood TLC	1.00 (0.99-1.00)	> 0.05		
Serum Sodium	0.92 (0.87-0.97)	< 0.05		
Treatment with VD	0.92 (0.81-1.03)	> 0.05	0.46 (0.22-0.95)	< 0.05
CTP score	2.21 (1.69-2.89)	< 0.05	1.39 (1.00-1.95)	< 0.05
MELD score	1.34 (1.23-1.47)	< 0.05	1.60 (1.26-2.02)	< 0.05

PT: Prothrombin time; AST: Aspartate aminotransferase; VD: Vitamin D; CTP: Child Turcott Pugh; MELD: Model for end-stage liver disease.

Factors like treatment was also included into the model because we have observed that the levels of VD, calcium, and phosphorus were better in treatment group compared to control group.

DISCUSSION

We enrolled 101 patients in study; 51 and 50 patients in treatment and control group, respectively. Majority of patients were male in both treatment group and control groups. Mean age of presentation in both treatment group and control group was fourth decade. Ethanol was most common etiology of CLD followed by HBV infection. Alcohol is most common cause of CLD in many parts of India^[17]. In our study cohort, all patients had advanced decompensated cirrhosis (CTP-C) with CTP score of ≥ 10 . Almost all patients (100%) had ascites and 80% had jaundice. However, most of the previous studies have included patients of all three CTP classes^[10,18] (Table 5).

Prevalence of VD deficiency is very high among patients with CLD. Most of the studies have showed VD deficiency in more than two-third patients with CLD (Tables 5 and 6). In a study by Arteh *et al*^[19], VD deficiency was seen in more than 90% of patients with CLD, and at least one-third of them were suffering from severe VD deficiency. In another study, VD deficiency

or insufficiency was found in 87% of the patients with CLD. VD levels were significantly lower in patients with CLD (15.9 ng/mL) and in alcoholic liver disease^[13]. VD deficiency is common among patients with alcoholic liver disease^[8], hepatitis C^[20,21] and chronic hepatitis B (CHB) patients^[22]. In our patient cohort of decompensated CLD, 84.31% patients had deficiency of serum VD.

Studies have showed association of VD deficiency with the degree of liver dysfunction. Because of the association of VD deficiency with hepatic insufficiency and infections, authors have suggested the use of VD as a prognostic marker in the liver cirrhosis^[10,18]. In a study, VD deficiency in patients of CLD was proportional to the severity of liver dysfunction^[13]. Putz-Bankuti *et al*^[9] measured VD level of 75 patients of CLD and patients were followed for median duration of 3.6 years. The study showed a statistically significant inverse correlations of 25(OH) D levels with the degree of liver function (MELD score and CTP score). In a prospective cohort study ($n = 251$) done by Finkelmeier *et al*^[23], the mean serum concentration of VD was 8.93 ± 7.1 ng/mL, and 25(OH)D3 levels showed a inverse correlation with the MELD score.

Bacterial infections are the common cause of morbidity and mortality in patients with cirrhosis^[24]. VD deficiency is associated with bacterial infections in cirrhotic patients. In a non-randomized study done

Table 5 Vitamin D deficiency in chronic liver disease

Ref.	Disease (n)	Prevalence of VDD	Findings/conclusions
Ko <i>et al</i> ^[27] , 2016	Compensated CLD (n = 207)	80% overall; 35% < 10 ng/mL; 45% < 20 ng/mL	VDD (< 10 ng/mL) in advanced <i>vs</i> no advanced fibrosis: 53% <i>vs</i> 24% (<i>P</i> < 0.05)
Gevora <i>et al</i> ^[28] , 2014	HCV related CLD (n = 296)	82% < 80 nmol/L; 16% < 25 nmol/L	The inverse relationship noted between VD levels and viral load, liver fibrosis and treatment outcomes
Trépo <i>et al</i> ^[8] , 2013	ALD (n = 324)	59% < 10 ng/mL	VDD are significantly associated with increased liver damage and mortality
Kitson <i>et al</i> ^[21] , 2013	HCV-1 related CLD (n = 274)	48% < 75 nmol/L; 16% < 50 nmol/L	VD level is not associated with SVR or fibrosis stage, but VDD is associated with high activity grade
Arteh <i>et al</i> ^[19] , 2010	CLD (n = 113)	92% < 32 ng/mL	VDD in cirrhotics <i>vs</i> noncirrhotics: 30% <i>vs</i> 14% (<i>P</i> = 0.05)
Costa Silva <i>et al</i> ^[29] , 2015	Cirrhosis (n = 133)	70% < 30 ng/mL; 14% < 20 ng/mL	Significantly lower levels of VD were found at the time of acute decompensation
Savic <i>et al</i> ^[30] , 2014	ALD (n = 30)	67% < 50 nmol/L	Highest prevalence of VDD were seen in CTP-C patients (<i>P</i> < 0.05)
Corey <i>et al</i> ^[31] , 2014	ESLD (n = 158)	67% < 25 ng/mL	VDD is common among patients with ESLD awaiting LT
Putz-Bankuti <i>et al</i> ^[9] , 2012	Cirrhosis (n = 75)	53% < 20 ng/mL	VD levels are inversely correlated with MELD and CTP scores (<i>P</i> < 0.05)
Malham <i>et al</i> ^[32] , 2011	Alcoholic cirrhosis (n = 89)	85% < 50 nmol/L 55% < 25 nmol/L	VDD in cirrhosis relates to liver dysfunction rather than aetiology
Trépo <i>et al</i> ^[8] , 2015	Cirrhosis (n = 251)	92% Overall; 69% < 10 ng/mL; 24% < 20 ng/mL	VDD in decompensated cirrhosis are associated with infectious complications and mortality
Anty <i>et al</i> ^[10] , 2014	Cirrhosis (n = 88)	57% < 10 ng/mL	Severe VDD is a predictor of infection [OR = 5.44 (1.35-21.97), <i>P</i> < 0.05]
Stokes <i>et al</i> ^[18] , 2014	Cirrhosis (n = 65)	86% < 20 ng/mL	VD levels is an independent predictors of survival [OR = 6.3 (1.2-31.2); <i>P</i> < 0.05]
Fernández Fernández <i>et al</i> ^[13] , 2016	CLD (n = 94)	87% < 30 ng/mL or < 20 ng/mL	VD supplementation significantly improves CTP score
Zhang <i>et al</i> ^[14] , 2016	Cirrhosis with SBP (n = 119)	100%	VD supplementation can up-regulate peritoneal macrophage VDR and LL-37 expressions and enhance defence against SBP
Rode <i>et al</i> ^[26] , 2010	CLD (n = 158)	64% 25-54 nmol/L; 14% < 25 nmol/L	VDD improves with oral VD supplementation and VD levels fall without supplementation
Present study	Decompensated cirrhosis (n = 101)	84% < 20 ng/mL	VD levels improved with VD supplementation. VD supplementation may increase the survival probability of patients of decompensated cirrhosis

To convert results into SI units: ng/mL × 2.5 = nmol/L. VD: Vitamin D; VDD: Vitamin D deficiency; SBP: Spontaneous bacterial peritonitis; LT: Liver transplant; ALD: Alcoholic liver disease; ESLD: End stage liver disease.

by Anty *et al*^[10], severe VD (<10 ng/mL) deficiency was seen in 56.8% of cirrhotic patients. As compared with the others, the severe VD deficient patients had significantly more infection rate (54% *vs* 29%, *P* < 0.05). A severe VD deficiency was a predictor of infection [OR = 5.44 (1.35-21.97); *P* < 0.05] independently of the CTP score [OR = 2.09 (1.47-2.97); *P* < 0.05]. VD exerts its antimicrobial effect through VD receptor (VDR), and LL-37, a VD-dependent antimicrobial peptide. In a study by Zhang *et al*^[25] the ascites with spontaneous bacterial peritonitis (SBP) group showed significantly higher levels of both VDR and LL-37 mRNA expressions in peritoneal leukocytes than the ascites without SBP group (*P* < 0.05). Vitamin supplementations in patients of VD deficient patients have showed up-regulation of VDR and LL-37 in patients with SBP^[14].

The association between VD deficiency and increased mortality is still controversial. Low 25-OH VD level has been reported to be associated with increased mortality in patients with CLD^[8]. Studies have showed increased risk of complication and mortality in VD deficient patients with CLD. In a prospective study (*n* =

251), Finkelmeier *et al*^[23] has showed significantly lower 25(OH) D3 levels in the patients with decompensated cirrhosis and infectious complications compared to patients without complications. Low 25(OH) D3 was associated with mortality in univariate and multivariate Cox regression models. Stokes *et al*^[18] identified low VD levels and MELD scores as independent predictors of survival (*P* < 0.05) in patients with advanced liver cirrhosis. VD level of 6.0 ng/mL was determined as optimal cut-off for discriminating survivors from nonsurvivors. In a study, age- and gender-adjusted relative risk (95%CI) was 6.37 (1.75-23.2) for hepatic decompensation and 4.31 (1.38-13.5) for mortality within the first *vs* the third 25(OH) D tertile (*P* < 0.05). However, after additional adjustment for CTP or MELD score, associations between VD levels and hepatic decompensation and mortality showed a non-significant trend^[9]. In our study cohort, VD level had no significant association with mortality. In this study, treatment with VD supplement was found significantly (*P* < 0.05) associated with survival of the patients over 6-mo. However, adjusted hazard ratio was < 1, implying the significance of this result is low.

Table 6 Vitamin D deficiency and liver disease

Ref.	Disease (n)	Prevalence of VDD	Findings/conclusions
Wong <i>et al</i> ^[22] , 2015	CHB (n = 426)	82% < 32 ng/mL	VDD is associated with adverse clinical outcomes
Bril <i>et al</i> ^[33] , 2015	NASH (n = 239)	31% < 30 ng/mL; 47% < 20 ng/mL	VD level is not associated the severity of NASH
Finkelmeier <i>et al</i> ^[34] , 2014	HCC (n = 200)	38% < 20 ng/mL; 35% < 10 ng/mL	VD levels negatively correlated with the stage of cirrhosis as well as with stages of HCC
Guzmán-Fulgencio <i>et al</i> ^[35] , 2014	HIV-HCV coinfection (n = 174)	16% < 25 nmol/L	VDD is associated with severity of liver disease F ≥ 2 [OR = 8.47 (1.88-38.3); P < 0.05] and A ≥ 2 [OR = 3.25 (1.06-10.1); P < 0.05]
Avihingsanon ^[36] , 2014	HCV (n = 331) HCV-HIV coinfection (n = 130)	< 30 ng/mL	Hypovitaminosis D is a predictor of advanced fibrosis [OR = 2.48 (1.09-5.67); P < 0.05]
El-Maouche <i>et al</i> ^[37] , 2013	HCV-HIV coinfection (n = 116)	41% < 15 ng/mL	VDD is not associated with significant liver fibrosis (METAVIR ≥ 2)
Terrier <i>et al</i> ^[38] , 2011	HIV-HCV coinfection (n = 189)	85% ≤ 30 ng/mL	Low VD level correlate with severe liver fibrosis
Petta <i>et al</i> ^[20] , 2010	HCV-1 (n = 197)	73% ≤ 30 ng/mL	Low VD is linked to severe fibrosis and low SVR on interferon-based therapy
Fisher <i>et al</i> ^[39] , 2007	Noncholestatic CLD (n = 100)	68% < 50 nmol/L, 23% 50-80 nmol/L	VDD is common in noncholestatic CLD

To convert results into SI units: ng/mL × 2.5 = nmol/L. VD: Vitamin D; VDD: Vitamin D deficiency; CHB: Chronic hepatitis B; NASH: Non-alcoholic steatohepatitis; NAFLD: Non-alcoholic fatty liver disease; HCC: Hepatocellular carcinoma.

Current clinical guidelines address the issue of VD supplementation for bone disease in liver cirrhosis and cholestatic disorders^[16]. Role of VD supplementation to prolong survival in VD deficient liver cirrhotic patient is not known. A few studies have showed that VD supplementation may prolong life span in older people^[11,12]. Authors have showed oral VD supplementation replenishes VD levels^[26], improves CTP score^[13], and enhance defence against SBP^[14]. In a study by Fernández Fernández *et al*^[13], after VD supplementation, significant improvements were observed in functional status assessed by the MELD and CTP score ($P < 0.05$). In our study cohort, over the period of time VD level was improved in treatment group compared to control group. We observed a decline in CTP and MELD score in both the groups over the period of 6-mo time, which can be explained by supportive therapy. However, we did not observed a greater decline in CTP score and MELD score in the treatment group compared to control group. Results of our study showed statistically non-significant trend in greater survival (69% vs 64%; $P > 0.05$) and longer survival (155 d vs 141 d; $P > 0.05$) in treatment group compared to control group. We did not find a study suggesting increase in survival of patients of VD deficient decompensated CLD after therapy with VD.

We also reviewed the data on the prevalence and the role of VDD in patients with liver disease. A concise literature review of VDD in patients with noncholestatic liver disease is summarized in Tables 5 and 6^[27-39].

The dose of VD and mode of administration in VD deficient/insufficient patient of CLD is not clear. Lim *et al*^[40] suggested periodic monitoring of VD in patients with CLD. Therapy is required in those with VD levels < 30 ng/mL, which includes administration of 5000 IU of vitamin D3 daily or 50000 IU of vitamin D2 or D3 weekly for 3 mo, followed by 1000 IU/d indefinitely. In a systemic review authors have recommended that

vitamin D3 be used for supplementation over vitamin D2. A single VD doses ≥ 300000 IU are most effective at increasing VD levels^[41]. Although both oral and intramuscular administration routes are effective and safe, intramuscular administration is more effective in increasing VD levels^[42,43]. In our study, we used intramuscular cholecalciferol 300000 IU as loading dose and 800 IU/d oral as maintenance dose along with 1000 mg oral calcium supplementation.

There are a few limitations of our study, each of which are the relatively small sample size, single center study, and lack of an age and sex matched VD deficient control population without cirrhosis. We did not study the relationship of parathyroid hormone level on serum calcium and phosphorous levels. Impact of VD supplementation in various etiologies of CLD was also not assessed because of small number of patients in each sub-group. Therefore, the findings of this study need to be confirmed in larger multicenter studies.

In conclusion, VD deficiency is very common in patients of decompensated CLD. Replenishment of VD along with calcium supplementation may improve survival in patients with decompensated liver cirrhosis.

COMMENTS

Background

Function of vitamin D (VD) is not limited to bone mineral metabolism. The role of VD has also been implicated in the pathogenesis and treatment of infections, autoimmune, cardiovascular, and degenerative diseases and several types of cancer. Liver plays important role in conversion of inactive VD into active 25-hydroxy VD (25-OH VD). Vitamin D deficiency (VDD) is very common in patients of chronic liver disease (CLD). The role of VDD in the natural history of non-cholestatic CLD is not well described. Recently, VD deficiency is found to be associated with complications of CLD.

Research frontiers

The data regarding the association of VDD with complications and prognosis in patients with CLD are limited. The available literature suggests the association

of VD deficiency with degree of liver fibrosis and functional liver dysfunction. There is increasing focus to find the relationship between the VD level and prognosis in patients with CLD. There are few studies suggesting the role of oral VD supplementation in replenishing VD levels, improving CTP score, and enhancing defence against spontaneous bacterial peritonitis in patients with CLD. More studies are necessary to establish the prognostic role of VDD in decompensated CLD. Current clinical guidelines address the issue of VD supplementation for bone disease in liver cirrhosis and cholestatic disorders. Effect of VD replenishment in increasing survival of patients with VD deficient decompensated cirrhosis has to be elucidated.

Innovations and breakthroughs

The current trial was designed to assess the VDD as a prognostic factor for survival in patients with decompensated CLD and effect of replenishment of VD on all-cause mortality in decompensated liver cirrhosis. Authors also performed concise review of role of VDD in patients with noncholestatic liver disease. In this study, there is suggestion that replenishment of VD may improve survival in patients with VD deficient decompensated cirrhosis compared to similarly-treated controls.

Applications

The results of the study add important scientific information on prognostic role of VD deficiency in patients with decompensated cirrhosis. This study provides evidence supporting the investigation of VD supplementation in improving prognosis in VD deficient patients with decompensated cirrhosis.

Terminology

Vitamin D deficiency: Patient with serum vitamin D level < 20 ng/mL is defined as vitamin D deficient.

Peer-review

The manuscript is well written and covers an area of current clinical interest. The data is presented and described well and comes from a reasonably sized cohort of patients.

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P- Reviewer: Lalor P, Sirin G S- Editor: Kong JX L- Editor: A
E- Editor: Lu YJ



Multiple endocrine neoplasia 2B: Differential increase in enteric nerve subgroups in muscle and mucosa

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Institutional review board statement: The study was performed under ethics approval to do further research on samples collected for diagnostic pathology. Royal Children's Hospital Ethics Committee approval number 23081B and 24105.

Informed consent statement: The patient and his family gave written consent for the surgery where biopsies were collected for pathological assessment and verbal consent for the samples to be used in this study.

Conflict-of-interest statement: None of the authors has a conflict of interest as the surgery was performed for clinical indications and with informed consent and the histologic analysis was done with commercial reagents.

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Manuscript source: Unsolicited manuscript

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Received: November 17, 2016
Peer-review started: November 22, 2016
First decision: February 30, 2017
Revised: May 24, 2017
Accepted: July 14, 2017
Article in press: July 17, 2017
Published online: August 15, 2017

Abstract

Multiple endocrine neoplasia 2B (MEN2B) is a rare syndrome caused by an activating mutation of the *RET* gene, leading to enteric gangliomatosis. This child presented with constipation at 1-mo old, was diagnosed with MEN2B by rectal biopsy at 4 mo, had thyroidectomy at 9 mo and a colectomy at 4 years. We studied the extent of neuronal and nerve fibre proliferation and which classes of enteric nerves are affected by examining the colon with multiple neuronal antibodies. Resected transverse colon was fixed, frozen, sectioned and processed for fluorescence immunohistochemistry labelling with antibodies against TUJ1, Hu, ChAT, NOS, VIP, SP and CGRP and cKit. Control transverse colon was from the normal margin of Hirschsprung (HSCR) colon (4-year-old) and a child with familial adenomatous polyposis (FAP, 12 year). Myenteric ganglia were increased in size to as wide as the circular muscle. There was a large increase in nerve cells and nerve fibres. ChAT-, NOS-, VIP- and SP-immunoreactive nerve fibres all increased in the myenteric ganglia. NOS-IR nerves preferentially increased in the muscle, while VIP and SP increased in submucosal ganglia and mucosal nerve fibres. The density of ICC was normal. RET overactivation in MEN2B lead to a large increase in intrinsic nerve fibres in the myenteric and submucosal ganglia, with a relative increase in NOS-IR nerve fibres in the circular muscle and VIP and SP in the submucosal

ganglia and mucosa. The changes were associated with severe constipation resulting in colectomy at 4 years.

Key words: Enteric nervous system; *RET*; Neuroganglioma; MEN2B

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Core tip: Multiple endocrine neoplasia (MEN) 2B is a rare anomaly caused by an activating mutation of the *RET* known to produce enteric gangliomatosis. Thyroidectomy is performed to avoid cancer and the colon removed to overcome the constipation. This child presented with constipation very early (1-mo-old) and had subtotal colectomy at 4 years. The classes of nerves affected in MEN2B colon have not been studied before. We used immunohistochemistry with multiple antibodies. There was a massive increase in intrinsic nerve fibres in myenteric and submucosal ganglia, with a differential increase in types of nerve fibres in the muscle and mucosa.

Hutson JM, Farmer PJ, Peck CJ, Chow CW, Southwell BR. Multiple endocrine neoplasia 2B: Differential increase in enteric nerve subgroups in muscle and mucosa. *World J Gastrointest Pathophysiol* 2017; 8(3): 142-149. Available from: URL: <http://www.wjgnet.com/2150-5330/full/v8/i3/142.htm> DOI: <http://dx.doi.org/10.4291/wjgp.v8.i3.142>

INTRODUCTION

Multiple endocrine neoplasia (MEN) 2B is a rare autosomal dominant anomaly characterised by marfanoid appearance, ganglioneuromatosis and gastrointestinal disorders^[1-4]. There is a very high risk of medullary carcinoma of the thyroid (MTC) which increases with age, and a moderate risk of developing pheochromocytoma^[3]. MTC develops in infancy and originally determined the prognosis for MEN2B patients. In the last 15 years, it has been recognised that MTC is curable by surgical removal of the thyroid under 1-year-old^[4].

The genetic anomaly in *MEN2B* is an activating mutation of *RET*^[5-7], mostly M918T^[5,8]. The *RET* gene codes for a membrane-bound tyrosine kinase receptor that is activated by glial-derived neurotrophic factor (GDNF), that regulates growth and development of the peripheral, autonomic and enteric nervous systems^[9-11]. In *MEN2B*, activation of *RET* results in hyperganglionosis in the nervous system including the enteric nervous system^[1,12,13].

Constipation is present in many *MEN2B* patients^[1,2,7,12,14-16], and they may present in the neonatal period with symptoms suspiciously like Hirschsprung disease (HSCR)^[2,16-20]. Indeed, *MEN2B* can be diagnosed in the neonatal period, by the presence of neurons among the enlarged nerves in submucosal ganglia in standard rectal biopsies^[16,18,21]. Intestinal ganglioneuromatosis develops in an age-dependent

fashion and not all develop severe constipation necessitating surgery. Removal of the thyroid does not alter the effect that *RET* activation has on the nervous system. We could not find any reports of the changes in the enteric nervous system post thyroidectomy. Also, despite numerous reports of hyperganglionosis, there are only a few histological images of the *MEN2B* intestine^[12,15,16,19]. Most of the images are haematoxylin and eosin and while they clearly show enlarged ganglia, they do not reveal the nerve fibres or how neuronal subtypes are affected in *MEN2B*. Here we examined the enteric nervous system in bowel from a child with severe constipation from birth, who had a colectomy at 4 years.

Choline acetyl transferase (ChAT) and nitric oxide synthase (NOS) are present in 95% of myenteric neurons (ChAT⁺/NOS⁻ 48%, ChAT⁺/NOS⁺ 43%, ChAT⁺/NOS⁺ 4%, ChAT⁻/NOS⁻ 5%)^[22,23]. Vasoactive intestinal peptide (VIP) is present in inhibitory motor and secretomotor neurons and substance P (SP) is in sensory, excitatory motor neurons and secretomotor neurons in the myenteric and submucosal ganglia^[23,24]. In this study we examined enteric nerves in samples taken from a child 3.5 years after thyroidectomy. We labelled full thickness bowel with antibodies against all these different molecules to determine if all of the neuron types were increased. We compared the labelling in the *MEN2B* case to 2 controls: Bowel from a patient with familial adenomatous polyposis (FAP) and the normal margin of bowel in a patient operated for HSCR. To determine if changes were specific to nerves, we also labelled the interstitial cells of Cajal (ICC), cells that have a different developmental origin to neurons (they arise from the mesenchyme)^[25].

CASE REPORT

This child (now 14 years) presented with chronic constipation at 1 mo (in 2003) and was investigated for HSCR with a rectal biopsy at 4 mo. A preliminary diagnosis of *MEN2B* was made from the mucosal biopsy stained for acetylcholinesterase and showing thickened nerve fibres with nerve cells present in the submucosal ganglia and we reported on this analysis in 2006^[18]. Genetic analysis confirmed the *RET* M918T mutation and diagnosis of *MEN2B* and thyroidectomy was performed at 9 mo. At 4-year-old, the boy underwent subtotal colectomy for management of severe constipation. Terminal ileum and transverse colon were fixed and frozen. Blocks were sectioned (5 µm) and sections were processed for fluorescence immunohistochemistry^[26,27] using a bank of antibodies to label nerve cells and fibres, different subtypes of neurons and ICC. Antibodies were chosen to label all nerve fibres (Tuj1), nerve cell bodies (Hu), the major excitatory (ChAT and SP) and inhibitory (NOS and VIP) neurons and nerve fibres and extrinsic sensory nerve fibres (CGRP) in the myenteric plexus and muscle layers, and the major secretomotor nerves (SP and VIP)

Table 1 Antibodies used in the study

Molecule	Company	Dilution
Primary antibody		
Goat anti c ChAT	AB5966 Chemicon International, Temecula, CA, United States	1:100
Rabbit anti nNOS	AB 5380, Chemicon International, Temecula, CA, United States	1:1000
Rabbit anti VIP	NCL VIPp, Novocastra Laboratories, Newcastle-upon-Tyne, United Kingdom	1:200
Rabbit anti SP	18-0091, Zymed Laboratories, South San Francisco, CA, United States	1:50
Goat anti CGRP	1720-9-7, AbD Serotec, Biorad	1:1000
Rabbit anti-human cKit	Polyclonal CD117, Dako	1:200
Secondary antibody		
Goat anti rabbit Alexa 488	Molecular Probes, ThermoFisher Scientific A-11012	1:400
Donkey anti rabbit Alexa 488	Molecular Probes, ThermoFisher Scientific, A-21206	1:200
Goat anti mouse Alexa 488	Molecular Probes, ThermoFisher Scientific, A11001	1:400
Donkey anti goat Alexa 568	Molecular Probes, ThermoFisher Scientific, A11057	1:400

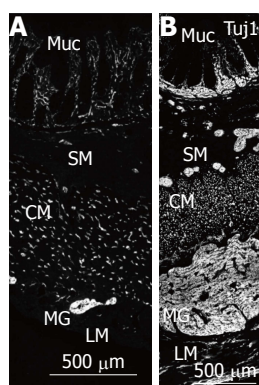


Figure 1 Nerve fibres in normal colon and MEN2B colon. Tuj1 immunoreactivity showing nerve fibres in full thickness biopsy of the transverse colon cut with circular muscle in cross section. A: Control patient (4-year-old, HSCR normal margin) showing multiple nerve fibres in the mucosa (Muc) within and at the base of the crypts, small ganglia in the submucosa (SM), nerve fibres in cross section in the circular muscle (CM) and myenteric ganglia (MG) between the CM and longitudinal muscle layer (LM); B: MEN2B patient showing greatly increased amount of labelling and increase in size of MG and SM ganglia. The MG is increased in diameter to a thickness similar to the circular muscle. The density and number of nerve fibres in the CM is higher than control. The nerve fibres in the Muc are greatly increased. The SM contains many large ganglia near the CM, near the Muc and in between. Neurons are not distinguishable because this antibody only displays nerve fibres. Note A and B are at the same magnification. CM: Circular muscle; LM: Longitudinal muscle; MG: Myenteric ganglia.

in the muscosa.

Cryostat sections (10 μ m thick) were air-dried at 4 $^{\circ}$ C overnight and then incubated overnight at room temperature with primary antibody (Table 1) followed by 2 h in secondary antibody (Table 1), rinsed and mounted in (Mowiol 4-88 (Hoechst/Aventis) in 25% glycerol/0.1 mol/L Tris HCl, with 2.5% DABCO (1,4-diazabicyclo-[2,2,2]-octane, Sigma). Negative controls excluded primary and secondary antibodies. Staining was covered by Royal Children's Hospital Ethics Committee approval 23081B and 24105.

Sections were imaged on a Leica CSLM confocal microscope using 488 nm excitation with variable filters. For each antibody, the entire thickness of the colon wall was imaged to locate nerve fibres in the longitudinal and circular muscle, myenteric and submucosal ganglia, submucosa and mucosa (Figure 1). Quantitation was

performed using NIH Image J. The thickness of the ganglia and muscle layers was measured in 24 sections from MEN2B patient and 15 sections from the controls. The density of nerve fibres labelled with each marker was measured in circular muscle cut in transverse section as previously described^[26,27] in 3 sections for each antibody.

Normal paediatric bowel is rarely collected, so we used full-thickness biopsies of the normal ganglionic region of colon from 1 child with HSCR (4 years), the same age as the MEN2B child, and sigmoid colon from 1 child (12 years) with familial adenomatous polyposis (FAP).

In the MEN2B colon, Tuj1-immunoreactivity (Tuj1-IR) showed overgrowth of enteric nerves in all layers (Figure 1). Myenteric ganglia were very large, with diameters equal to the thickness of the circular muscle layer (Figure 2A). The diameter of the myenteric ganglia was 5-fold more than control ($P < 0.0001$). The longitudinal muscle layer was significantly thicker than control ($P < 0.01$), while the circular muscle thickness was less than in controls ($P < 0.05$). Nerve fibres (Tuj1) in circular muscle were increased so they comprised 25% of the muscle area, compared with 10% in controls (Figure 2A). The submucosal ganglia were enlarged and there was a great increase in nerve fibres in the lamina propria (Figure 1B). The thickness of muscle layers and size of ganglia in the 2 "controls" were not statistically different, so we combined the results in the quantitation of different neuron types.

Hu-immunoreactivity (IR) shows neuronal cell bodies (Figure 3). In myenteric ganglia, there was an increase from 20 to 76 neurons per high power field. As the size of the ganglia increased, nerve fibres also increased greatly and the density of neurons (neurons/ mm^2 of ganglion area) decreased from 1.5/ mm^2 to 0.2/ mm^2 . In the submucosa ganglia, neurons increased from 2-3 to 5-10 per ganglia (per high power field) with a great increase in nerve fibres (Figure 3).

In control and MEN2B, NOS-IR was present in neurons in myenteric ganglia with nerve fibres abundant in the muscle (Figure 4), with no labelling in mucosa. NOS nerve fibre density increased 2 fold in the circular

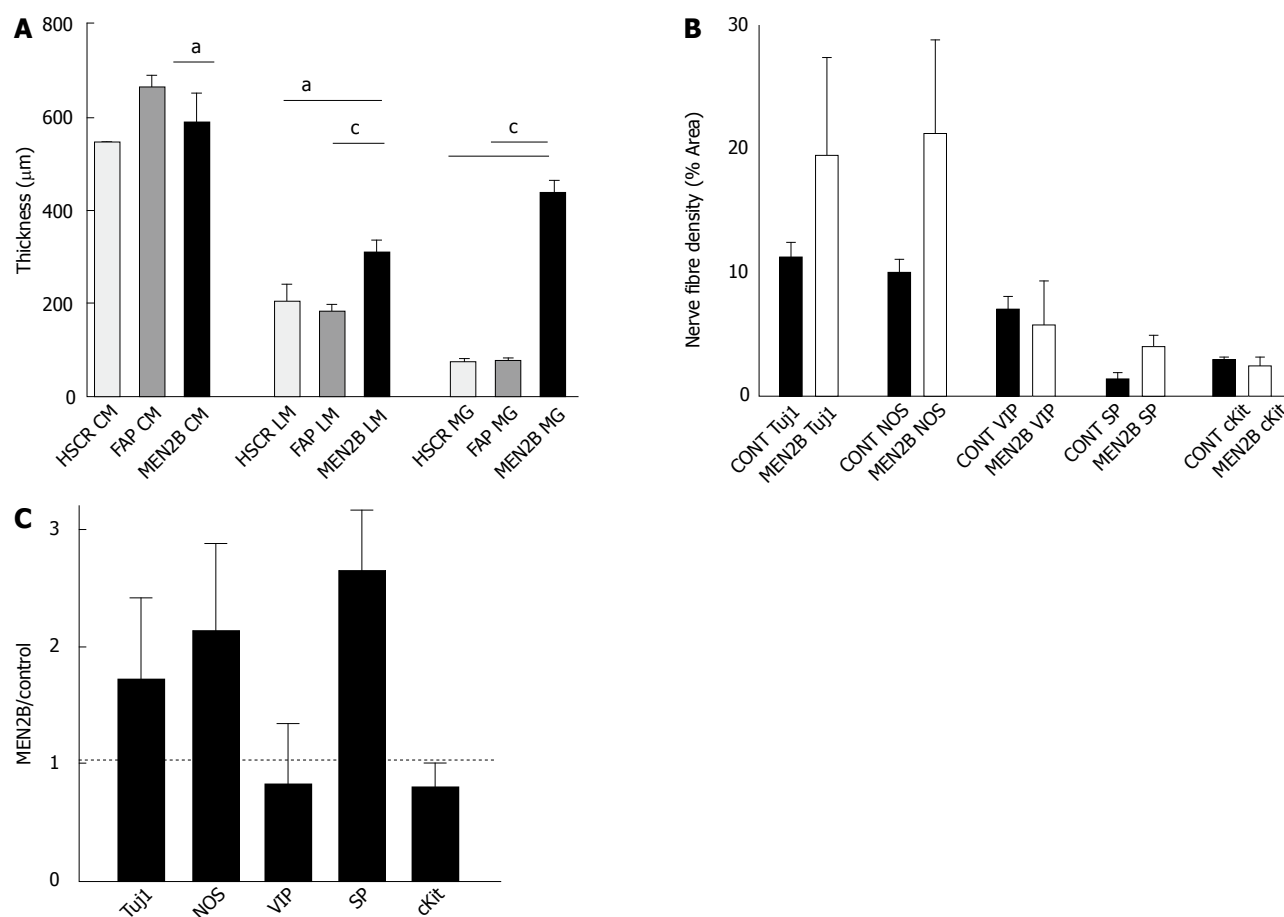


Figure 2 Thickness of muscle layers and ganglia and density of nerve fibres in normal colon and MEN2B colon. A: Comparison of thickness (mm) of muscle and myenteric ganglia in colon from Control (HSCR and FAP) and MEN2B patient. Graph shows mean and SEM. HSCR $n = 6$, FAP $n = 12$, MEN2B $n = 24$, $^aP < 0.05$, $^cP < 0.001$; B: Nerve fibre and ICC density in circular muscle in transverse colon from Control (HSCR and FAP combined, $n = 3$) and MEN2B patient ($n = 3$). Percent area of circular muscle containing immunoreactive pixels; C: Relative nerve fibre and ICC density in the circular muscle in the MEN2B patient ($n = 3$) relative to Control patients (HSCR and FAP combined, $n = 3$). Labelling with Tuj1, NOS, VIP, SP and cKit (for interstitial cells of Cajal).

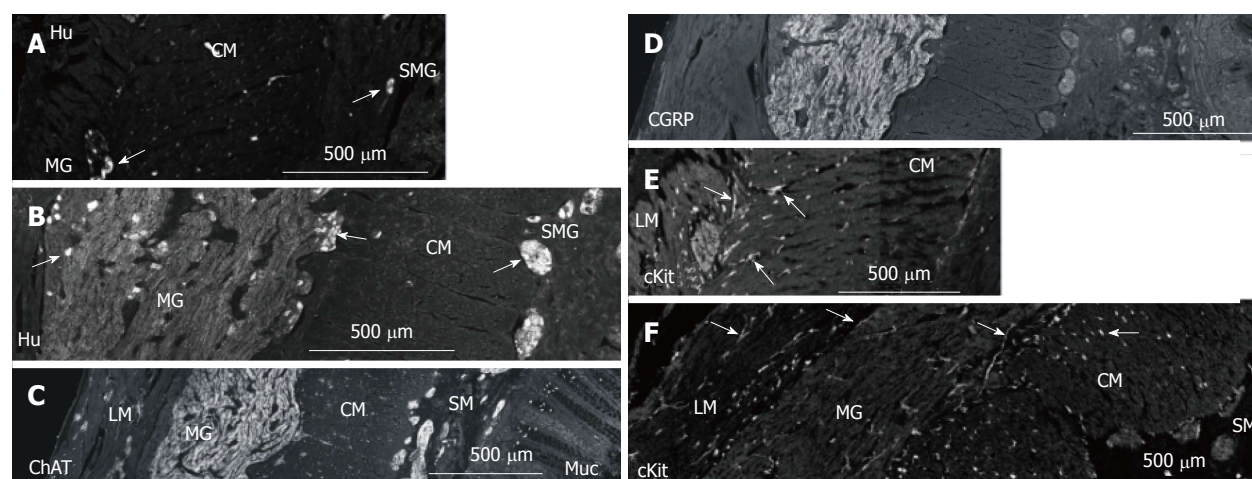


Figure 3 Nerve cells in myenteric and submucosal ganglia in normal colon and MEN2B colon. A: Hu immunoreactivity showing nerve cell bodies in transverse colon cut with circular muscle in cross section. Control patient (HSCR) showing myenteric ganglia (MG) neurons and submucosal ganglia; B: MEN2B patient at same magnification showing greatly enlarged MG containing 41 neurons and multiple large ganglia in the submucosa (SMG) containing 2-6 neurons. Note most of the increase in size of the MG is due to nerve fibres; C: Choline acetyltransferase (ChAT) immunoreactivity in transverse colon of MEN2B patient. Note many large ganglia in the submucosa (SM); D: Calcitonin gene-related peptide (CGRP) immunoreactivity in transverse colon of MEN2B patient. CGRP labels extrinsic sensory nerves. Note most of labelling is in the myenteric ganglia; E: ICC in the muscle in normal colon (HSCR); F: MEN2B colon. cKit-immunoreactivity shows ICC in the muscle and around the MG (arrows) and shows mast cells in the mucosa and submucosa. Mast cells are round cells with no process while ICC are elongate cells with processes. ICC numbers are similar in control and MEN2B. The longitudinal muscle (LM), myenteric ganglia (MG), circular muscle (CM), submucosa (SM), submucosal ganglia (SMG) and mucosa (Muc) are shown from left to right.

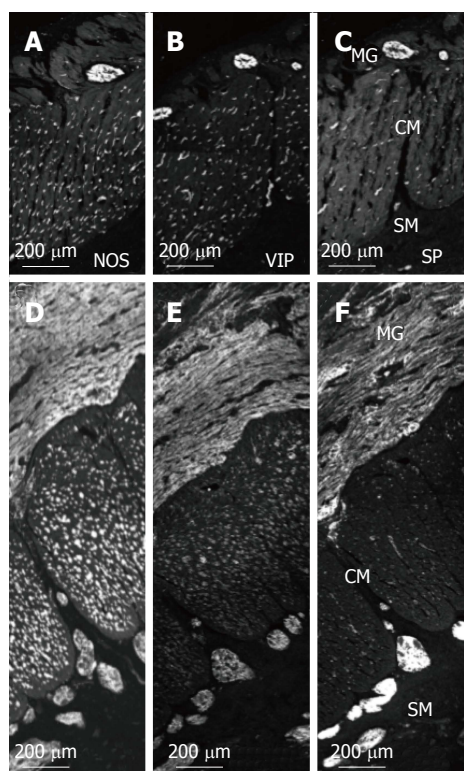


Figure 4 NOS, VIP and SP nerve fibres in myenteric and submucosal ganglia and circular muscle in normal colon and MEN2B colon. A: NOS-; B: VIP-; C: SP-immunoreactivity in Control (HSCR) patient; D: NOS-; E: VIP-; F: SP-immunoreactivity in MEN2B patient. Note all images are at the same magnification. The enlarged myenteric ganglia (MG) contain many NOS, VIP and SP nerve fibres with NOS at the highest density. The submucosal (SM) ganglia also contain all 3 labels, but SP is most intense and extremely bright in MEN2B. In the circular muscle (CM), NOS is the most abundant and highly increased in MEN2B. Full thickness biopsy of the transverse colon cut with circular muscle in cross section.

muscle (Figure 2). VIP-IR nerve fibres increased in the ganglia (Figure 4E), and mucosa (Figure 5) but proportionally did not change in density in the muscle (Figure 2). The increased density of NOS fibres in MEN2B accounted for the significant increase in density of all nerve fibres (Figure 2B).

In the MEN2B bowel, SP-IR showed increased nerve fibres in myenteric and submucosal ganglia (Figure 4) and in the mucosa (Figure 5). The relative amount of SP-IR fibres supplying the circular muscle was greater than in normal colon (Figure 2C).

ChAT-IR was high in nerve fibres in the enlarged myenteric and submucosal ganglia with relatively little labelling in the circular muscle or mucosa (Figure 3C). CGRP-IR was also high in the myenteric ganglia but low in the muscle, submucosa and mucosa (Figure 3D).

c-Kit-IR labelled a relatively normal number of ICC in the muscle layers and mast cells in the lamina propria (Figure 3E and F).

At follow-up at 14 years of age, this child is well. Early recognition and treatment (thyroidectomy and colectomy) have enabled normal growth. In addition to colectomy, he has had neuromas removed from his tongue and has nerves growing across his cornea that

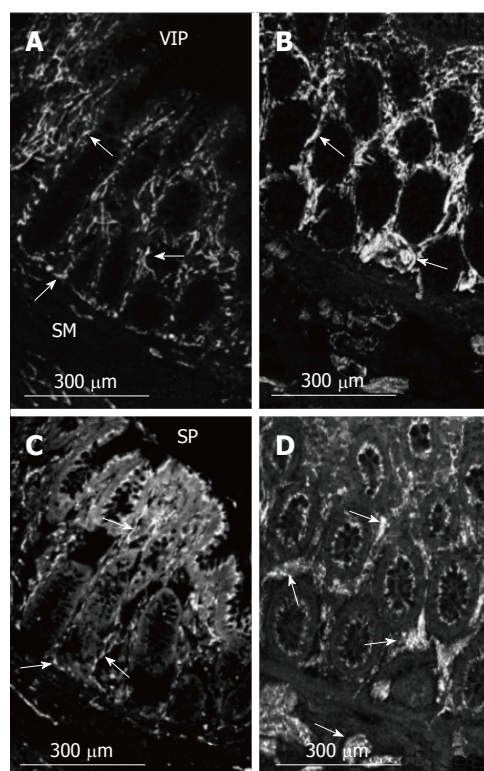


Figure 5 VIP and SP nerve fibres in the mucosa in normal colon and MEN2B colon. VIP-immunoreactivity in (A) Control (HSCR) and (B) MEN2B patient. Crypts in transverse colon. VIP immunoreactive nerve fibres (arrows) are plentiful at the base and within the arms of the crypts in the control. There is a large increase in the numbers of VIP-IR nerve fibres and brightness of labelling in the crypts. SP-immunoreactivity in (C) Control (HSCR) and (D) MEN2B patient. Background labelling is higher with SP with labelling of epithelial cells. Varicose nerve fibres are visible (arrows) in a similar pattern to VIP. The thickness and brightness of the nerve fibres is greatly increased within the crypts and in the submucosa in MEN2B. Note all images are at the same magnification.

are monitored 6 monthly.

DISCUSSION

We established the normal ganglion size and area of nerve fibres in the circular muscle in 2 children (aged 4 and 12 years), then compared the area of ganglia and nerve fibres in a 4-year-old child who presented as an infant with constipation and genetic confirmation of MEN2B. We used a range of antibodies to identify nerve cells, fibres, nerve subgroups, and ICC, and found that in MEN2B ganglioneuromatosis, the overgrowth in myenteric ganglia was both nerve cells and fibres with most subpopulations of enteric nerves involved. There was an increase in nerve cell bodies in the submucosal ganglia, with large bundles of nerve fibres in the submucosa reminiscent of HSCR.

In the aganglionic region of Hirschsprung colon, extrinsic nerve fibres form thick cables in the submucosa and myenteric layer. This is thought to be because the lack of nerve cells provides no sites for the extrinsic nerves to synapse onto. In MEN2B submucosa, the nerve fibres also form large cables but with nerve cells present in many ectopic ganglia^[18,20]. The presence

of neurons among the enlarged nerve fibres has been reported previously as the diagnostic criterion to distinguish MEN2B from Hirschsprung's disease^[18].

Myenteric interneurons and excitatory motor and sensory neurons that send processes to the muscle and secretomotor neurons in the submucosal ganglia that send processes to the mucosa are cholinergic^[22,23]. There was a high level of ChAT labelling in the myenteric ganglia but not in the muscle. The antibody against ChAT only labels proximal processes (*i.e.*, nerve fibres near the cell body)^[28] and so does not display the nerves in the muscle or mucosa. Identifying the cholinergic nerve fibres in the muscle and mucosa requires labelling with vesicular acetyl choline (VAcHT) or high affinity choline transporter (CHT)^[28] and was not done in this study. This should be further investigated in future studies.

Nerve fibres containing the inhibitory neurotransmitters, nitric oxide and VIP, were unevenly distributed, with NOS prominent in the muscle layers and VIP prominent in the lamina propria. NOS is present in inhibitory motor neurons in the ganglia, forming connections within the ganglia and sending process into the muscle layers. More NOS-synthesizing fibres in the myenteric layer would increase the inhibitory pathways within the ganglia and more in the muscle layer might inhibit contraction or lead to relaxation. NOS immunoreactive neurons are normally sparse in the submucosal ganglia and their fibres innervate the inner muscle layer. NOS-immunoreactive nerve fibres are not normally present in the muscosa.

SP levels were especially high in the submucosal ganglia and mucosa. Thus, RET overactivation increased all nerve fibres in the myenteric ganglia, NOS in the circular muscle, and VIP and SP in the submucosal ganglia and mucosa. Previous studies have shown histology of ganglia and staining with neuron specific enolase (NSE)^[12,15,19] but only one other study has reported labelling for specific neurotransmitters^[20]. Previously, in seromuscular biopsies from 2 other MEN2B patients, we noted a decrease in SP in the circular muscle. This may reflect differences between patients in types of nerves that increase^[20].

ICC arise from the mesoderm^[25]. In this patient there was normal density of ICC suggesting that they are not influenced by RET. Interestingly, ICC numbers were not decreased in the MEN2B patient despite severe constipation. Reduced numbers of ICC are often associated with bowel dysmotility and chronic constipation^[29,30].

Mouse models of the human *RET*^{MEN2B} gene have produced thyroid and sympathetic ganglioneuroma phenotypes but to date have not reproduced intestinal gangliomatosis^[31]. Thus it is difficult to investigate what the molecular mechanism of the *RET*^{MEN2B} affects in the enteric nervous system. We can speculate that the activating mutation in *RET*^{MEN2B} overrides an "OFF" switch that is normally induced by synapse formation and normally inhibits neuron proliferation and nerve

fibre formation. This results in overproduction of nerve fibres in all layers of the bowel.

Different types of neurons differentiate at different times and environmental cues change over time. Neurturin, a GDNF family member, may play a role in development of cholinergic nerves^[32]. There is particular interest in determining the factors that induce NOS expression in neuronal stem cells^[32], as optimal conditions for differentiation will be needed for potential neuronal transplantation to treat HSCR. MEN2B shows that too much RET activation is not advantageous.

In this patient, overactivation of RET lead to a large increase in neurons and nerve fibres in the myenteric and submucosal ganglia, muscle and mucosa. This patient had a relative increase in NOS-IR nerve fibres in the circular muscle and of VIP-IR and SP-IR nerve fibres in the submucosal ganglia and mucosa. The *RET* gene test was performed after finding the enlarged ganglia in the rectal biopsies at 4 mo old and was positive resulting in a pre-emptive thyroidectomy at 9 mo. The samples studied were collected at 4 years during a partial colectomy. This study indicates that the neuronal growth continues due to the RET activation, independent of thyroid presence. So although removing the thyroid does prevent the MTC^[4], the children require ongoing monitoring and maintenance and are not problem free. The child has 6-monthly screening for thyroid with annual ultrasound, annual review of corneal nerves and bowel function. He last had removal of tongue ganglia in 2015 and was seen 3 recently (May 2017) with no new nodules needing treatment. He is now 14 and doing well.

COMMENTS

Case characteristics

A 4-year-old boy with chronic intractable constipation since a few weeks after birth, diagnosed with multiple endocrine neoplasia 2B (MEN2B) via rectal biopsy to exclude Hirschsprung (HSCR) has partial colectomy.

Clinical diagnosis

MEN2B with proven *M918T-RET* mutation (activating).

Differential diagnosis

Hirschsprung's disease was main differential, investigated in rectal biopsy at 4 wk of age, showing enlarged nerve fibres and enlarged ganglia rather than lack of enteric neurons.

Laboratory diagnosis

Immunohistochemistry showed a large increase in intrinsic nerve fibres in both myenteric and submucosal ganglia, with a relative increase in NOS-IR nerve fibres in the circular muscle and VIP in the submucosal ganglia. GENE analysis identified *M918T-RET* mutation.

Pathological diagnosis

MEN2B caused by *RET* gene mutation.

Treatment

Thyroidectomy at 9 mo to prevent medullary thyroid carcinoma, partial colectomy at 4 years to overcome constipation, removal of gangliomas from the tongue. Continued monitoring of thyroid and corneal nerves.

Related reports

MEN2B is a rare syndrome caused by an activating mutation in the *RET* gene, which produces medullary thyroid cancer, adrenal pheochromocytoma enlarged nerve fibres and ganglia, including overgrowth of the enteric nerves.

Term explanation

Nitric oxide synthase (NOS) is present in inhibitory motor nerves and interneurons in the myenteric and submucosal ganglia; VIP (vasoactive intestinal peptide) is also present in secretomotor nerves in the submucosal ganglia and mucosa.

Experiences and lessons

Detailed immunohistochemistry has enabled us to understand the effects of an activating mutation of the *RET* gene in MEN2B, with the relative increase in inhibitory neurotransmitters (NOS and VIP) likely related to the clinical symptoms of intractable constipation.

Peer-review

Strengths: Endeavours to characterise morphological and immunohistochemical changes of MEN2B associated intestinal ganglioneuromatosis. Found that M918T-induced RET activation led to a large increase in intrinsic nerve fibres in myenteric and submucosal ganglia, specifically NOS and VIP. There is paucity of data on enteric neuronal content in MEN2B other than that ganglia are enlarged.

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World Journal of Gastrointestinal Pathophysiology is now indexed in PubMed, PubMed Central.

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NAME OF JOURNAL
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ISSN
ISSN 2150-5330 (online)

LAUNCH DATE
April 15, 2010

Frequency
Quarterly

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PUBLICATION DATE
November 15, 2017

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Paneth cells in intestinal physiology and pathophysiology

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Author contributions: Gassler N contributed to the study idea, literature research, manuscript writing, and final revision.

Conflict-of-interest statement: None.

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Manuscript source: Invited manuscript

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Received: January 24, 2017

Peer-review started: January 26, 2017

First decision: May 2, 2017

Revised: July 28, 2017

Accepted: August 15, 2017

Article in press: August 16, 2017

Published online: November 15, 2017

Abstract

Small intestinal mucosa is characterised by villus forming connective tissues with highly specialised surface lining epithelial cells essentially contributing to the establishment of the intestinal border. In order to perform these diverse functions, spatially distinct compartments of epithelial differentiation are found along the crypt-villus axis, including Paneth cells as a highly specialised cell type. Paneth cells locate in crypts

and assist undifferentiated columnar cells, called crypt base columnar cells, and rapidly amplifying cells in the regeneration of absorptive and secretory cell types. There is some evidence that Paneth cells are involved in the configuration and function of the stem cell zone as well as intestinal morphogenesis and crypt fission. However, the flow of Paneth cells to crypt bottoms requires strong Wnt signalling guided by EphB3 and partially antagonised by Notch. In addition, mature Paneth cells are essential for the production and secretion of antimicrobial peptides including α -defensins/cryptdins. These antimicrobials are physiologically involved in shaping the composition of the microbiome. The autophagy related 16-like 1 (ATG16L1) is a genetic risk factor and is involved in the exocytosis pathway of Paneth cells as well as a linker molecule to PPAR signalling and lipid metabolism. There is evidence that injuries of Paneth cells are involved in the etiopathogenesis of different intestinal diseases. The review provides an overview of the key points of Paneth cell activities in intestinal physiology and pathophysiology.

Key words: Antimicrobial peptide; Paneth cell; Crohn's disease; Microbiome; Small intestine

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Core tip: Paneth cells physiologically locate in small intestinal crypts. Wnt signalling promotes their differentiation and movement into crypts, whereas Notch activities antagonise Paneth cell maturation. The cells essentially contribute to crypt morphogenesis and intestinal homeostasis, sharpening the microbiome by secreting antimicrobial peptides, like defensins, and crypt fission. There is increasing molecular evidence that Paneth cells disorders are strongly involved in the pathophysiology of several intestinal diseases including ileal Crohn's disease and necrotising enterocolitis.

Gassler N. Paneth cells in intestinal physiology and pathophysiology. *World J Gastrointest Pathophysiol* 2017; 8(4): 150-160
Available from: URL: <http://www.wjgnet.com/2150-5330/full/v8/i4/150.htm> DOI: <http://dx.doi.org/10.4291/wjgp.v8.i4.150>

INTRODUCTION

The human small intestine is a multi-layered organ with complex cellularity. The intestinal mucosa is characterised by villus forming connective tissues with highly specialized surface lining epithelial cells. The epithelium is highly dynamic and interacts with the underlying mesenchyme and the luminal content. In order for these diverse functions to be performed, spatially distinct compartments of epithelial differentiation are found along the crypt-villus axis (CVA). Stem cells located in the crypts are responsible for the high throughput of surface lining cells within a few days. Histomorphologically, the CVA is a very characteristic mucosal feature and fundamental in explaining the structure and function of the surface lining epithelia. At present, four important cell types are defined, namely absorptive (enterocytes), mucosecreting (goblet cells), enteroendocrine, and Paneth cells^[1]. Some other epithelial and non-epithelial cell types are found at the mucosal surface, including M-cells, brush/tuft/caveolated cells, and several types of intraepithelial lymphocytes.

Enterocytes are most common in the surface epithelium and are responsible for digestion and absorption of nutrients as well as in forming the intestinal border. They are assisted by mucosecreting/goblet cells which produce several mucine types which are chemically different along the intestinal tract. Goblet cells increase in number from oral to aboral as the stool becomes increasingly compacted. Enteroendocrine cells represent the third important cell type and comprise a highly specialised chemosensory system involved in sensing the energy balance and reserve of an individual. A detailed classification of enteroendocrine subtypes is possible by the specific intestinal hormones that are produced and the assistance of Notch signalling in enteroendocrine cell differentiation^[2,3].

Paneth cells, first described by Gustav Albert Schwalbe in 1872, originate directly from intestinal stem cells^[4]. In contrast to other secretory cell types, Paneth cells are physiologically found at the bottom positions of small intestinal crypts (Figure 1). The unique histomorphological feature implicates special functions of Paneth cells in cellular homeostasis as well as in the establishment and configuration of the mucosal barrier as a physical and highly organised immune interface. Recently, some molecular mechanisms underlying secretory disorders of Paneth cells were identified strongly related to intestinal diseases, e.g., ileal Crohn's disease and necrotising enterocolitis. The important etiopathological findings were addressed and summarised with the term "Paneth's disease" and introduced in the literature^[4].

In the following paragraphs, important aspects of Paneth cell physiology and pathophysiology are reviewed. The data clearly demonstrate that Paneth cells are a highly specialized cell type strongly involved in assisting to sharpen and maintain of the microbiome as well as in the establishment of the stem cell niche

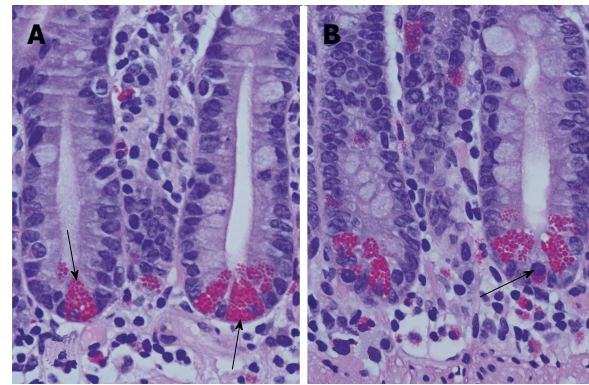


Figure 1 Normal small intestinal crypts with basal orientated Paneth cells. A: Paneth cells are characterised by their apical located granules (arrows). Between Paneth cells undifferentiated progenitor cells are found. In the normal *Lamina propria mucosae* a mixed population of immune cells and stroma resident cells is found; B: Occasionally, Paneth cells at the bottom of small intestinal crypts are mixed up with enteroendocrine cells (arrow). They are characterised by basal located granules. In the upper part of the crypt, a mitotic figure is shown.

and promotion of cellular renewal and mucosal morphogenesis. Consequently, Paneth cell disorders are involved in the pathophysiology of intestinal diseases.

PANETH AND STEM CELL NETWORK

The small intestinal epithelium renews within 3-6 d. The extraordinary rate of cell renewal is driven by a vigorous proliferation within crypts and a highly dynamic movement of epithelial columns toward the villus tip. The intestinal epithelia descend from a distinct stem cell zone located in small intestinal crypts. The zone consists of Paneth cells and 4 to 6 independent intestinal adult stem cells adjacent to rapidly cycling progenitors in the upper part of intestinal crypts.

The stem zone model is orientated on the morphological finding of crypt base columnar cells (CBC cells). These undifferentiated cycling cells are intermingled with Paneth cells and are hierarchically followed by Mix cells located directly above the Paneth cells^[5-7]. Mix cells are assumed to be strongly amplifying precursors of the different epithelial cell lines including Paneth cells.

In contrast to the stem cell zone model, a +4 position model has been suggested^[8]. The model was substantiated by the finding that severe radiation sensitivity exists in the +4 position^[9]. In this location, active cell cycling is found and radiation sensitivity indicates sufficient protection of the stem cell compartment from genetic damage. In the proposed model, injured +4 position stem cells are replaced by earlier generations of transit amplifying (TA) cells with a better repair capacity and asymmetric segregation of old and new DNA strands^[10]. Some parallels exist between the both models including definition of a slow and a rapid cycling cell type and an assisting role of Paneth cells in maintaining stem cell behaviour.

Maturing Paneth cells migrate downward into small intestinal crypts, where they reside for 3-6 wk^[11]. Paneth

cells escape from the crypt bottom by cellular fragmentation and phagocytosis from *lamina propria mucosae* infiltrating macrophages. There is experimental evidence that Wnt signalling and the expression of Wnt target genes are essential in the configuration and function of the stem cell zone including establishment of rapidly cycling TA cells^[4,12-14]. In a current model, an increasing gradient of Wnt activity directed into the small intestinal crypt is proposed reflecting the governing action of adjacent mesenchymal cells that release Wnt proteins. At the base of crypts, β -catenin is enriched in the nuclei of progenitor cells implying a strong response to Wnt signalling. The Wnt gradient is crucial for a graded expression of EphB2 and EphB3 acting as cell-sorting receptors along the CVA^[15]. In addition, graded Wnt activity is essential in the differentiation of Paneth cells with accumulation of large granules in the cytoplasm. Terminal differentiation of Paneth cells exists at the crypt bottom, where Wnt activity is maximised and several Wnt target genes are expressed^[16]. It has to be stressed that the expression pattern of Wnt target genes, especially *Lgr5/Gpr49*, is essential in the establishment of the small intestinal crypt stem cell zone including CBC cells interspersed between the maturing Paneth cells^[10]. Recently, intestinal organoids were used to visualize the short-range Wnt gradient in the intestinal stem cell niche^[17].

Paneth cells are important players in the intestinal stem cell niche, but they are identified as nonessential constituents. In a mouse model deficient in *Math1* (*Atoh1*), which totally and permanently lacks Paneth cells, *Lgr5*⁺ CBCs occupied the full crypt base, showed strong proliferation, and generated differentiated progeny over months^[18]. The increased proliferation along the CVA was associated with an increase in crypt height in the duodenum (13%) and ileum (15%). Enhanced or unaffected levels of Wnt target transcripts of genes associated with cell proliferation such as *CD44*, *Myc*, and *Ccnd1* were found. Using a different strategy of intestinal *Math1* deficiency, functional intestinal stem cells were found after Paneth cell ablation^[19]. In this study, *ex vivo* and *in vivo* experiments were performed to clarify the role of mesenchyme in the establishment of the stem cell niche. In the *ex vivo* setting, *Math1*-deficient crypt cells were not able to self-renew or survive indicating for extraepithelial, mesenchymal signals necessary for stem cell activities. Using caspase-8 deleted mesenchyme-free organoids, differentiation of Paneth cells was found^[20].

The Wnt dependency of Paneth cell differentiation is modified by other signalling cascades including the hierarchical Notch. The expression of Notch receptors is found in CBCs, and Paneth cells express the corresponding ligands^[21]. Consequently, Paneth cells are postulated as a source of Notch signalling to maintain CBC functions and crypt activities. It is suggested that some CBC functions unrelated to secretory metaplasia may require a Paneth cell source of Notch signalling.

Inhibition of Notch by pharmacological inactivation of the γ -secretase, an enzyme that is involved in Notch signalling, is already established. In dibenzazepine (DBZ)

- treated wild-type black-6 mice, a significant increase in secretory differentiated cells located throughout small intestinal crypts is found. After DBZ treatment, small intestinal crypts are lined by orthotopic Paneth cells and transdifferentiated cells with morphological features mixed from Paneth cells and mucus-retaining cells (Figure 2). The change in secretory differentiation is called secretory metaplasia.

PANETH CELL METAPLASIA

In distinct intestinal disorders, the physiology of Paneth cells is disturbed. In general, true injuries/diseases of Paneth cells should be separated from such intestinal disorders, where differentiation of Paneth cells is necessary to compensate cellular stress or misdirected mucosal differentiation/tissue homeostasis. The important histomorphological findings include loss, transdifferentiation or metaplasia of Paneth cells.

Metaplasia is defined as the occurrence of differentiated cells in a histomorphological location, where they are physiologically not found. Paneth cell metaplasia is seen throughout the gastro-intestinal tract, but frequently manifests in the stomach and is associated with different intestinal injuries (Figure 3). In addition, Paneth cell metaplasia is found in extra-gastro-intestinal sites, like the lung and tracheobronchial system, pancreatobiliary tract, and the uro-genital tract, but is a rarity in the nasopharyngeal system. It has been postulated that Notch may be involved in forms of pre-cancerous, metaplastic conditions in the stomach, which typically results from a loss of parietal cells and chronic inflammation. The expression of *Math1*, which is physiologically not found in normal gastric mucosa, has been shown to be upregulated in intestinal metaplasia of the stomach^[22,23].

Paneth cell loss is a hallmark of special types of Crohn's disease and is also found in acute inflammation like graft vs host disease (GvHD) grade II and III and ischemic tissue damage^[20,24]. The phenomenon of transdifferentiation, whereby Paneth cells are replaced by lysozyme-producing mucus cells, is described in inflammatory small intestinal disorders and in celiac disease. There is some phenomenological evidence that Paneth cell transdifferentiation could be due to re-programmed stem cells^[25].

At present, it is well established that the molecular framework underlying the process of regular Paneth cell differentiation and maturation strongly depends on Wnt activity^[16]. In contrast to the crypt physiology, the role of Wnt signalling in the development of Paneth cell metaplasia is only marginally elucidated. However, there is some evidence of Wnt activity as a driving force in metaplasia. In an experimental study with the constitutive expression of a β -catenin-Lef1 fusion protein under control of a lung-endoderm-specific promoter from the surfactant protein C gene transgenic lungs included cells expressing marker genes strongly characteristic of intestinal Paneth cells^[26]. The data strongly supports the view that hyperactive Wnt signalling could be crucial

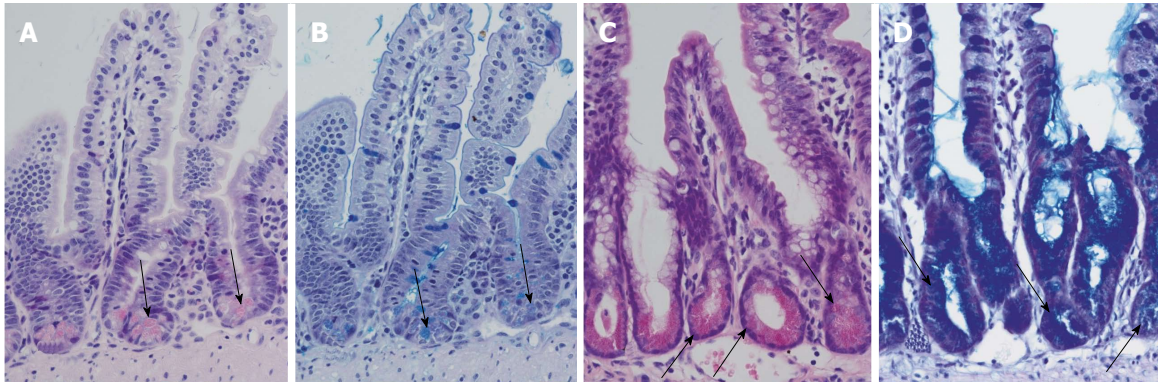


Figure 2 Differentiation of Paneth cells and Notch inhibition. Normal ileal mouse mucosa with normal crypts and Paneth cells: HE staining (A) and alcian-PAS staining (B). Arrows indicate Paneth cells. Ileal mouse tissues after treatment with dibenzazepine show an increase in secretory cells in the crypts with differentiation of Paneth cell-like epithelia (arrows): HE staining (C) and alcian-PAS staining (D).

in stem cell lineage commitment and the generation of intestinal metaplasia. In the lung study, where lysozyme was used as a marker protein of mature Paneth cells, increased Wnt activity was associated with diminished lysozyme expression in metaplastic cells^[26]. This interesting finding is highly suggestive that Wnt activity is an important prerequisite for Paneth cell metaplasia, but additional factors and supportive signalling mechanisms are necessary. The auxiliary components necessary for Paneth cell differentiation and maturation probably differs between tissues. For example, inflammation seems to induce Paneth cell metaplasia/hyperplasia in the colon, but not in the small intestine^[27]. The intestinal microbiome is suggested as an important variable in signalling activities and the expression level of several intestinal pathways and regulates phenomena including inflammation and cellular differentiation.

PANETH CELLS AND ANTIMICROBIAL PEPTIDES

In the intestine, a plethora of microbes is found including autochthonous long-term colonisers and allochthonous transient microorganisms. One important function of commensal microbiota is host defence through the promotion of the mucosal immune system and inhibition of pathological microbes. Another function is the nutrition of the host, because the bacteria have the capacity to ferment components of the diet. The synthesised short-chain fatty acids (SCFAs), vitamins, and amino acids are essentials in organising host physiology and energy balance. SCFAs act as signalling molecules and highly specialised free fatty acid receptors (FFARs) exist in the intestinal epithelium. FFARs contribute to the chemosensory intestinal system and differ in their molecular structure, ligand specificity, and functional properties^[28]. There is evidence that SCFAs are able to induce glucagon-like peptide-1 release from intestinal cells. The molecular link is important to display the integral role of the intestinal microbiome in the regulation host's energy homeostasis. Metabolic disorders and obesity are

associated with changes in the gut microbiota^[29].

In the intestine, a complex molecular system is established to maintain microbiome - host homeostasis and to shape the composition of microbes colonising the intestine. For this purpose, Paneth cells sufficiently express and secrete different types of antimicrobial peptides (AMPs) which are important host-defence substances in the communication between host and microbiome. The secretory capacity of Paneth cells is reflected by the cytoarchitecture with apical clustering of large secretory granules. Ultrastructurally, the endoplasmic reticulum (ER) is hyperplastic and associated with a well-developed Golgi network. Upon prosecretory stimuli like bacterially derived Toll-like receptor ligands the cytosolic calcium content increases *via* KCa3.1, a calcium-activated potassium channel, and leads to granule secretion^[30-33].

Defensins are the major AMP family and contain six cysteines involved in intramolecular disulphide bonds^[34,35]. Two major subfamilies of defensins exist. The α -defensins are strongly synthesised by Paneth cells and neutrophils, whereas β -defensins with constitutive HBD-1 and inducible HBD-2 are found in different types of cells including enterocytes^[36]. In mice, α -defensins are named cryptidins or cryptidins which consist of six major isoforms. Among those isoforms, variant 4 is the most microbicidal peptide^[37]. In addition to α -defensins, Paneth cell granules contain lysozyme as another potent host-defence molecule. The biosynthesis of active α -defensins depends on proteolytic processing. In murine Paneth cells, matrix metalloprotease 7 (MMP7) is found cleaving defensin precursor molecules to active α -defensins^[38,39]. Disturbed proteolytic activity as found in MMP7 knockout mice is associated with a hampered intestinal microbiome and an increased distribution of bacterial pathogens^[40]. In contrast to mice, trypsin is found in human Paneth cells as an activating protease for prodefensins and the antimicrobial protein Reg3A^[41]. The human α -defensins type 5 (HD5) and 6 (HD6) are essential in host protection from intestinal pathogens. Using a HD5 transgenic mouse model with physiological defensin levels, animals were resistant to enteric *Salmonella* infections due to reduced viability of bacteria, diminished bacterial translocation,

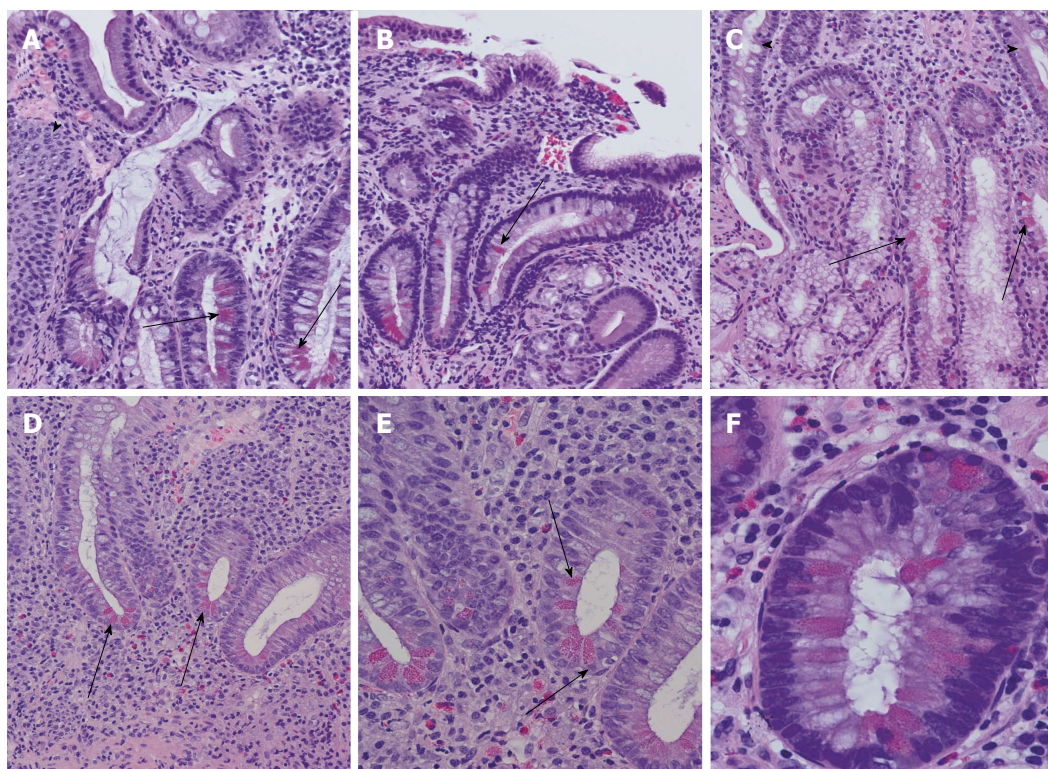


Figure 3 Examples of Paneth cell metaplasia throughout the intestinal tract. A: Paneth cell metaplasia (arrows) in Barrett mucosa. Squamous epithelia of the oesophagus are marked with an arrowhead; B: Chronic atrophic gastritis with Paneth cell metaplasia (arrow); C: Paneth cell metaplasia (arrows) of Brunner's gland. In the upper part, small intestinal mucosa and secretory ducts are shown (arrowheads); D: Colon mucosa in ulcerative colitis with disturbed crypt architecture, increased numbers of stroma infiltrating inflammatory cells, and Paneth cell metaplasia (arrowheads); E: Higher magnification of ulcerative colitis-associated Paneth cell metaplasia (arrows) in colon mucosa as demonstrated in (D); F: Paneth cell metaplasia (arrows) in tubular adenoma of the colon with low-grade dysplasia.

and increased survival after lethal *Salmonella* infections^[42]. In the same model, evidence of a direct role of α -defensins in regulating and shaping the small intestinal microbiome was shown^[40]. In the intestine of HD5 transgenic animals the number of commensals was changed with an increase in *Bacteroides*, a decrease in *Firmicutes*, and the loss of segmented filamentous bacteria (SFB), designated *Candidatus arthromitus*, accompanied by an absence of Th17 cell differentiation. SFB are host specific intestinal symbionts belonging to the *Clostridiaceae* displaying differentiation of filaments that interact intimately with the surface lining intestinal epithelia. Secreted proteins are expressed by SFB including different ADP-ribosyltransferases and a myosin-cross-reactive antigen, all probably involved in modifying the host response and post-natal maturation of the gut immune system^[43,44]. Recently, an SFB-host cell co-culturing system was established producing viable infectious particles, which can colonise intestinal mucosa with the induction of an immune response^[45]. Regional variation in the expression and secretion of Paneth cell AMPs along the intestinal tract are well-balanced by the colonising activity of SFB with the establishment and shaping the intestinal microbiome^[46].

The α -defensin HD6 secreted by human Paneth cells acts in a way that is completely different to HD5. The HD6 is antibacterial by the configuration of self-assembled peptide nanonets and formation of nanofibrils^[47,48].

Targeted bacteria surrounded by the fibrils and nanonets are not able to invade the intestinal mucosa. The HD6 self-assembling function is essentially based on the presence of histidine-27, which forms a salt bridge necessary for multimerising the peptide. The important Paneth cell produced α -defensins HD5 and HD6 can be further classified by their unique functions. The antimicrobial HD5 activity with disruption of bacterial membranes is called "harpoon" activity, whereas HD6 represents so-called "net forming" activities^[49]. In summary, HD5 acts antimicrobially and exhibits lectin-like properties, whereas HD6 entraps microbes and blocks host cell invasion^[50,51].

In addition to defensins, Paneth cells are able to secrete other AMPs including lysozyme, secretory phospholipase A2 (sPLA2), RegIII, angiogenin 4, and cathelicidins^[52-54]. Among the AMPs, RegIII proteins are important in antibacterial defence, and murine RegIII γ shares 65% identity with human RegIII α . RegIII proteins belong to the family of C-type lectin regenerating islet-derived proteins and bind glycan chains of peptidoglycans on the cell wall of gram-positive bacteria. In contrast to other C-type lectin AMPs as mannose-binding lectin, the complement recruitment domains are not constantly expressed in RegIII suggesting a direct anti-bactericidal function^[55].

The AMP LL-37 belongs to the cathelicidin family and acts in a similar way as HD5 by puncturing holes in microbial membranes. LL-37 mediates antimicrobial

activities in addition to immunological functions *via* various cellular receptors^[56]. LL-37 is an inducible AMP, because LL-37 expression increases in the presence of SCFAs. Consequently, the protein is predominantly found in the transit-amplifying zone in colonic crypts, when compared with small intestinal mucosa^[57]. In quantitative terms, the ratio of HD5 to HD6 is about 3:1, whereas HD5 expression levels are higher by a factor of up to 100 than those of lysozyme and sPLA2^[4].

Paneth cells are secretory cells with the excretion of a plethora of molecules including several types of AMPs^[50,52-54]. The secretory function depends on a well-adapted ER, which is controlled and smoothed by autophagy. Disruption of autophagy by the deletion of the unfolded protein response transcription factor X-box binding protein-1 results in ER stress, Paneth cell impairment, and spontaneous inflammation resembling special variants of Crohn's disease^[58]. There is experimental evidence that lipids and lipid metabolising enzymes are involved in the signalling cascade for exocytosis of granules from Paneth cells^[59]. Expression profiling revealed a panel of target genes and related proteins including lipoprotein lipase, apolipoprotein A-IV, stearoyl-CoA desaturase-1, adiponectin, and leptin. Mapped pathways include PPAR signalling, statin pathway, adipocytokine signalling, and polyunsaturated fatty acid biosynthesis. It has been speculated that the lipid-associated exocytosis is an additive mechanism to the chemosensory system of FFARs and enteroendocrine cells to perform synergisms between the intestinal microbiome and host metabolism.

The release of AMPs from Paneth cells into the intestinal mucus and lumen is initiated by stimulation of pattern recognition receptors (PRRs) located on intestinal surfaces. The class of PRRs includes Toll-like receptors (TLRs) activated by lipopolysaccharide and nucleotide-binding oligomerisation domain-containing molecules (NODs), which recognise muramyl dipeptide. Independently of PRRs, bacterial cell wall glycolipids are able to stimulate the release of defensins by Paneth cells. The myeloid differentiation primary response gene (MyD88) acts as an important cytoplasmic TLR limiting bacterial penetration into the intestinal mucosa^[4].

In summary, Paneth cells are essential for the establishment of a mucus layer enriched with AMPs resting on the surface lining epithelia. The composition of AMPs differs along the intestine, implicating diverse functions in communication with the intestinal microbiome.

PANETH CELLS AND INTESTINAL DISEASES

High numbers of *Lamina propria mucosae* infiltrating leukocytes with different types of specialised lymphocytes, plasma cells, mast cells, monocytes, and eosinophiles is a very characteristic histomorphological feature of intestinal mucosa. The balanced correspondence of the immune cells with the intestinal microbiome *via* the surface

epithelium is a prerequisite for intestinal homeostasis. In consequence, an inflammatory response with a further increase in leukocytes including neutrophils is very common in intestinal disorders and is established in the majority of intestinal diseases. There are remarkable differences in the quantity and quality of infiltrating leukocytes between the underlying intestinal disorders. The differences of leukocytes are helpful to classify the basal disease using morphological, immunohistochemical, and functional techniques.

In addition to resting Paneth cells, infiltrating leukocytes are an important transient source for AMPs. They are assisted by metaplastic Paneth cells, found in the large intestine and stomach. In intestinal inflammation, sPLA2, which is physiologically not found in the colon, is expressed by metaplastic Paneth cells^[60,61]. There are experimental data demonstrating a direct role of the microbiome in regulating the defensin production and secretion. Using *Nod2* knockout mice in co-housing experiments, the wild-type microbiome was able to regulate defensin secretion to physiological levels^[62]. The inflammation-related expression profile of AMPs includes some characteristics indicating a defined intestinal disease^[63,64]. For example, β -defensin types 2, 3, and 4 are increased in ulcerative colitis but not in Crohn's disease^[65]. In summary *Nod2* is highly expressed in ileal Paneth cells that essentially contribute to the regulation of ileal microbiota through the secretion of AMPs^[66].

As outlined above, Paneth cells are the main source of AMPs and acts in stabilising the stem cell zone. Consequently, Paneth cells are investigated as a site of origin for intestinal inflammation. This point of view seems of high relevance concerning the pathogenesis of inflammatory bowel diseases (IBD). A fundamental feature of ileal Crohn's disease is a reduced expression of HD5 and HD6. The finding is sometimes paralleled by a reduced Paneth cell number, but may be also a result of an injured microbiome^[4,36,67].

An in-detail analysis of Paneth cells in Crohn's disease revealed subgroups of the disease characterised by unique molecular, morphological, and clinical features. The clinical phenotype of ileal Crohn's disease is especially associated with Paneth cell injuries. There was first evidence from loss-of-function mutations in *NOD2* (SNP 13), a gene that is strongly expressed by Paneth cells^[66,68-70]. Subsequently, the molecular mechanisms underlying several injuries of Paneth cells were described and linked to (ileal) Crohn's disease. One important finding was low Wnt signalling activity in ileal Crohn's disease with diminished Tcf-4 and reduced secretion of defensins^[71,72]. Another milestone was the identification of defective granule exocytosis from Paneth cells with diminished levels of defensins due to abnormal autophagy in homozygosity for the risk allele autophagy related 16-like 1^[59,73]. The molecular characterisation of disturbed autophagy and unresolved ER stress due to genetically and environmentally controlled dysfunction of unfolded protein response (UPR) was the breakthrough in understanding the underlying mechanisms for

the threshold for the development of (ileal) Crohn's disease^[58,59,74].

In addition to Paneth cells, enterocytes are an additional source to produce AMPs including Reg proteins, defensins, and cathelicidins^[50,52-54]. This fact is of high relevance to physiologically compensate the perinatal period prior to the establishment of Paneth cells and to pathologically balance Paneth cell disorders^[75]. Paneth cells are found in the prenatal small intestine, but the number of cells is highly variable. In view of this observation, insufficient Paneth cell maturation is in discussion as an important variable in the pathogenesis of necrotising enterocolitis. The multifactorial disease is preferentially found in premature infants. Clinically, a sudden onset and high mortality in babies born after 35 weeks' gestation are characteristic.

At present, two different pathophysiological models for the development of necrotising enterocolitis are in discussion; the top-down and the bottom-up hypothesis^[76]. In the top-down scenario, bacterial invasion is found in the villus that triggers thrombosis of small arteries via activation of platelet-activating factor (PAF). In contrast to the top-down scenario, bacterial translocation at the crypt basis with consecutive activation of Paneth cells and arterial thrombosis are the key points in the bottom-up model. In very preterm neonates, the time period between birth and onset of the necrotising enterocolitis is frequently long. In this time maturation of the intestinal immune system is found, a pre-requisite for the initiation and development of enterocolitis^[77]. In animal models, toxic Paneth cell ablation and gastral infection with *Klebsiella pneumoniae* is able to induce necrotising enterocolitis in a bottom-up scenario^[78]. The variable number of Paneth cells found in human necrotising enterocolitis is discussed as enterocolitis associated necrobiosis and does not contradict the bottom-up model^[79,80]. It has to be stressed that the immense damage to intestinal tissues in necrotising enterocolitis is not plausible from the thrombosis of small arteries. Therefore, a two-hit model has been proposed including at first ischemic damage of the intestinal mucosa. In a second step, reperfusion is found with uncontrolled activation of signalling cascades and enzymes aggravating the tissue damage^[81].

Barrett mucosa is frequently found in the oesophageal junction and is due to chronic gastroesophageal reflux. Histomorphologically, the epithelial cells display intestinal differentiation with goblet cells and aberrant mucus retention^[82]. In contrast to complete intestinal metaplasia, brush border and Paneth cells are not commonly found in Barrett mucosa. However, there is evidence of Paneth cell metaplasia in Barrett oesophagus (Figure 3A). The phenomenon is found in about 30% of the cases^[83]. The frequency of Paneth cells in Barrett mucosa is inversely correlated to the development of epithelial dysplasia. In long-segment Barrett oesophagus, the metaplasia is more frequent than in short-segment lesions. The persistence of Barrett mucosa when Paneth cell metaplasia is found could be due to a dys-balanced Notch and Wnt signalling^[84,85]. There is no evidence that Paneth cell metaplasia is

associated with the progression of Barrett mucosa to dysplasia and adenocarcinoma^[83].

In GvHD the intestinal tract is frequently affected and Paneth cell loss is a hallmark of the acute inflammation associated with changes in the composition of the microbiome^[86,87]. Due to the loss of Paneth cells a striking disease of α -defensins is found favouring a shift from commensal to pathologic microbiota^[88]. The dysbiotic shift is assumed to be crucial for severe septic complications in GvHD. In particular, the diminished number of Paneth cells correlates with higher disease severity and poor treatment response in patients suffering from GvHD^[89]. Enteroendocrine cells have been recently discovered as sensors of the intestinal microbiome. The cells are involved in the pathogenesis of intestinal disorders and express specific receptors which can respond to bacterial products^[90]. In germ-free mice, the number of enteroendocrine cells is drastically reduced^[91], whereas an increase in microbiome metabolites such as in GvHD is inductive for secretion of immunomodulatory enteroendocrine hormone peptides^[92]. The inductive mechanisms could be a pathophysiological link for hyperplasia of enteroendocrine cells in GvHD.

PANETH CELLS AND MORPHOGENESIS

Crypt fission, the division of a single crypt into two daughters, is fundamental in intestinal tissue expansion and morphogenesis, but is also found in tumourigenesis driving the clonal expansion of mutant adenomatous crypts^[93-96]. A specific cellular arrangement in the intestinal stem cell niche is observed that controls crypt fission. In a recent model, Paneth cells and CBCs (Lgr5+) are both importantly involved in crypt morphogenesis^[97]. The findings summarise the data from intestinal organoids, where Paneth cells are essentially involved in crypt budding^[98,99], and as mice lacking intestinal Paneth cells, which are able to sufficiently repair crypt injuries^[21,100]. The morphogenic activities of Paneth cells depend on Wnt signalling and redundant sources contribute^[101,102]. Important for the crypt fission model is the observation that Paneth cells adhere to their substrate more strongly than other crypt cells. In view with this strong evidence is given that so-called "mislocalised" Paneth cells change the symmetry of fission in determining the site of fission^[97].

CONCLUSION

Paneth cells are important secretory cells in the small intestinal mucosa. They are found as metaplastic cells in several other locations. Important secretory products of Paneth cells belong to the AMPs. They are essential for the dialogue between the microbiome and the host. In addition to Paneth cell functions in controlling the microbiome, the cells are functionally and structurally involved in forming the stem cell zone of small intestinal crypts and involved in morphogenesis of the CVA. In the last decade, molecular mechanisms of different Paneth cell injuries have been identified and correlated with intestinal

diseases. There is evidence from these experiments that ileal Crohn's disease as well as necrotising enterocolitis are strongly associated with diseased Paneth cells. The important role of Paneth cells in intestinal physiology and pathophysiology strongly supports the view that these cells are the guardian of small intestinal crypts.

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P- Reviewer: Hokama A, Wejman J, Xu WX **S- Editor:** Kong JX
L- Editor: A **E- Editor:** Lu YJ



Basic Study

Pharmacological inhibition of diacylglycerol acyltransferase-1 and insights into postprandial gut peptide secretion

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Author contributions: Maciejewski BS, Manion TB and Steppan CM substantially contributed to the conception and design of the study, analysis and interpretation of data; Maciejewski BS and Manion TB contributed to the study acquisition; Maciejewski BS and Steppan CM drafted the article and made critical revisions related to the intellectual content of the manuscript and approved the final version of the article published.

Supported by Pfizer Inc.

Institutional review board statement: All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of Pfizer Inc.

Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of Pfizer Inc. (IACUC protocol number: GTN-16169-2011).

Conflict-of-interest statement: All authors were employees of Pfizer Inc. during the conduct of these studies. To the best of our knowledge, no conflict of interest exists.

Data sharing statement: No additional data are available.

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Manuscript source: Invited manuscript

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Received: January 29, 2017

Peer-review started: February 13, 2017

First decision: July 10, 2017

Revised: July 25, 2017

Accepted: September 4, 2017

Article in press: September 5, 2017

Published online: November 15, 2017

Abstract**AIM**

To examine the role that enzyme Acyl-CoA:diacylglycerol acyltransferase-1 (DGAT1) plays in postprandial gut peptide secretion and signaling.

METHODS

The standard experimental paradigm utilized to evaluate the incretin response was a lipid challenge. Following a lipid challenge, plasma was collected *via* cardiac puncture at each time point from a cohort of 5-8 mice per group from baseline at time zero to 10 h. Incretin hormones [glucagon like peptide-1 (GLP-1), peptide tyrosine-tyrosine (PYY) and glucose dependent insulinotropic polypeptide (GIP)] were then quantitated. The impact of pharmacological inhibition of DGAT1 on the incretin effect was evaluated in WT mice. Additionally, a comparison of loss of DGAT1 function either by genetic ablation or pharmacological inhibition. To further elucidate the pathways and mechanisms involved in the incretin response to DGAT1 inhibition, other interventions [inhibitors of dipeptidyl peptidase-IV (sitagliptin), pancreatic lipase (Orlistat), GPR119 knockout mice] were evaluated.

RESULTS

DGAT1 deficient mice and wildtype C57/BL6J mice were

lipid challenged and levels of both active and total GLP-1 in the plasma were increased. This response was further augmented with DGAT1 inhibitor PF-04620110 treated wildtype mice. Furthermore, PF-04620110 was able to dose responsively increase GLP-1 and PYY, but blunt GIP at all doses of PF-04620110 during lipid challenge. Combination treatment of PF-04620110 and Sitagliptin in wildtype mice during a lipid challenge synergistically enhanced postprandial levels of active GLP-1. In contrast, in a combination study with Orlistat, the ability of PF-04620110 to elicit an enhanced incretin response was abrogated. To further explore this observation, GPR119 knockout mice were evaluated. In response to a lipid challenge, GPR119 knockout mice exhibited no increase in active or total GLP-1 and PYY. However, PF-04620110 was able to increase total GLP-1 and PYY in GPR119 knockout mice as compared to vehicle treated wildtype mice.

CONCLUSION

Collectively, these data provide some insight into the mechanism by which inhibition of DGAT1 enhances intestinal hormone release.

Key words: Glucagon-like peptide-1; Peptide tyrosine-tyrosine; Glucose independent insulinotropic peptide; Acyl-CoA:diacylglycerol acyltransferase-1; Incretin

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Core tip: Pharmacological Inhibition of diacylglycerol acyltransferase-1 (DGAT1) and insights into postprandial gut peptide secretion" describes studies that evaluate the effects of loss of DGAT1 function either pharmacologically or genetically on the incretin response. We demonstrate a synergistic effect on the incretin response with the combination of a DGAT1 inhibitor and sitagliptin, a dipeptidyl peptidase-IV inhibitor. Additional studies performed address the molecular mechanism by with pharmacological inhibition of DGAT1 results in increased gut peptide secretion. These data provide insight into the role of DGAT1 in the intestinal hormone release and its potential as a drug target for the treatment of type 2 diabetes.

Maciejewski BS, Manion TB, Stepan CM. Pharmacological inhibition of diacylglycerol acyltransferase-1 and insights into postprandial gut peptide secretion. *World J Gastrointest Pathophysiol* 2017; 8(4): 161-175 Available from: URL: <http://www.wjgnet.com/2150-5330/full/v8/i4/161.htm> DOI: <http://dx.doi.org/10.4291/wjgp.v8.i4.161>

INTRODUCTION

Metabolic abnormalities are major risk factors for cardiovascular disease, obesity and type 2 diabetes mellitus (T2DM)^[1]. Impaired glucose uptake, glycogen synthesis and glucose oxidation can result from abnormal amounts of triglyceride (TG) in the blood and non-

adipose tissues leading to potential insulin resistance^[2-6]. Gut hormones are known to play an important role in glucose and lipid absorption. These gut peptides include glucagon like peptide-1 (GLP-1), peptide tyrosine-tyrosine (PYY) (both produced by L cells) and glucose dependent insulinotropic polypeptide (GIP) (produced by K cells). These peptides are secreted as a complex nutrient sensing system to control glucose homeostasis^[7]. An impaired incretin effect in diabetic patients is most likely responsible for defective insulin release^[7] as levels of GLP-1 and GIP can be reduced as much as 54% in T2DM patients^[8]. GLP-1 is responsible for conveying glucose competence to beta-cells^[9] and up-regulates genes involved in insulin secretion such as glucokinase and GLUT2^[10,11]. GLP-1 mimetics like exenatide-4 and enhancers like dipeptidyl peptidase-IV (DPP-IV) inhibitors have also shown promise in elevating incretin levels to promote insulin release, decrease fasting glucose levels and further influence weight loss^[12-16]. Furthermore, levels of GLP-1 and GIP can be augmented by lipid delivered to the intestinal lumen and can also be further altered by the degree of fatty acid (FA) saturation delivered^[17-19]. The data supports the notion that endogenous lipids and their enzymatic modifiers potentially play an important role in the release of incretin hormones from the gut.

Acyl-CoA:diacylglycerol acyltransferase-1 (DGAT1) is one of the key enzymes responsible for the breakdown and reassembly of hydrolyzed lipid products 2-monoacylglycerol (2-MG) and FA. Diacylglycerol (DG) produced from acyl coenzyme A by A:monoacylglycerol acyltransferase (MGAT) is esterified by DGAT1 to resynthesize TG for repackaging and entry into systemic circulation^[20,21]. The systemic deletion of DGAT1 as well as its acute pharmacological inhibition has been shown to elicit a blunted post absorptive lipid profile^[22-27] with promise in modulating postprandial incretin levels^[28-30]. DGAT1 deficient mice are phenotypically lean, resistant to diet-induced obesity and hepatic steatosis, exhibit increased insulin and leptin sensitivity, have a reduced tissue TG concentration and delayed chylomicron release with lipid treatment and high fat diet^[28,31-35]. In the enterocytes DGAT1 inhibition modulates lipid absorption and alters FA/TG circulation resulting in increased levels of GLP-1^[29,30]. Intestinal DGAT1 deletion during a lipid challenge in mice has also exhibited increased levels of GLP-1, PYY and decreased GIP^[36]. Furthermore, pharmacological inhibition of DGAT1 has been shown to alter the temporal and spatial pattern of lipid absorption in the small intestine as well as alter the type of lipids released into circulation^[37] which could change the pattern of enteroendocrine cell secretion of gut hormones in the small intestine. While the evidence regarding the importance of the DGAT1 enzymatic downstream effect on incretin release is growing, the mechanism of action still remains elusive.

One possible regulator of gut hormone release are G-protein coupled receptors such as GPR119. GPR119 is expressed predominantly in pancreatic beta-cells and intestinal L cells and are known to stimulate GLP-1

release^[38]. This receptor may promote glycemic control *via* the incretin effect as well as insulin regulation^[39]. Agonists to this G-protein have been shown to reduce food intake and decrease body weight in rodents^[40] while GPR119 deficient mice have reduced fasting plasma GLP-1 and impaired glucose tolerance^[39]. Furthermore, increased FA after meal ingestion such as oleoylethanolamides are known activators of GPR119^[39,41,42]. The monoacylglycerol species 2-oleoyl glycerol, an activator of the GPR119 receptor and diacylglycerol precursor, was presented to elevate plasma GLP-1 and GIP levels in humans after oral administration^[43]. This evidence provides a potential role of GPR119 using certain FA signals to promote gut hormone and insulin release, but further investigation is needed to understand this mechanism.

In the current study, we evaluated the impact of pharmacological inhibition of DGAT1 on the incretin effect during a lipid challenge. We used different genetic rodent models and pharmacological interventions including DGAT1 deficient mice, GPR119 deficient mice, DPP-IV inhibitor Sitagliptin (Merck) and pancreatic lipase inhibitor Orlistat (Roche) in combination with PF-04620110, a pyrimidooxazepinone, a competitive DGAT1 inhibitor with a K_i of 94 nmol/L in mice^[27,29] to study incretin release into systemic circulation. These data further confirm the important role DGAT1 plays in postprandial incretin hormone release and provides insight as to molecular mechanism by which this occurs.

MATERIALS AND METHODS

In vitro assay

The discovery of PF-04620110 has been reported previously^[27] and is a potent and selective small molecule inhibitor of DGAT1 with 100-fold selectivity vs human DGAT2, ACAT1, AWAT1, AWAT2, MGAT2, MGAT3 and mouse MGAT1. Briefly, the ability of PF-04620110 to inhibit recombinant human (38 nmol/L), rat (94 nmol/L) and mouse (64 nmol/L) DGAT1 enzymatic activity was determined by measuring the incorporation of [³H]n-decanoyl Coenzyme A into DG to form TG. Additionally, in a cell-based assay in intestinal derived HT-29 cells, PF-04620110 (IC₅₀ approximately 39 nmol/L) inhibits the incorporation of ³H-glycerol into TG^[27].

Mice

C57BL/6J male mice (5-6 wk of age) (Jackson Laboratories), B6.129S4-Dgat1^{tm1Far} male mice (DGAT1 knockout mice, 5-6 wk of age) (Jackson Laboratories) and GPR119 male mice (GPR119 knockout mice, 10-12 wk of age) (Charles River) were allowed *ad libitum* access to water and normal chow (5001, Purina) on a 6am-6pm light/dark cycle. All procedures were approved by the Institutional Animal Care and Use Committee and all animals received humane treatment according to the criteria stated by the National Academy of Sciences National Research Council (NRC) publication 86-23, 1985.

TG tolerance test in mice

Mice were randomized according to body weight on the

day of experimentation with 5-8 mice per group per timepoint. The mice were fasted for three hours prior to a single oral dose of vehicle (0.5% methylcellulose) or PF-04620110 at 10, 1, 0.1 or 0.01 mg/kg. Thirty minutes after PF-04620110 dosing, the animals were administered 5 mL/kg corn oil (Sigma) by oral gavage. Sitagliptin (Merck) and Orlistat (Roche) were administered one hour prior to the corn oil bolus at 10 mg/kg and 25 mg/kg respectively. Blood was obtained in EDTA/aprotinin/DPP-IV inhibitor treated tubes *via* cardiac puncture just prior ($t = 0$) to corn oil administration and at 1, 2, 4, 6, 8 and 10 h post corn oil administration. Plasma samples were collected for incretin analysis.

Incretin analysis

Solid phase extraction was used to clean up plasma samples for incretin analysis. In brief, EDTA/DPP-IV/aprotinin treated plasma and carbon stripped serum were ran through Oasis HLB LP (60 mg) extraction plates (Waters). Samples were washed 3 times through the extraction plate with 100:1 water/trifluoroacetic acid mixture. A (60/40/1) mixture of acetonitrile/water/trifluoroacetic acid was used for the final elution and collection. The samples were placed on an Argonaut SDE Dry-Dual apparatus under 50 L/min N₂ pressure at 40 °C and allowed to dry overnight. The samples were then reconstituted in 300 µL of assay buffer and processed according to protocol for active GLP-1 (EGLP-35K, Linco), total amide GLP-1 (K110FAC-1, Meso Scale Discovery), PYY and GIP (RGT-88K-02, Milliplex map gut hormone panel immunoassay, Millipore).

Statistical analysis

Analysis of incretin levels involved using a Student's *t*-test (Microsoft Excel) or ANOVA (GraphPad Prism) to compare treated vs control groups at each time point. Group comparisons were conducted at the 5% significance level.

RESULTS

DGAT1 deficient mice and administration of a single dose of PF-04620110 increases postprandial plasma GLP-1 levels

To understand the role of DGAT1 on the incretin effect, fasted DGAT1 deficient mice and wildtype C57/BL6J mice were challenged with a corn oil bolus and plasma levels of total and active GLP-1 were measured for 2 h post lipid administration. Levels of both active and total GLP-1 in the plasma were increased 1.9-fold in DGAT1 wildtype mice from 1.13 pmol/L to 2.14 pmol/L for active GLP-1 and 5.11 pmol/L to 9.94 pmol/L for total GLP-1 (Figure 1A and B). A statistically significant increase was detected in DGAT1 knockout mice of 3.1-fold from 1.41 pmol/L to 4.49 pmol/L for active GLP-1 and 7.23 pmol/L to 24.24 pmol/L for total GLP-1 (Figure 1A and B). We then utilized a selective and potent DGAT1 inhibitor, PF-04620110 to compare pharmacological inhibition to genetic loss of function. Levels of active and total GLP-1 in the plasma were increased 2.3-fold (active GLP-1)

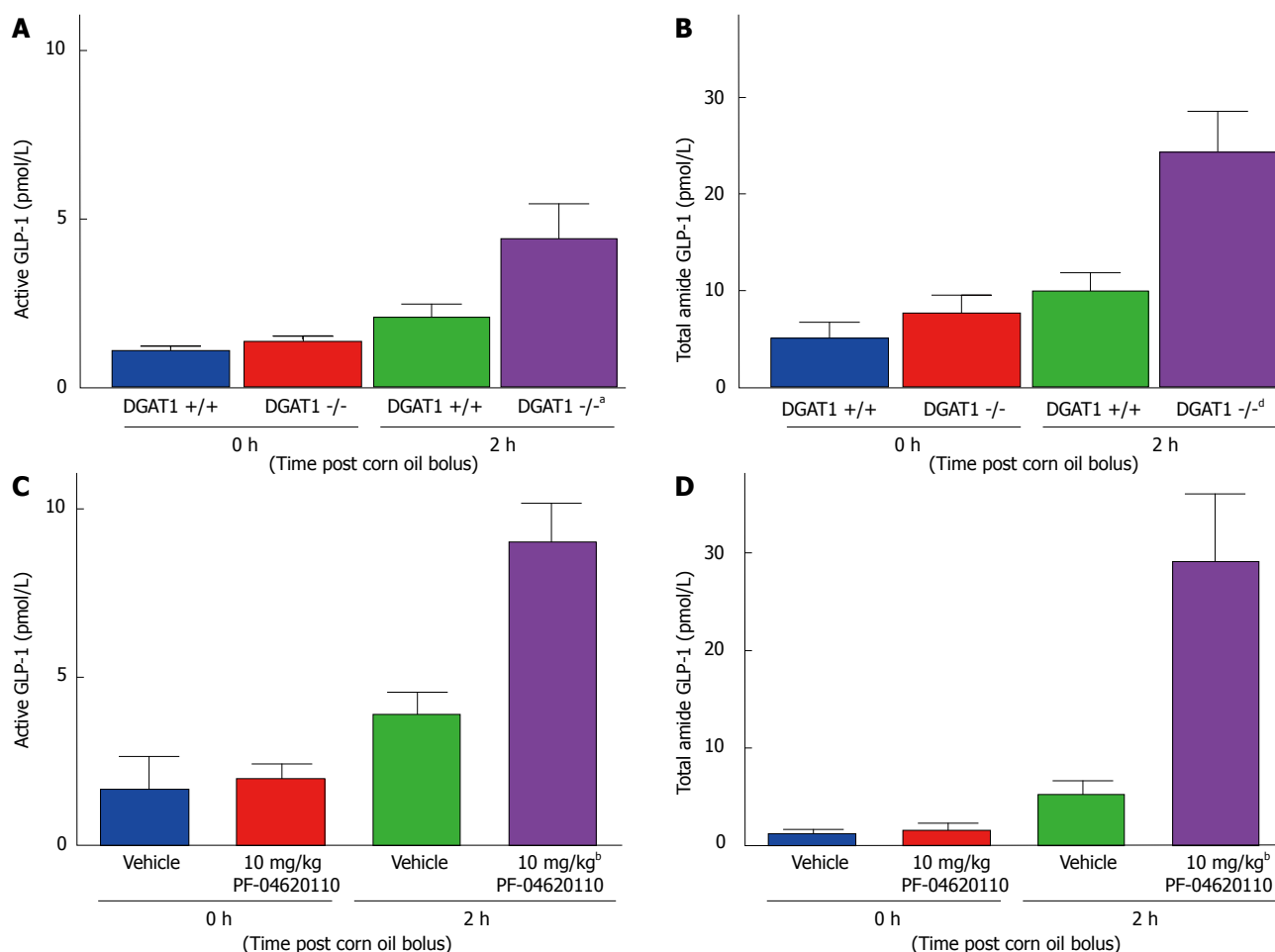


Figure 1 Diacylglycerol acyltransferase-1 knockout and acute administration of a diacylglycerol acyltransferase-1 inhibitor, PF-04620110 increases postprandial plasma glucagon like peptide-1 levels in mice. Fasted male DGAT1 knockout (-/-), wildtype (+/+) and C57BL/6J mice were administered either vehicle or 10 mg/kg of PF-04620110 by oral gavage ($n = 8$ per group). Thirty minutes post compound administration mice received a bolus of corn oil (challenge). Blood was collected just prior to bolus (time 0) and at 2 h afterwards for determination of active/total GLP-1 concentrations. (A) Active GLP-1 (pmol/L) and (B) total GLP-1 in DGAT1 -/- and +/+ mice following challenge. (C) Active GLP-1 (pmol/L) and total GLP-1 (D) in C57BL/6J mice treated with vehicle or PF-04620110 following challenge. DGAT1 -/- compared to DGAT1 +/+ and vehicle compared to PF-04620110 treatment at 2 h timepoint (^a $P < 0.05$, ^b $P < 0.01$, ^c $P < 0.001$ Student's t -test). Values are means $\pm$ SEM. DGAT: Diacylglycerol acyltransferase-1; GLP-1: Glucagon like peptide-1.

from 1.71 pmol/L to 3.96 pmol/L and 4.1-fold from 1.36 pmol/L to 5.48 pmol/L (total GLP-1) in vehicle treated wildtype mice 2 h post corn oil bolus. This response was further augmented as evidence by 4.5-fold increase in active GLP-1 from 2.03 pmol/L to 9.08 pmol/L and 17.2-fold increase in total GLP-1 from 1.71 pmol/L to 29.35 pmol/L with DGAT1 inhibitor PF-04620110 treated mice 2 h post corn oil bolus (Figure 1C and D). Acute pharmacological inhibition of DGAT1 *via* PF-04620110 administration resulted in a greater increase of active and total GLP-1 as compared to DGAT1 deficient mice, 1.4 and 14.1 fold, respectively in the context of postprandial lipid challenge.

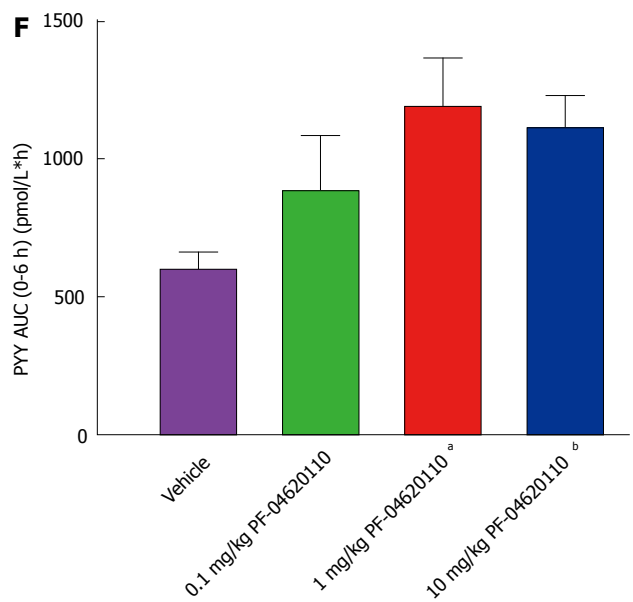
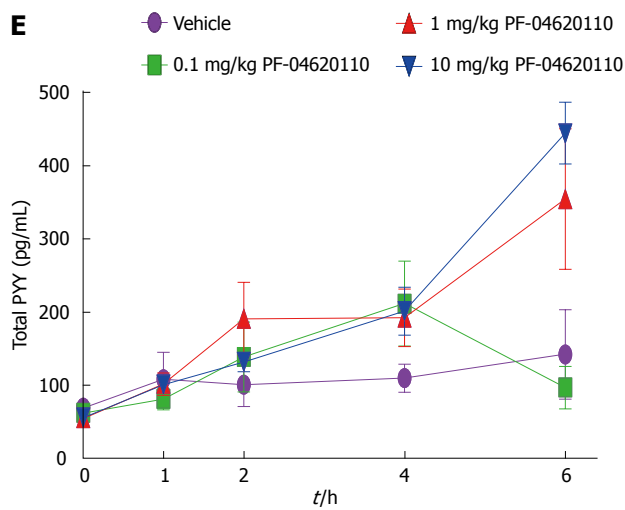
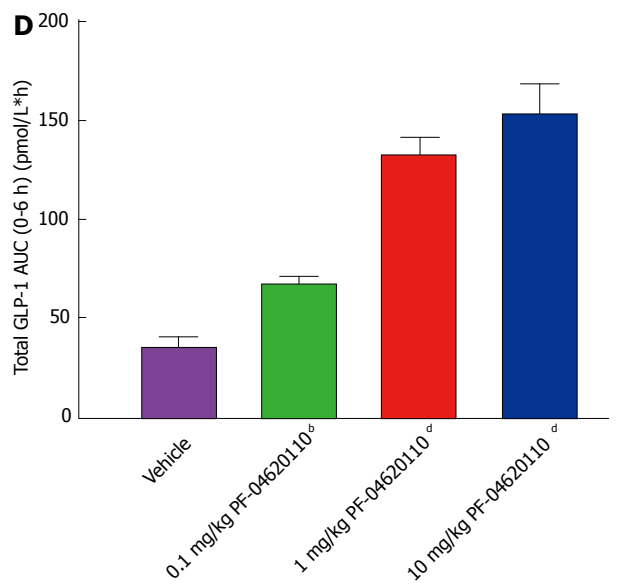
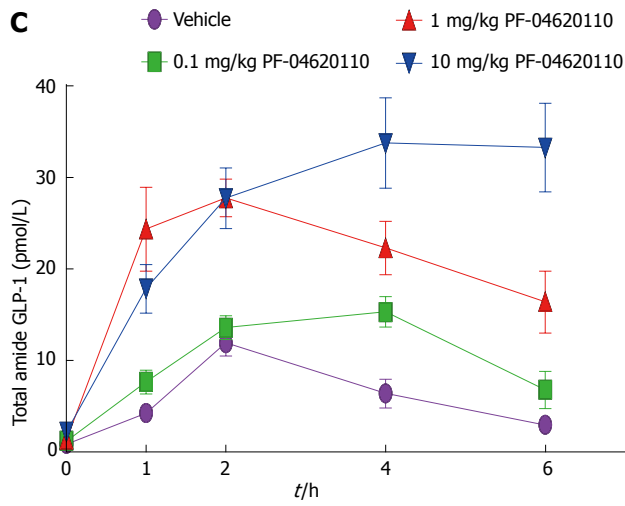
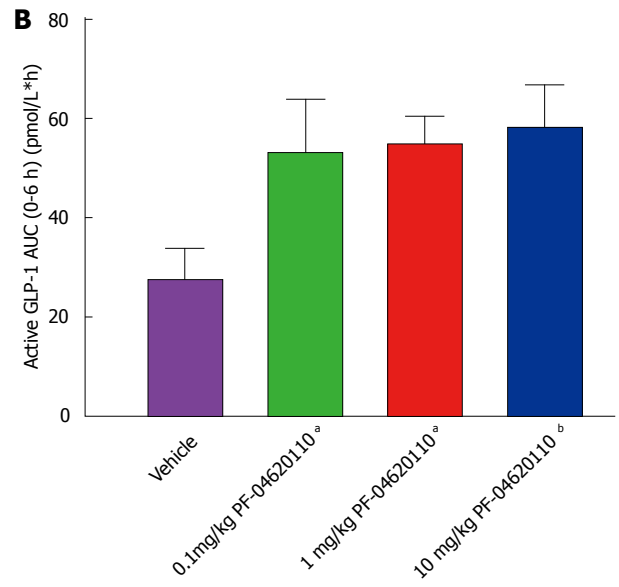
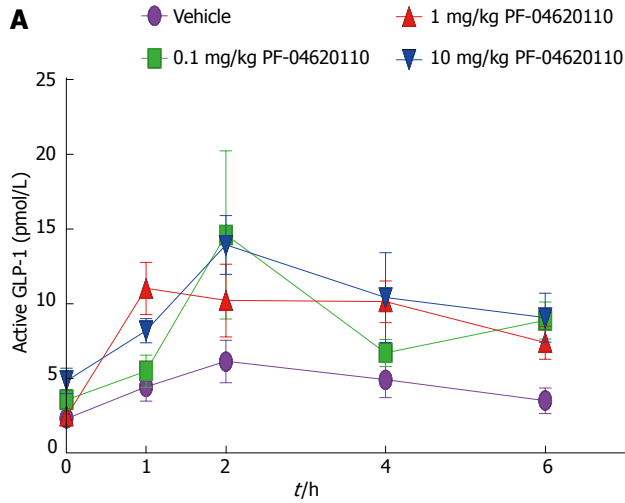
PF-04620110 administration in mice alters postprandial incretin hormone levels in a dose responsive manner

We then explored if pharmacological inhibition of DGAT1 is dose responsively altering gut hormones over a 6-h period. We monitored the levels of active and total GLP-1, total PYY and total GIP in the plasma over time following

an oral gavage of corn oil. Levels of active GLP-1 AUC were significantly increased almost 2-fold higher in all drug groups of over the 6-h period with peak levels seen at 2 h, the highest level of lipid excursion during this challenge (Figure 2A and B)^[37]. Levels of total GLP-1 were increased with increasing dose of PF-04620110 over time with a maximum 4.3-fold increase in AUC at 10 mg/kg of PF-04620110 compared to vehicle (Figure 2C and D). Levels of total PYY were also increased with increasing dose of PF-04620110 over time with a highest significant difference compared to vehicle at 1 and 10 mg/kg doses (Figure 2E and F). Interestingly, levels of GIP were significantly blunted with all drug doses over the 6-h postprandial excursion compared to vehicle (Figure 2G and H).

Combined administration of PF-04620110 and Sitagliptin synergistically enhance the postprandial active GLP-1 response in mice

Next, we hypothesized that administration of a DGAT1



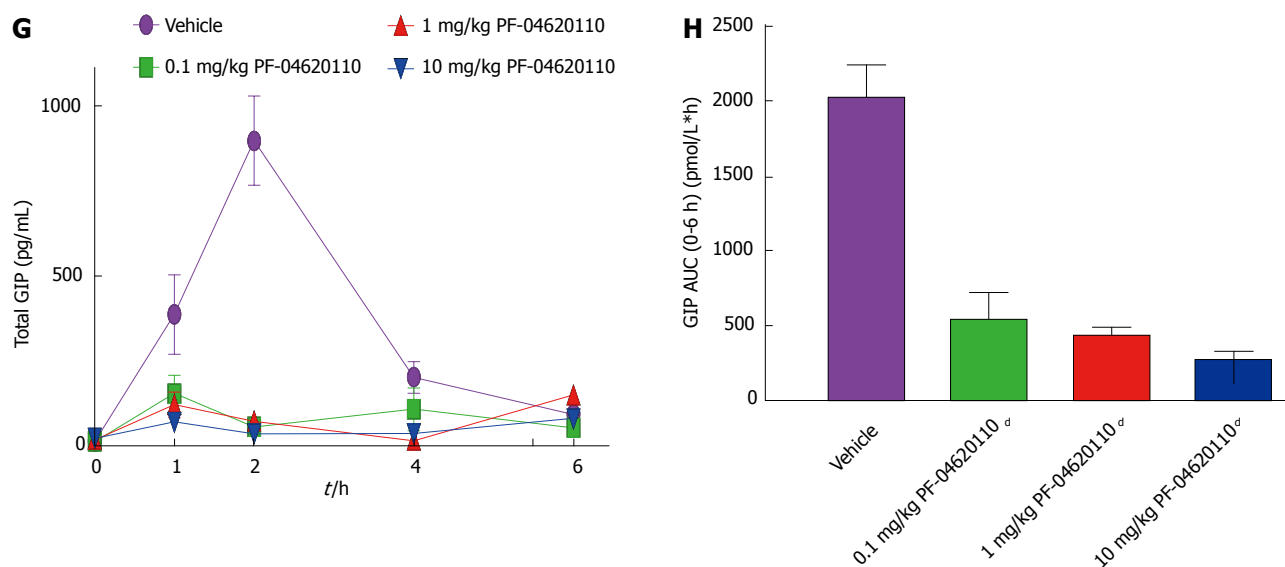


Figure 2 Pharmacological diacylglycerol acyltransferase-1 inhibition alters Incretin effect postprandially in a dose responsive manner. Fasted male C57BL/6J mice were administered vehicle or 10, 1, 0.1 or 0.01 mg/kg of PF-04620110 by oral gavage ($n = 8$ per group). Thirty minutes post compound administration mice received a bolus of corn oil (challenge). Blood was collected just prior to bolus (time 0) and at 1, 2, 4 and 6 h afterwards for determination of active/total GLP-1, PYY and GIP concentrations. (A) Active GLP-1 (pmol/L), (C) total GLP-1, (E) total PYY (pg/mL) and (G) total GIP (pg/mL) in C57BL/6J mice treated with vehicle or PF-04620110 following challenge. AUC values for (B) active GLP-1, (D) total GLP-1, (F) total PYY and (H) total GIP. Diacylglycerol acyltransferase-1 (DGAT1) inhibitor treatment compared to vehicle (^a $P < 0.05$, ^b $P < 0.01$, ^c $P < 0.001$ ANOVA). Values are means $\pm$ SEM. GLP-1: Glucagon like peptide-1; GIP: Glucose dependent insulinotropic polypeptide.

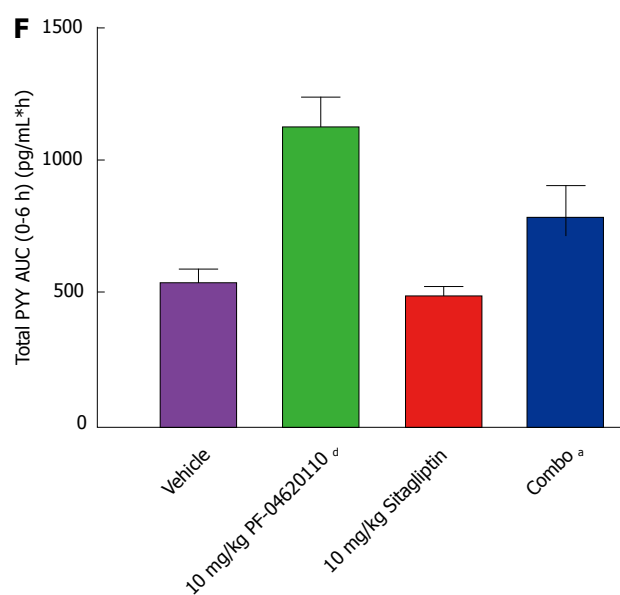
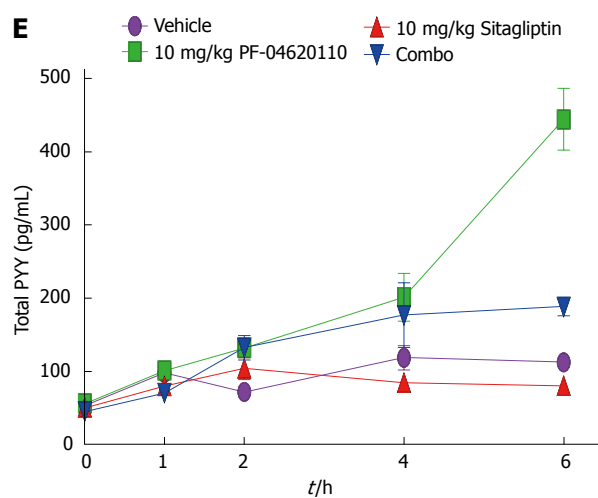
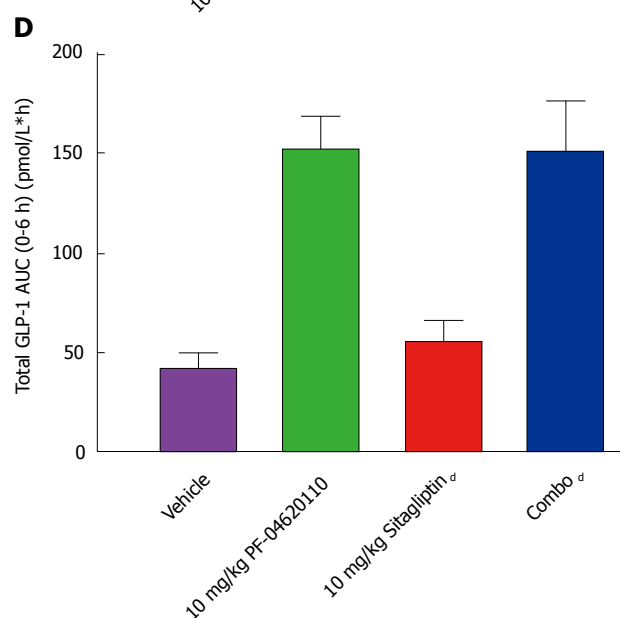
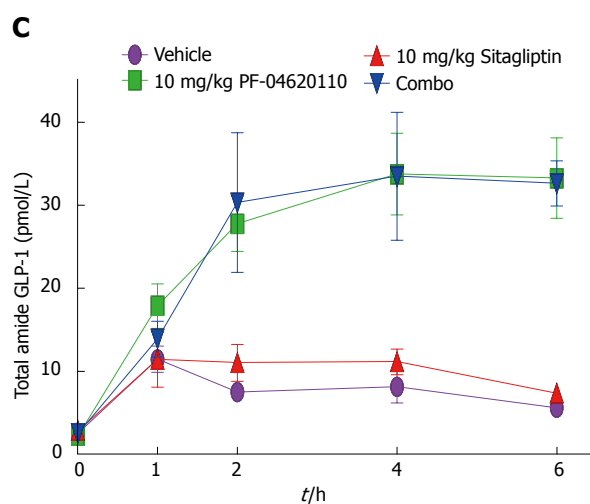
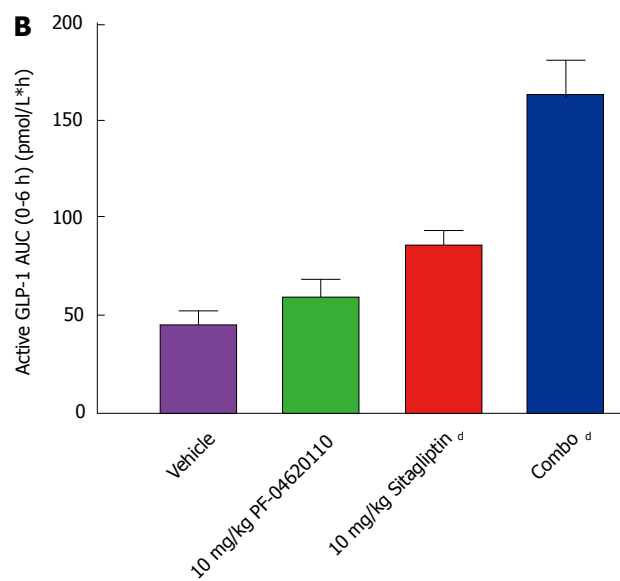
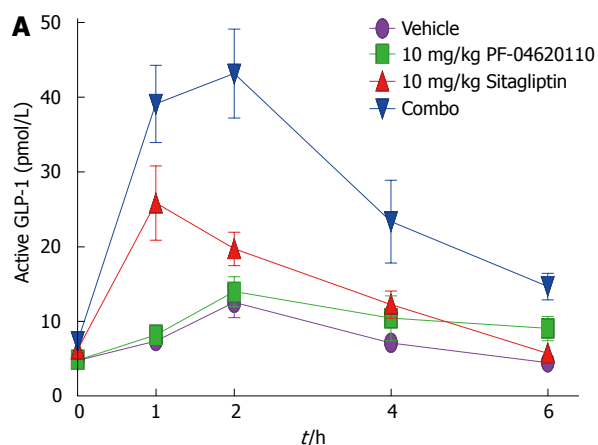
inhibitor which is stimulating increased GLP-1 and PYY secretion in combination a DPP-IV inhibitor which is blocking the clearance pathways of those gut hormones would result in synergy on plasma gut hormone levels. To test this hypothesis, we monitored the levels of active and total GLP-1, total PYY and total GIP in the plasma over a 6-h period following an oral gavage corn oil following the administration of either vehicle, PF-04620110, Sitagliptin or a fixed dose combination of PF-04620110 and Sitagliptin.

Levels of active GLP-1 AUC were modestly increased with singular administration of 10 mg/kg PF-04620110 1.3-fold 6 h AUC relative to vehicle treated mice from 18.48 pmol/L*h to 36.93 pmol/L*h and increased significantly with Sitagliptin 1.9-fold at 10 mg/kg (Figure 3A and B). The fixed dose combination resulted in 6 h AUC of 136.6 pmol/L*h or a 3.7-fold increase (Figure 3A and B). Levels of total GLP-1 AUC were significantly and identically increased over the 6-h excursion by 3.5-fold in both 10 mg/kg dose of PF-04620110 and combined administration of PF-04620110 and Sitagliptin compared to vehicle (Figure 3C and D). Levels of total GLP-1 were not changed with Sitagliptin therapy alone. Levels of total PYY AUC were significantly increased with PF-04620110 by 2-fold and combined administration of PF-04620110 and Sitagliptin by 1.5-fold over time compared to vehicle (Figure 3E and F) with no changes in Sitagliptin alone compared to vehicle treatment. Levels of GIP AUC were significantly blunted with PF-04620110 by 4.1-fold and combined administration of PF-04620110 and Sitagliptin 6-fold over time compared to vehicle (Figure 2E and F) and no changes in Sitagliptin alone compared to vehicle treatment. Synergistically, inhibition of DGAT1 and

inhibition of DPP-IV enhance postprandial levels of active GLP-1.

Orlistat suppresses altered postprandial incretin response seen with DGAT1 inhibitor PF-04620110 in mice

To gain mechanistic insight as to how DGAT1 inhibition is eliciting an enhanced incretin response, we tested if dietary lipid absorption is required. Therefore, we examined the postprandial incretin effect while inhibiting the absorption of fats from the diet using a pancreatic lipase inhibitor. Orlistat was administered to mice treated with and without PF-04620110 and monitored over a 10-h period with corn oil administration. Levels of active GLP-1 AUC were significantly increased over the 10 h excursion by 1.6-fold with PF-04620110 treatment alone, but no change in active GLP-1 AUC levels with Orlistat or the combination of Orlistat and PF-04620110 compared to vehicle (Figure 4A and B). Similar effects were seen with total GLP-1 AUC and PYY AUC levels (Figure 4D-F). Levels of total GLP-1 AUC were increased 3.8-fold and PYY AUC levels increased 2.1-fold by PF-04620110 compared to vehicle. Total GLP-1 AUC decreased 1.8-fold with Orlistat and 1.6-fold with Orlistat/PF-04620110 combination treatment (Figure 4C and D). Total PYY AUC significantly decreased 2.2-fold with Orlistat treatment and 1.7-fold combined Orlistat/PF-04620110 treatment compared to vehicle (Figure 4E and F). Total GIP AUC levels were significantly decreased 1.6-fold by PF-04620110, 4.2-fold by Orlistat and 3.9-fold by combination of PF-04620110 and Orlistat compared to vehicle (Figure 4G and H). The combination of this data suggests that inhibiting



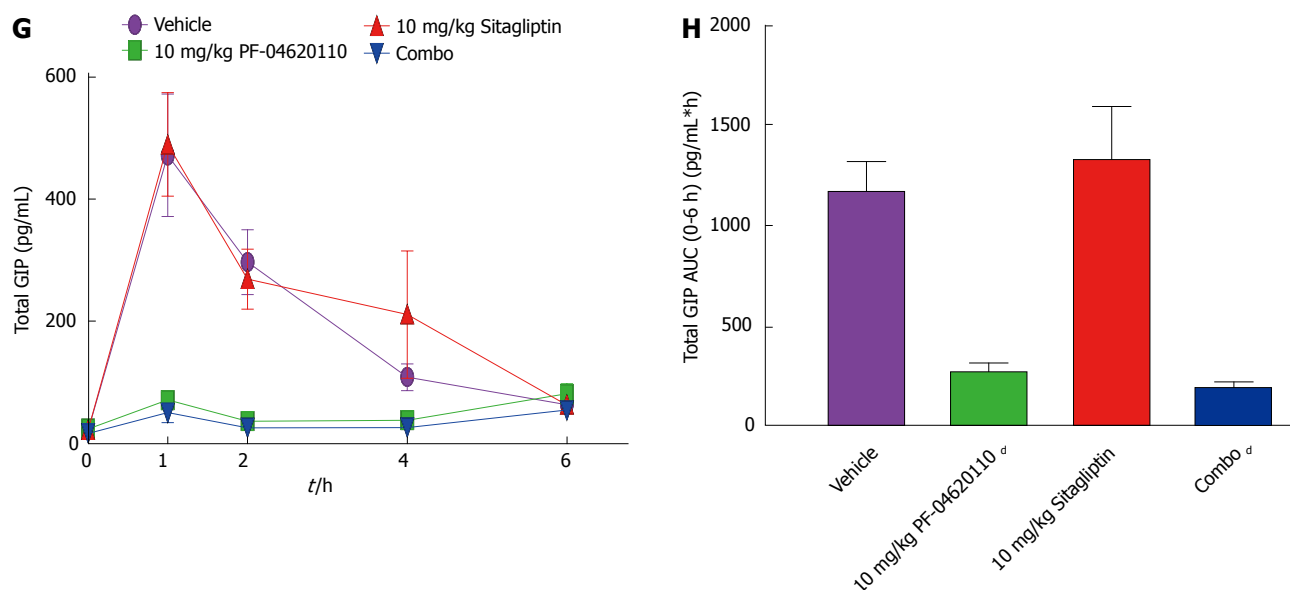
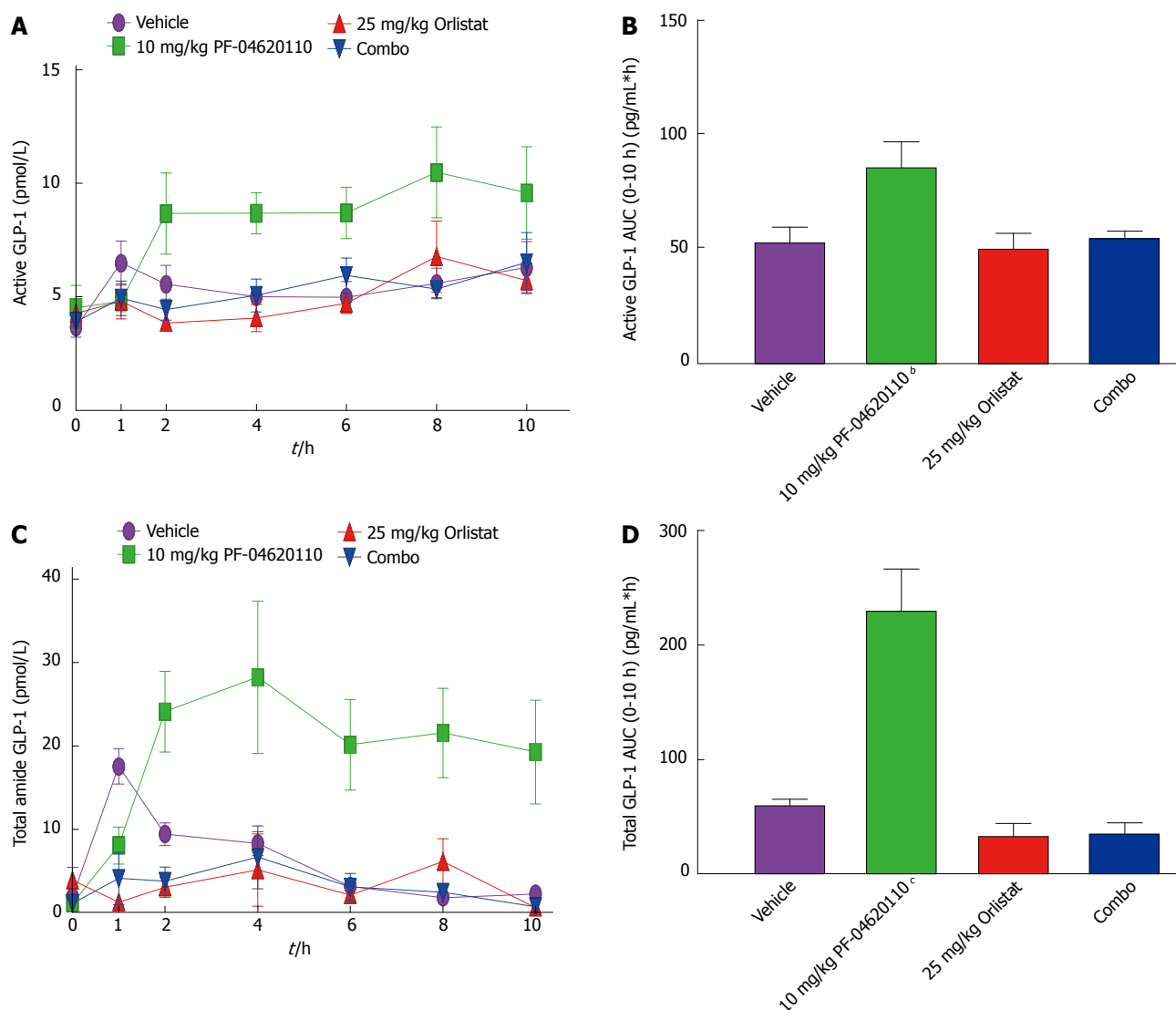


Figure 3 Incretin effect enhanced postprandially over time with combination pharmacological DPP-IV and diacylglycerol acyltransferase-1 inhibition. Fasted male C57BL/6J mice were administered vehicle, 10 mg/kg of PF-04620110 or Sitagliptin by oral gavage ($n = 8$ per group). Thirty minutes post compound administration mice received a bolus of corn oil (challenge). Blood was collected just prior to bolus (time 0) and at 1, 2, 4 and 6 h afterwards for determination of active/total GLP-1, PYY and GIP concentrations. (A) Active GLP-1 (pmol/L), (C) total GLP-1, (E) total PYY (pg/mL) and (G) total GIP (pg/mL) in C57BL/6J mice treated with vehicle or PF-04620110 following challenge. AUC values for (B) active GLP-1, (D) total GLP-1, (F) total PYY and (H) total GIP. DGAT1 inhibitor treatment compared to vehicle (^a $P < 0.05$, ^b $P < 0.01$, ^c $P < 0.001$ ANOVA). Values are means $\pm$ SEM. DGAT: Diacylglycerol acyltransferase-1; GLP-1: Glucagon like peptide-1; PYY: Peptide tyrosine-tyrosine.



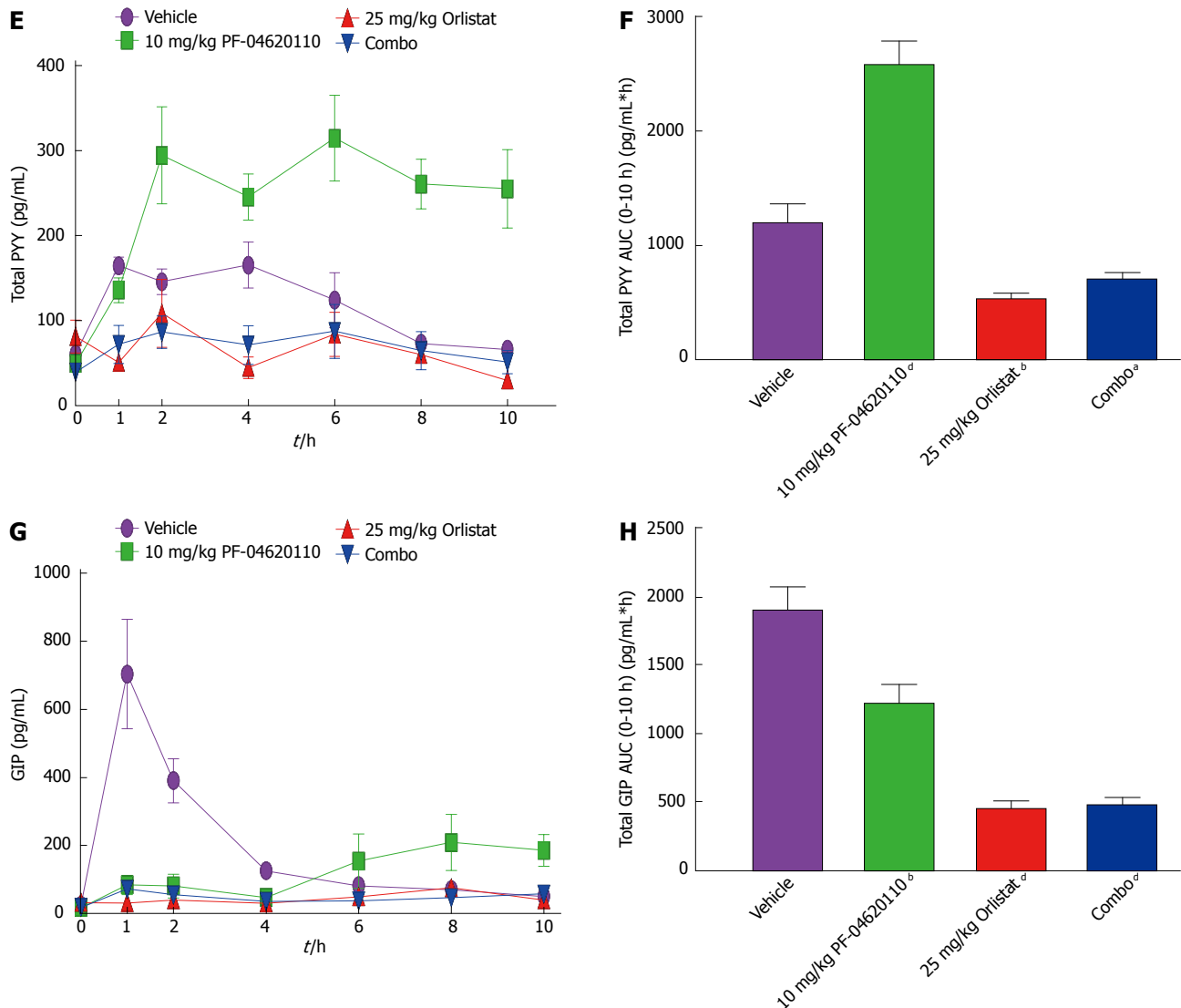


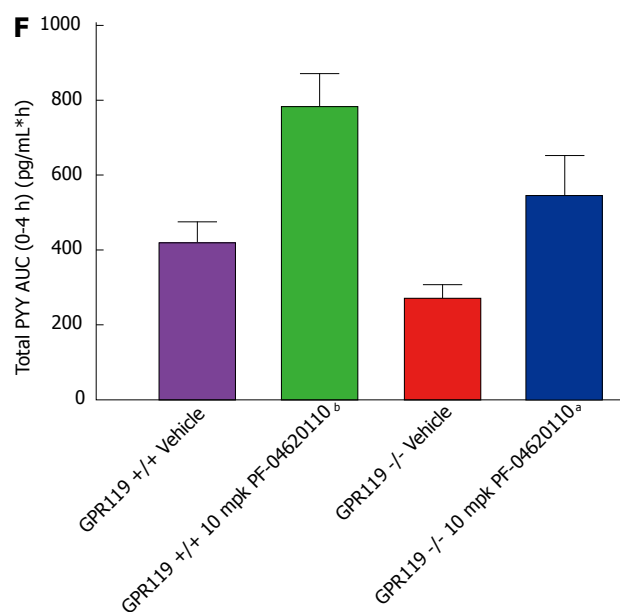
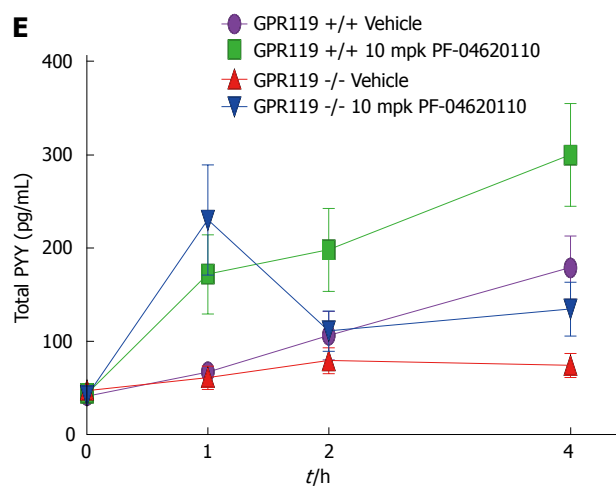
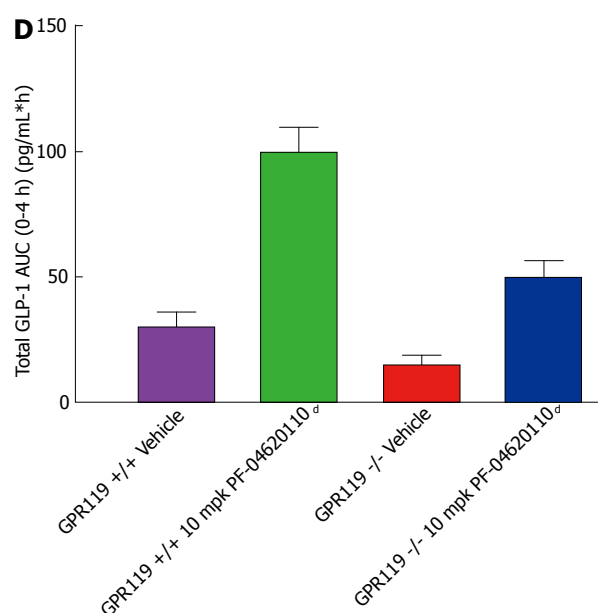
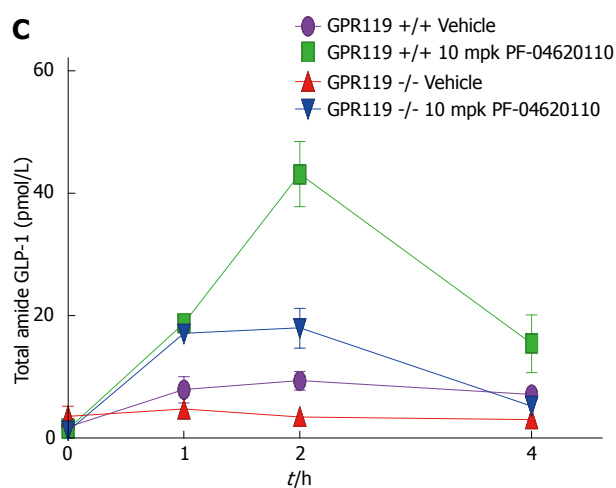
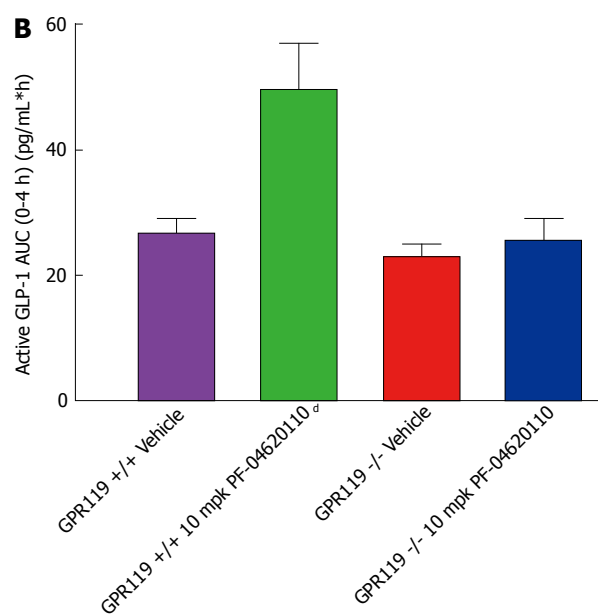
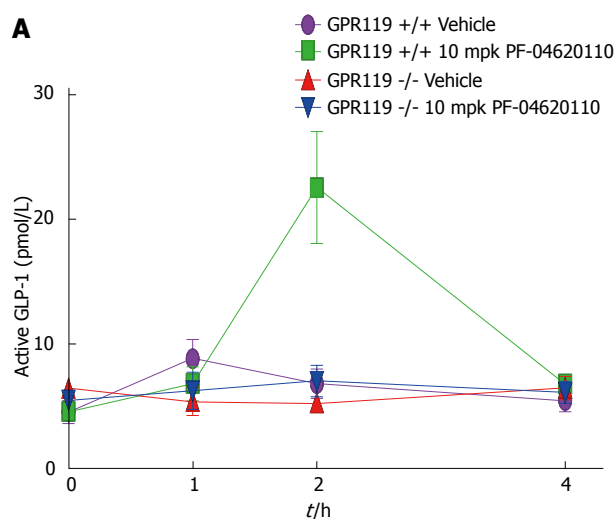
Figure 4 Incretin effect suppressed postprandially over time with combination orlistat and pharmacological diacylglycerol acyltransferase-1 inhibition. Fasted male C57BL/6J mice were administered vehicle, 10 mg/kg of PF-04620110 or orlistat (xenical) by oral gavage ($n = 8$ per group). Thirty minutes post compound administration mice received a bolus of corn oil (challenge). Blood was collected just prior to bolus (time 0) and at 1, 2, 4 and 6 h afterwards for determination of active/total GLP-1, PYY and GIP concentrations. (A) Active GLP-1 (pmol/L), (C) total GLP-1, (E) total PYY (pg/mL) and (G) total GIP (pg/mL) in C57BL/6J mice treated with vehicle or PF-04620110 following challenge. AUC values for (B) active GLP-1, (D) total GLP-1, (F) total PYY and (H) total GIP. DGAT1 inhibitor treatment compared to vehicle (^a $P < 0.05$, ^b $P < 0.01$, ^d $P < 0.001$ ANOVA). Values are means $\pm$ SEM. DGAT: Diacylglycerol acyltransferase-1; GLP-1: Glucagon like peptide-1; PYY: Peptide tyrosine-tyrosine.

pancreatic lipase blocks the effects of DGAT1 inhibition on postprandial incretin levels.

GPR119 deficiency suppresses altered postprandial incretin response seen with PF-04620110 administration in mice

To examine the incretin effect with whole body knockout of GPR119, GPR119 knockout and wildtype mice were administered with vehicle or PF-04620110 and monitored over a 4-h period during a lipid challenge. Levels of active GLP-1 AUC were significantly increased as expected over the 4-h excursion by 1.9-fold with PF-04620110 treatment in wildtype mice, but no change in active GLP-1 AUC levels was observed in vehicle treated knockout mice and PF-04620110 treated knockout mice compared to wildtype vehicle treated mice (Figure 5A

and B). Total GLP-1 AUC levels were increased 3.4-fold in wildtype mice treated with PF-04620110, decreased 2-fold in vehicle treated knockout mice and significantly increased 1.2-fold in PF-04620110 treated knockout mice compared to vehicle treated wildtype mice (Figure 5C and D). Similarly, levels of PYY AUC were increased 1.8-fold in PF-04620110 treated wildtype mice, slightly decreased by 1.5-fold in vehicle treated knockout mice and significantly increased by 1.3-fold in PF-04620110 treated knockout mice compared to vehicle treated wildtype mice (Figure 5E and F). Notably, increased levels of total GLP-1 and PYY in GPR119 knockout mice treated with PF-04620110 were lower than wildtype mice treated with PF-04620110. Total GIP AUC levels were significantly decreased 4.5-fold by PF-04620110 treated wildtype mice, 3.8-fold by vehicle treated knockout mice



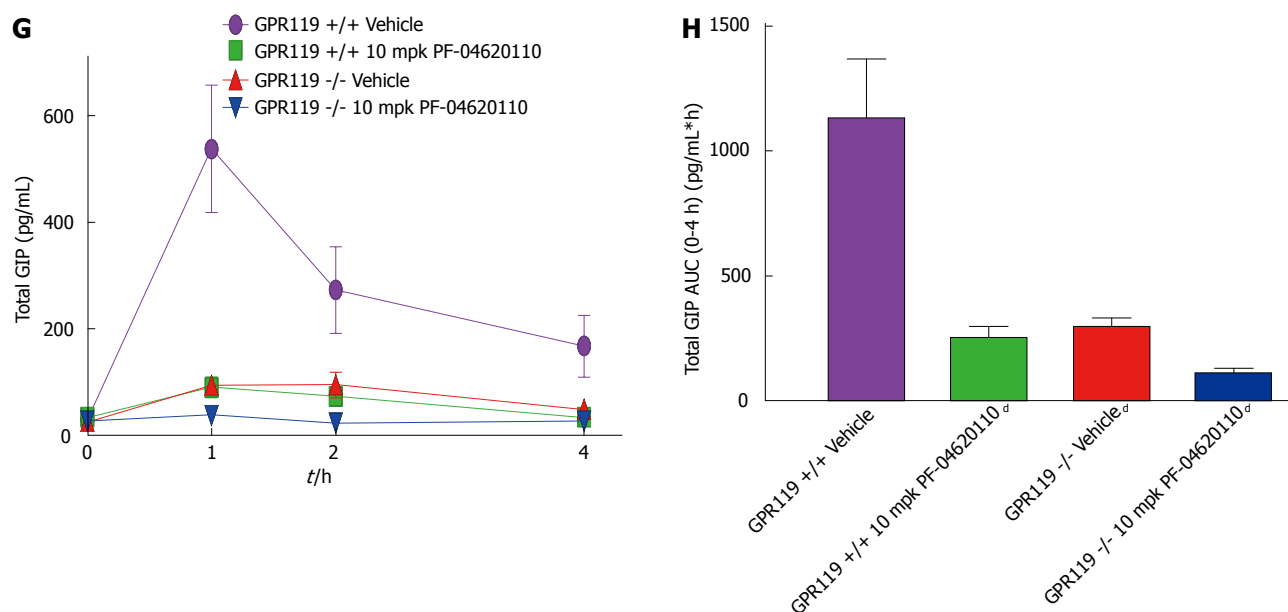


Figure 5 Incretin effect suppressed postprandially over time with pharmacological diacylglycerol acyltransferase-1 inhibition in GPR119 $-/-$ mice. Fasted male GPR119 $-/-$ and $+/+$ mice were administered vehicle or 10 mg/kg of PF-04620110 by oral gavage ($n = 8$ per group). Thirty minutes post compound administration mice received a bolus of corn oil (challenge). Blood was collected just prior to bolus (time 0) and at 1, 2, 4 and 6 h afterwards for determination of active/total GLP-1, PYY and GIP concentrations. (A) Active GLP-1 (pmol/L), (C) total GLP-1, (E) total PYY (pg/mL) and (G) total GIP (pg/mL) in C57BL/6J mice treated with vehicle or PF-04620110 following challenge. AUC values for (B) active GLP-1, (D) total GLP-1, (F) total PYY and (H) total GIP. Treatments compared to vehicle $+/+$ ($^{\circ}P < 0.05$, $^bP < 0.01$, $^dP < 0.001$ ANOVA). Values are means $\pm$ SEM. DGAT: Diacylglycerol acyltransferase-1; GLP-1: glucagon like peptide-1.

and 9.9-fold by PF-04620110 treated knockout mice compared to vehicle treated wildtype (Figure 5G and H). These data indicate that the altered incretin response with PF-04620110 inhibition is partially inhibited by knocking out GPR119.

DISCUSSION

In this study, we evaluated the role of DGAT1 in the secretion of gut derived hormones during a post-prandial lipid challenge. Previously, we have demonstrated that pharmacological inhibition of DGAT1 using PF-04620110 inhibits TG and vitamin A absorption in mice as well as delay gastric emptying, increase fecal fat and temporally shift triglyceride absorption downstream in the small intestine^[37]. These observations led us to further investigate how DGAT1 inhibition impacts the release of gut derived hormones. DGAT1 deficient mice have been previously demonstrated to have increased postprandial levels of circulating GLP-1 and our DGAT1 knockout mouse data is consistent with these studies^[44]. Interestingly, DGAT1 inhibition with PF-04620110 when compared to DGAT1 deficient mice results in a further elevation of GLP-1 and PYY plasma levels while attenuating GIP. The increase in GLP-1 and PYY occurs in a dose dependent manner. To elevate circulating levels even further, we tested the combined effect of DPP-IV (Sitagliptin) and DGAT1 (PF-04620110) inhibition for synergistic effects in mice following administration of inhibitors. Sitagliptin alone increases active GLP-1 but in combination prolongs the circulating active GLP-1 elevated by PF-04620110.

Due to our previous observations of the role of DGAT1 in the temporal and spatial absorption of dietary lipids, we speculated if the "signal" generated by DGAT1 inhibition to enhance gut hormone secretion was generated in the lumen of the gut or intracellularly within the enterocytes. DGAT1 is expressed on enterocytes whereas incretins are secreted from enteroendocrine L and K cells. To gain some insight, we used a pancreatic lipase inhibitor which blocks the luminal absorption of dietary lipids. Coadministration of Orlistat completely blunts the ability of PF-04620110 to elevate GLP-1 and PYY levels demonstrating a requirement of luminal TG degradation and lipid absorption. Thereby, these data suggest DGAT1 inhibition enhances the incretin response from the lumen and breakdown of lipids in the lumen are upstream of the effects of DGAT1 inhibition. We then hypothesize the mechanism by which DGAT1 inhibition effects incretin release is by increasing luminal long chain fatty acids and 2-MAG which could signal *via* GPR119 on enteroendocrine cells. To understand if GPR119, whose ligand, 2-monoacylglycerol is presumably being elevated *via* DGAT1 inhibition^[45], is playing a role in the ability of PF-04620110 to elevate gut hormones, we utilized GPR119 deficient mice. GPR119 deficient mice were treated with PF-04620110 and subjected to a TG tolerance test. GLP-1, PYY and GIP were all reduced postprandially and drastically reduced the PF-04620110 profile of GLP-1, PYY and GIP. The decrease in GLP-1 AUC demonstrated in GPR119 knockout mice in either vehicle or PF-04620110 treated mice is 50% of GPR119 wildtype mice, suggestive that effects of DGAT1 inhibition on gut hormone secretion

are partially dependent on GPR119. We speculate that additional long chain fatty acid/2-MAG GPCRs are also activated by DGAT1 inhibition. Collectively, these data suggest DGAT1 inhibition requires upstream TG hydrolysis and is partially mediated by altering GPR119.

The augmented plasma GLP-1 and attenuation of GIP observed with genetic deficiency and pharmacological inhibition of DGAT1 in rodents is in agreement with previously reported studies^[28,29,44-46]. It has been hypothesized that the delay in gastric emptying and temporal shift in intestinal lipid absorption in these rodent models can contribute to increased GLP-1 and attenuation of GIP^[47]. The reduction in GIP secretion *via* DGAT1 inhibition is potentially affecting K cell signaling by increasing lipid transit bypassing the proximal portion of the intestine. Apical L cell stimulation *via* GLP-1 and PYY release would also be enhanced with increased gut transit as more lipids end up further down the GI tract. The PF-04620110 mediated increase in PYY is consistent with other studies as well as being increased in DGAT1 deficient mice, but the mechanism of action is not well understood^[44,45]. PYY secreting L cells are located predominantly in the lower portion of the gut (ileum and colon)^[48]. Since it is clear inhibiting the resynthesis of TG through DGAT1 inhibition is causing an increase in PYY concurrent to GLP-1 release, it is most likely occurring through an ancillary pathway.

The incretin response is altered *via* the lipid load as well as the degree of FA saturation presented to the K and L cell population in the intestine^[17]. The dose responsiveness of the enhanced gut hormone response following DGAT1 inhibition suggests the "signal" is potentially the generation of ligands for fatty acid GPCRs. The exact molecular mechanism of how inhibition of DGAT1 in the enterocytes results in impacts on enteroendocrine cells within the intestine remains to be fully understood. While DGAT1 plays a major role in endogenous TG synthesis, it does not rule out other enzyme contributions as residual TG synthesis is present in DGAT1 deficient mice and other enzymes like DGAT2 could compensate for the absence of DGAT1^[49]. One limitation to these experiments includes measuring these gut hormones only 6 h post corn oil administration in mice. It is possible that the GIP signal returns at later time points when the lipid load reaches the distal sections of the intestine. This has been observed in DGAT1 inhibitor treated canines where GIP signaling returned after 4 h^[45].

The prolonged incretin signal produced by the combination of DPP-IV inhibitor Sitagliptin and PF-04620110 is in agreement with a published study using a different DGAT1 inhibitor^[45]. In humans, the DPP-IV inhibitor Vildagliptin singularly increased active GLP-1 and GIP circulation while decreasing PYY, glucose and increasing insulin in the presence of intraduodenal fat infusion^[50]. While we see similar effects with Sitagliptin alone in our mouse study, the addition of PF-04620110 is able to further enhance active GLP-1 indicating the dose combination could be beneficial in a clinical setting.

Increased PYY circulation seen with combination of PF-04620110 and Sitagliptin compared to vehicle could also potentially have long term effects of suppressed appetite and weight loss, but further studies are needed to understand this combined compound effect. The notion that a fixed dose combination therapy of a DPP-IV and DGAT1 inhibitors would provide additional therapeutic benefit than either moiety alone is supported by these studies.

Combined oral dosing of the pancreatic lipase inhibitor Orlistat and PF-04620110 results in a blocking of GLP-1, PYY and GIP release into circulation. This provides further evidence that the DGAT1 inhibitor induced gut hormone response is being mediated by a lipid or lipid derived signal emanating from within the enterocyte. Fatty acids being used as gut peptide signaling have been studied previously with the use of Orlistat to block the initial breakdown of fat in the intestinal lumen^[51,52]. This combined dosing also seems to be blocking all other methods of TG synthesis in the enterocytes since there is complete gut peptide inhibition, giving us more confidence that lipids or their derivatives are the likely secretagogue signal.

Increasing FFA in L cell rich areas of the intestine could signal G-protein coupled receptors (GPCR) with GLP-1 and PYY secretagogues capabilities. To this end, we investigated the administration of PF-04620110 to mice deficient in GPR119, a GPCR present in both beta-cells and intestinal L cells which are activated by FFA including 2-MAG^[39]. Incretin levels and PYY were drastically decreased in these GPR119 deficient mice even in the presence of PF-04620110. This suggests that lacking GPR119 cannot be rescued by PF-04620110 and the effects of DGAT1 inhibition on gut hormone secretion are partially dependent on GPR119 and potentially could be acting as a GPR119 inhibitor by limiting lipid exposure to enteroendocrine cells. It is hypothesized that GPR119 may promote glycemic control *via* various incretin mechanisms and is known to be co-expressed in GLP-1 producing L cells^[39]. GPR119 agonists *in vivo* have been shown to increase GLP-1 and GIP as well as reverse these effects in GPR119 deficient mice^[39]. Furthermore, other GPCRs have the capability of using FA signals *via* incretin response to induce insulin secretion including GPR120 and GPR40^[41,42]. It is possible that the lipid signals created by DGAT1 are affecting other GPCRs indicated by the slight increase in GLP-1 and PYY seen in the GPR119 knockout mice with PF-04620110 administration. Future studies exploring the combined effects of GPR119 agonists and DGAT1 inhibitors on gut hormone secretion might result in an additive response.

The importance of gut hormones and their role in glucose and lipid absorption only become more significant with the ongoing development of novel gut hormone altering pharmaceuticals. Incretin mimetics and incretin enhancers have become staples in the battle against T2DM and continue to offer hope towards controlling hyperglycemia and insulin secretion with varying degrees of side effects. Understanding the

mechanism behind what alters the secretion of gut hormones could help to determine if combinatorial therapy could be beneficial or detrimental in controlling side effects or further increase the benefits seen with current dosing regimens. Pharmacological inhibition of DGAT1 can postprandially increase incretin hormones and PYY. Furthermore, combined dosing of a DPP-IV inhibitor with DGAT1 inhibition can synergistically extend the benefits seen with DGAT1 inhibition. Collectively these data provides new insights for the impact of pharmacological DGAT1 inhibition and future long term studies in T2DM patients.

COMMENTS

Background

Incretin hormones such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP) are released from the enteroendocrine cells into the circulation in response to dietary nutrition. Peptide tyrosine-tyrosine is also a gut released hormone involved in energy homeostasis. Impaired incretin effect in type 2 diabetics could in part be responsible for defective insulin release, beta-cell failure and often have alterations in fat metabolism, in particular elevated plasma fatty acids and triglycerides. Acyl-CoA:diacylglycerol acyltransferase 1 (DGAT1), catalyzes the final step in triacylglycerol formation. In the intestine, DGAT1 is the predominate enzyme in the re-esterification of dietary TG and its inhibition or deletion in rodents has increased levels of GLP-1 in circulation. While the evidence regarding the importance of the DGAT1 enzymatic downstream effect on incretin release is growing, the mechanism of action still remains elusive.

Research frontiers

Previous intestinal studies have hinted towards FA signaling molecules and their involvement in incretin hormone release, but the mechanisms behind this release remains not fully understood.

Innovations and breakthroughs

This study is the first to evaluate how DGAT1 inhibition manipulates lipid signaling in the gut and its involvement in manipulating G-protein coupled receptor signaling such as GPR119 in enteroendocrine cells to alter the incretin effect.

Applications

Examining oral lipid absorption in mice has been a proven way to relate to human intestinal physiology and the ability to delay lipid absorption with DGAT1 inhibitors is a way to study how lipid signals in the gut effect incretin hormone release. Utilizing genetic ablation mouse models and different drug combination therapies to study intestinal absorption can provide us more insight into the mechanism by which inhibition of DGAT1 enhances intestinal hormone release and its relevance to humans.

Terminology

Type 2 diabetes mellitus is a complex disease caused by a variety of issues that ultimately leads to insulin resistance and uncontrolled levels of glucose in the blood. The incretin effect is a complex nutrient sensing system consequential of food ingestion put in place to control glucose homeostasis and ultimately leads to insulin secretion. This effect is made up of gut hormones GLP-1 and GIP produced predominantly by enteroendocrine cells of the gut lumen.

Peer-review

The experimental work presented in the manuscript is well designed and of good quality, data are presented in the logical manner.

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P- Reviewer: Akiba Y, Efanov AM **S- Editor:** Ji FF **L- Editor:** A
E- Editor: Lu YJ



Basic Study

Effects of commercially produced almond by-products on chemotherapy-induced mucositis in rats

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Author contributions: Whittaker AL, Zhu Y, Howarth GS, Bastian SEP and Wirthensohn MG designed the research; Whittaker AL, Zhu Y and Loung CS performed the research; Whittaker AL, Zhu Y and Loung CS analyzed the data; Whittaker AL, Zhu Y, Howarth GS, Bastian SEP and Wirthensohn MG wrote the paper.

Supported by Horticulture Australia Limited.

Institutional review board statement: The use of animals for teaching, research or experimentation is regulated by State legislation - the South Australian Animal Welfare Act 1985. The University of Adelaide is licensed under the Act to acquire and use animals only when approval has been granted by its Animal Ethics Committee (AEC). No animal may be held or used for any purpose until written approval has been obtained from the AEC. Internal approval for this study was obtained from the AEC.

Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the Animal Ethics Committee of the University of Adelaide (protocol number: s2014-162) and conducted in accordance with the provisions of the Australian Code for the Care and Use of Animals for Scientific Purposes.

Conflict-of-interest statement: The authors have no conflict of interest.

Data sharing statement: No additional data are available.

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Manuscript source: Unsolicited manuscript

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Received: March 17, 2017

Peer-review started: March 20, 2017

First decision: May 3, 2017

Revised: May 20, 2017

Accepted: June 30, 2017

Article in press: July 3, 2017

Published online: November 15, 2017

Abstract**AIM**

To determine if almond extracts reduce the severity of chemotherapy-induced mucositis as determined through biochemical, histological and behavioural markers.

METHODS

Intestinal mucositis is a debilitating condition characterized by inflammation and ulceration of the gastrointestinal mucosa experienced by cancer patients undergoing chemotherapy. Certain bioactive plant products have shown promise in accelerating mucosal repair and alleviating clinical symptoms. This study evaluated almond extracts for their potential to reduce the severity of chemotherapy-induced mucositis in Dark Agouti rats. Female Dark Agouti rats were gavaged (days 3-11) with either PBS, almond hull or almond blanched water

extract at two doses, and were injected intraperitoneally with 5-fluorouracil (5-FU-150 mg/kg) or saline on day 9 to induce mucositis. Burrowing behavior, histological parameters and myeloperoxidase activity were assessed.

RESULTS

Bodyweight was significantly reduced in rats that received 5-FU compared to saline-treated controls ($P < 0.05$). Rats administered 5-FU significantly increased jejunal and ileal MPO levels (1048%; $P < 0.001$ and 409%; $P < 0.001$), compared to healthy controls. Almond hull extract caused a pro-inflammatory response in rats with mucositis as evidenced by increased myeloperoxidase activity in the jejunum when compared to 5-FU alone (rise 50%, 1088 ± 96 U/g *vs* 723 ± 135 U/g, $P = 0.02$). Other extract-related effects on inflammatory activity were minimal. 5-FU significantly increased histological severity score compared to healthy controls confirming the presence of mucositis (median of 9.75 *vs* 0; $P < 0.001$). The extracts had no ameliorating effect on histological severity score in the jejunum or ileum. Burrowing behavior was significantly reduced in all chemotherapy-treated groups ($P = 0.001$). The extracts failed to normalize burrowing activity to baseline levels.

CONCLUSION

Almond extracts at these dosages offer little beneficial effect on mucositis severity. Burrowing provides a novel measure of affective state in studies of chemotherapy-induced mucositis.

Key words: Cancer; Mucositis; Burrowing; 5-Fluorouracil; Almond

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Core tip: In spite of their procyanadin content and antioxidant capacity the almond extracts tested failed to provide any ameliorating effect on chemotherapy-induced mucositis in rats as determined through biochemical and histological evaluation. Currently studies utilising animal models of gastrointestinal disease largely fail to assess measures of patient affect. In order to achieve successful translational outcomes assessment of patient experience is important since these disease conditions are often self-limiting. Burrowing behaviour shows promise as a novel measure of affective state in studies of chemotherapy-induced mucositis.

Whittaker AL, Zhu Y, Howarth GS, Loung CS, Bastian SEP, Wirthensohn MG. Effects of commercially produced almond by-products on chemotherapy-induced mucositis in rats. *World J Gastrointest Pathophysiol* 2017; 8(4): 176-187 Available from: URL: <http://www.wjgnet.com/2150-5330/full/v8/i4/176.htm> DOI: <http://dx.doi.org/10.4291/wjgp.v8.i4.176>

INTRODUCTION

Patients undergoing chemotherapy treatment for

cancer frequently experience severe side-effects which can cause significant deterioration to quality of life^[1]. A common side-effect, affecting approximately 60% of patients is mucositis^[2]. The term mucositis refers to inflammation and ulceration of the gastrointestinal tract. Anatomical sites commonly affected are the buccal mucosa (oral mucositis), and the small intestinal mucosa (intestinal mucositis)^[3]. Histologically, intestinal mucositis is characterized by crypt cell apoptosis and hypo-proliferation, villous atrophy, and ulcerative lesions^[4]. These pathophysiological processes result in a range of clinical symptoms which may include pain, diarrhea, nausea and inappetence^[5]. The severe nature of the symptoms can lead to patient-requested reduction of chemotherapy dose. Additionally, intestinal mucositis can increase the frequency of peripheral parenteral nutrition prescription, which can predispose to high morbidity. Any reduction in therapeutic level has the potential to impact on tumor kill rates; hence the need to discover new treatments or preventative agents which will reduce mucositis side-effects^[6]. These treatments should not just provide symptomatic relief but target the pathogenesis of the condition^[7].

Almonds belong to the genus *Prunus* or *Amygdalus*. A number of fruit extracts from these genera have been investigated for their anti-cancer properties. Many have had moderate success in suppressing growth and reducing proliferation in *in-vitro* studies using various cancer cell lines^[8-11]. Almonds have also been determined to play a chemo-preventative role^[12,13], and to exhibit anti-inflammatory properties^[14]. It should be noted that cultivated (sweet) almonds differ in composition to bitter almonds^[15]. Bitter almonds contain amygdalin which releases hydrocyanic acid^[16]. Amygdalin has been widely proposed as a potential anti-cancer treatment. However, amygdalin use has been controversial due to the risk of cyanide poisoning, and its effectiveness has not been substantiated^[17].

The various health related benefits conferred through sweet almond consumption are presumed to be due to their content of arginine, fiber, mono- and polyunsaturated fat, vitamin E, and other nutrients. Presence of phenolic acids and polyphenols may also contribute to their health profile^[18]. Procyanidins are a class of polyphenolic compounds that have bioactive properties^[19]. Grape-seed extract has recently shown promise in the alleviation of intestinal injury in chemotherapy-induced mucositis, and at enhancing chemotherapeutic effectiveness in colon cancer cell lines^[19]. This beneficial effect is thought to be brought about through the action of the procyanidins. Almonds also contain procyanidins^[12], and thus they may similarly ameliorate the gut inflammation caused by chemotherapy administration.

This study was designed to assess whether two types of sweet almond extracts were safe and efficacious in the prevention of chemotherapy-induced mucositis damage in a rat model. Furthermore, we incorporated a behavioral measure of affective state (burrowing) into the study in order to determine validity of the technique, and

to provide a further measure of therapeutic outcome.

MATERIALS AND METHODS

Almond extract preparation

This study utilized two different by-products of the commercial almond production process; almond hull extract (AHE) and almond blanched water (ABW). Almond hulls (50 g; Riverland, South Australia) were extracted in 1250 mL of reverse osmosis treated water in a slow cooker. Temperature within the cooker increased from 21 °C to 80 °C over 5 h. Following this, the dry matter was maintained in water overnight until the solution was completely cooled. Extract was then stored at 4 °C until use. Almond blanched water was sourced from a different processing plant (Almond Co., Riverland, South Australia) and was taken directly from the process line and stored at 4 °C until use. The total polyphenol levels and antioxidant capacity of both sources were measured using the total polyphenol gallic acid equivalent test and L-ascorbic acid equivalent test described below.

Both extracts were prepared similarly by drying 1 mL of the stored extracts in a centrifuge dryer (45 °C, 3–4 h). Using the gravimetric method, the resultant dry matter was measured and re-dissolved in 1.5 mL Dulbecco's Modified Eagle's Minimum Essential Medium (DMEM) (Gibco BRL, Life Technologies Pty Ltd, Australia). This process generated concentrations of 24.6 mg/mL and 7.08 mg/mL of AHE and ABW, respectively.

Total polyphenol gallic acid equivalent test

Total phenolic content (TPC) was measured by the reaction between gallic acid and Folin-Ciocalteu reagent and described as gallic acid equivalent (TPC-GAE). Samples were added in triplicate to non-sterile 96 well plates and incubated with 100 µL Folin-Ciocalteu reagent for 5 min. Sodium carbonate solution (300 µL, 20% w/v) was added and further incubated for 4 h in the dark. The calibration curve was prepared as follows: 20 µL of gallic acid ethanol solution at concentrations of 0.23 mmol/L, 0.46 mmol/L, 0.92 mmol/L, 1.84 mmol/L, 3.68 mmol/L, 7.35 mmol/L. The plate was read at 765 nm by a spectrometer (BioRad UV, NSW, Australia). Measurement of TPC in the extracts was performed using 20 µL of the extract in place of the gallic acid. Data were expressed as gallic acid equivalents (TPC-GAE).

Antioxidant capacity l-ascorbic acid equivalent test

Antioxidant capacity was measured by the reaction between L-ascorbic acid and 1,1 diphenyl-2-picrylhydrazyl (DPPH). Samples (0.1 mL) were added in triplicate to non-sterile 96 well plates and incubated with 1.5 mL of DPPH ethanol solution (25.36 mmol/L) for 30 min in the dark. The calibration curve was prepared as follows: 0.1 mL of L-ascorbic acid ethanol solution as concentrations of 110.9 µmol/L, 221.8 µmol/L, 443.5 µmol/L, 887 µmol/L, 1774 µmol/L. The plate was read at 515 nm by a spectrometer (BioRad UV, NSW, Australia). Data were

expressed as ascorbic acid equivalents (AC-AAE).

Animals and experimental design

The animal protocol was designed to minimize pain or discomfort to the animals. Female DA/Arc rats (100–140 g, $n = 80$) were sourced from a barrier-maintained Specific-Pathogen Free production facility (Laboratory Animal Services, the University of Adelaide, Adelaide, SA, Australia). Following facility acclimatization they were housed individually in metabolic cages (Tecniplast, Exton, PA, United States). Room temperature was maintained at a constant 22 °C with a 12 h reversed light-dark cycle (lights off at 0800). Rats were provided *ad libitum* access to food (18% casein-based diet)^[20] and water purified by reverse osmosis. This study was approved by the Animal Ethics Committee of the University of Adelaide and conducted in accordance with the provisions of the Australian Code for the Care and Use of Animals for Scientific Purposes^[21].

Rats were stratified into 10 treatment groups ($n = 8$) on the basis of bodyweight and burrowing ability (see later) such that both covariates demonstrated similar mean values between groups. Treatments were: Phosphate-buffered saline (PBS) + saline injection, AHE 31.25 µg/mL + saline injection, AHE 1000 µg/mL + saline injection, ABW 31.25 µg/mL + saline injection, ABW 1000 µg/mL + saline injection, PBS + 5-fluorouracil (5-FU), AHE 31.25 µg/mL + 5-FU injection, AHE 1000 µg/mL + 5-FU injection, ABW 31.25 µg/mL + 5-FU injection, ABW 1000 µg/mL + 5-FU injection. The low and high dose extracts were referred to as (1) and (2) respectively. Dose selection was based on previous *in vitro* data (unpublished). Rats were acclimatized to metabolic cages on experimental days 0–2. On experimental days 3–11 rats were gavaged daily with 1 mL of PBS or almond extract dependent on group allocation. On day 9 rats were injected intraperitoneally with either saline or 5-FU (150 mg/kg, Mayne Pharma Pty Ltd. Mulgrave, Vic, Australia).

Daily measurements of bodyweight, food and water intake and urine and fecal output were performed. On day 12, rats were humanely killed by CO₂ asphyxiation followed by cervical dislocation. Visceral organs were weighed. Lengths and weights of the small intestine, duodenum and colon were recorded. All lengths were measured unstretched prior to emptying of content. Weights were subsequently measured after gastrointestinal content had been removed. Midsection samples (2 cm) of the gastrointestinal organs were collected into 10% buffered formalin for histological analysis. Further 4 cm samples were snap frozen in liquid nitrogen and stored at -80 °C for later biochemical analysis.

Burrowing assessment

The burrowing apparatus was adapted from Andrews *et al.*^[22] 2012. Burrows were hollow plastic tubes (200 mm length × 90 mm diameter) sealed at one end with a plastic cap. The open end was raised 60 mm above the cage floor by the use of a plastic stand in order to

minimize gravel loss when placed in the test cage. Each burrow was filled with 1 kg of gravel (7 mm pea-shingle decorative gravel; Tuscan Path, Hawthorn East, Victoria, Australia). The test cage was a standard open-top rat cage (590 mm × 385 mm × 200 mm; Tecniplast, Rydalmere, New South Wales, Australia) with the burrow placed to the rear of the cage. These cages were devoid of bedding substrate. In order to reduce the effects of cage novelty on propensity to perform burrowing behaviour, animals were always placed in the same cage for testing.

Rats were habituated to the test cages and burrowing apparatus in pairs on two consecutive days prior to experimental treatments (experimental days -2 and -1). To perform the test, rats were removed from their home cages and placed into the test cage for one hour. Following the test hour the remaining gravel in the burrow was weighed and the volume of gravel displaced calculated. Rats were assessed for level of baseline burrowing on experimental days 1 and 2. The mean of these two values was used to determine baseline burrowing level and to select animals into their treatment groups. Since, it had been previously shown that there was wide inter-individual variability in propensity to burrow^[23], burrowing ability was categorised as good or poor using the method described by Deacon 2006^[24]. Animals were then allocated into their treatment groups to include an equivalent number of good and poor burrowers in each group. Post-treatment burrowing was performed on experimental day 12. On test days burrowing was conducted between 1000 and 1200 h.

Myeloperoxidase activity assay

Small intestinal tissue samples (4 cm) of jejunum and ileum were thawed on ice and homogenized with 1.5 mL of phosphate buffer (10 mmol/L, pH 6.1) for 60 s until the solution was homogenous. Homogenates were stored at -80 °C until required for assay.

Level of myeloperoxidase (MPO) in the intestinal tissue was determined by a slight modification of the assay described by Krawisz *et al.*^[25]. Tissue homogenates were thawed on ice and centrifuged at 13000 g for 13 min. The supernatant was discarded and cell pellets were re-suspended in hexadecyltrimethyl ammonium bromide (0.5%, pH 6.0). Samples were then vortexed for 2 min and centrifuged at 13000 g for 3 min. Supernatants were reacted with *o*-dianisidine and absorbance measured at 450 nm at 1 min intervals for a period of 15 min using a microplate reader (Sunrise Microplate Reader, Tecan Austria GmbH, Grodig, Austria). Absorbance readings were used to calculate MPO activity using a pre-designed macro. Activity was expressed as units MPO per gram of tissue.

Histological analysis

The collected tissue samples were formalin-fixed for 24 h and then transferred to 70% ethanol. Transverse tissue sections (4 µm) were embedded in paraffin wax, H&E stained and viewed using a light microscope. The histological disease severity score was assessed semi-

quantitatively based on scoring of eight independent histological criteria using a slight modification to the method described by Howarth *et al.*^[26] 1996. The criteria scored were: villus blunting, crypt distortion, reduction in goblet cell number, dilation of lymphatics, thickening of the submucosa, thickening of the muscularis externa, enterocyte disruption and lymphocytic and polymorphonuclear cell infiltration. Each criterion was scored from zero (normal) to three (maximal damage) and expressed as a median score. Saline control rat intestinal tissue was used as a baseline reference to grade the criteria. Villus heights and crypt depths (40 villi and 40 crypts per section) were determined in the jejunal and ileal sections as described in Howarth *et al.*^[26]. All microscope-based analyses were performed in a blinded fashion using a light microscope (Nikon, ProgRes® CS, Tokyo, Japan) and image ProPlus software version 5.1 (Media Cybernetics, Silver Spring MD, United States).

Statistical analysis

Statistical analyses were conducted using PASW 21 (SPSS, Inc., Chicago, IL, United States) and Megastat Excel Add-In (version 10.2, McGraw-Hill Higher Education, New York, NY, United States). Data were tested for normality using the Shapiro-Wilk test. All parametric data including bodyweight, daily metabolic data, histological parameters, MPO and villus height and crypt depth, were compared using one-way analysis of variance (ANOVA) with a Tukey's *post-hoc* test. Burrowing behavior was analyzed using a 3-way ANOVA with treatment, injection type and time as fixed factors, both adjusted and unadjusted for baseline burrowing values. The histological disease severity score was analyzed by a Kruskal-Wallis test with a Mann Whitney *U*-test to determine between-group significance. Significance was determined at $P < 0.05$.

RESULTS

Phenolic content and anti-oxidant capacity

The phenolic content of AHE and ABW as measured by the polyphenol gallic acid equivalent test was 1.06 g/L and 0.48 g/L Respectively. Anti-oxidant capacity measured by the L-ascorbic acid equivalent test yielded values of 0.27 mg/mL for AHE, and 0.25 mg/mL for ABW.

Daily metabolic measures

Administration of 5-FU significantly increased urine output and reduced bodyweight and food intake compared to rats that received a saline injection (Table 1). Despite the increase in urine output a concomitant increase in water intake was not observed except in the group receiving 5-FU + ABW1. The treatments provided no advantage in terms of normalizing these clinical parameters in the chemotherapy-treated groups.

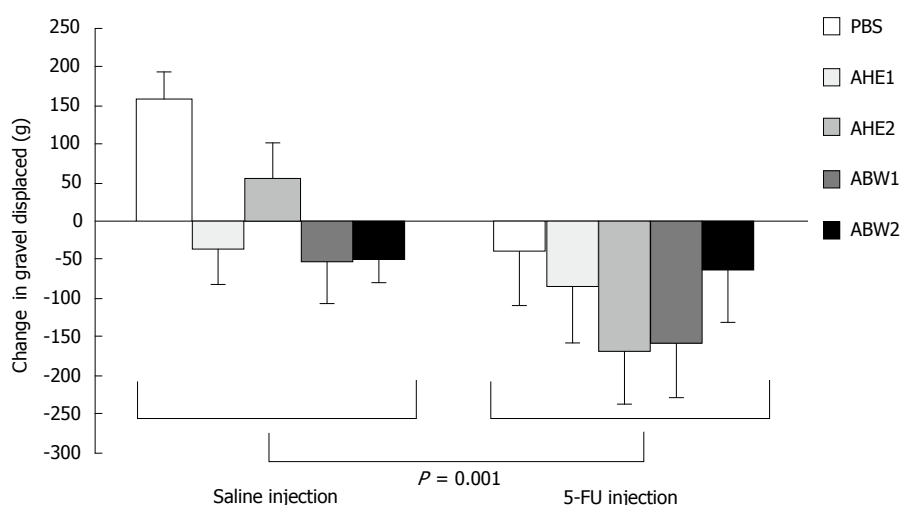
Burrowing behaviour

Simple main effects analysis demonstrated a change in mass of gravel displaced between baseline values and

Table 1 Effects of almond extracts on bodyweight change, food and water intake and urine and faeces output in saline-injected rats and in 5-fluorouracil injected rats from days 10-12

	Sal + PBS	Sal + AHE1	Sal + AHE2	Sal + ABW1	Sal + ABW2	5-FU + PBS	5-FU + AHE1	5-FU + AHE2	5-FU + ABW1	5-FU + ABW2
Body weight change with respect to day 9(%)	3.7 ± 0.6	4.0 ± 1.0	1.6 ± 0.9	2.3 ± 1.1	2.6 ± 0.9	-7.2 ± 1.5 ^b	-9.5 ± 1.9 ^b	-7.3 ± 1.3 ^b	-7.0 ± 1.1 ^b	-7.2 ± 1.2 ^b
Water intake (mL/kgbw)	256 ± 49	247 ± 28	184 ± 18	236 ± 22	200 ± 28	275 ± 27	244 ± 18	296 ± 22	348 ± 38 ^a	284 ± 28
Food intake (%bw)	6.7 ± 0.5	6.9 ± 0.5	6.9 ± 0.4	6.7 ± 0.6	6.4 ± 0.5	5.1 ± 0.3 ^b	5.2 ± 0.4 ^a	5.4 ± 0.3 ^a	4.9 ± 0.3 ^b	4.8 ± 0.3 ^b
Urine output (mL/kgbw)	103 ± 11	99 ± 12	98 ± 8	103 ± 9	89 ± 6	175 ± 19 ^b	202 ± 20 ^b	180 ± 25 ^b	215 ± 19 ^b	164 ± 18 ^b
Faeces output (g/kgbw)	39 ± 9	31 ± 5	40 ± 10	34 ± 6	24 ± 3	31 ± 4	35 ± 5	29 ± 6	31 ± 4	28 ± 5

Data are expressed as means ± SEM. 0.05. ^a*P* < 0.05 *vs* saline + PBS, ^b*P* < 0.01 *vs* saline + PBS. 5-FU: 5-fluorouracil; PBS: Phosphate-buffered saline; AHE: Almond hull extract; ABW: Almond blanched water.

**Figure 1** Effects of almond extracts on change in mass of gravel displaced between baseline values and 72 h following 5-fluorouracil or saline injection. Data are expressed as mean ± SEM. 5-FU: 5-fluorouracil; PBS: Phosphate-buffered saline; AHE: Almond hull extract; ABW: Almond blanched water.

72 h following chemotherapy injection between groups administered chemotherapy versus those receiving saline. Chemotherapy treated groups having a significantly greater, and more negative, change in mass of gravel displaced in comparison to all saline-treated groups [*F* (2, 68) = 7.514, *P* = 0.001; Figure 1]. However, there was no interaction between type of injection (5-FU or saline) and almond extract administered *F* (4, 68) = 1.41, *P* = 0.24.

Visceral organs

Administration of 5-FU significantly reduced thymus weight by 50% (*P* < 0.001), spleen weight by 22% (*P* = 0.01) and increased caecum weight by 38% (*P* = 0.002) compared to saline-injected rats. Whilst almond extracts did not prevent the occurrence of these 5-FU induced changes, in general they failed to produce any negative effects on organ weights (Table 2). However, ABW2 caused an increase in liver weight in advance of that seen in chemotherapy-treated control animals by ABW2 (15%, *P* = 0.005).

Similarly, 5-FU significantly reduced the length of the jejunum + ileum (5% reduction, *P* = 0.03) and colon (12% reduction, *P* = 0.02) (Table 3). A corresponding decrease in weight of the jejunum + ileum occurred

(9% reduction, *P* = 0.02) although a similar weight reduction in the colon was not evidenced, and in fact the contrary occurred with a general increase in weight in 5-FU treated animals. This weight increase showed significance in the groups treated with 5-FU and AHE2, ABW1 and ABW2 (respective *P* values 0.03, 0.001 and 0.009). This increase in colon weight showed a trend towards significance (*P* = 0.06) in the 5-FU + PBS treated animals in comparison with saline control animals (Table 3).

Myeloperoxidase activity

Injection of 5-FU caused a significant (*P* < 0.001) increase in MPO activity in the proximal jejunum and ileum of 5-FU + PBS treated rats (1048% and 409% respectively) compared to saline + PBS control animals (Figure 2).

In the jejunum AHE1 caused a statistically significant elevation in MPO activity in comparison with chemotherapy-treated control animals (50%, *P* = 0.02). This effect was not observed in the ileum. All other almond extracts had no ameliorating effect on 5-FU induced MPO activity, but did not worsen the effect. There was no significant difference in MPO activity between almond extract-treated animals and PBS-treated animals determining that almond extracts caused no adverse

Table 2 Effects of almond extracts on organ weights of female Dark Agouti rats 72 hr after 5-fluorouracil or saline injection

	Sal + PBS	Sal + AHE1	Sal + AHE2	Sal + ABW1	Sal + ABW2	5-FU + PBS	5-FU + AHE1	5-FU + AHE2	5-FU + ABW1	5-FU + ABW2	5-FU + AHE2
Thymus	20 ± 1	21 ± 1	19 ± 1	20 ± 1	21 ± 1	10 ± 2 ^b	10 ± 3 ^b	8 ± 1 ^b	8 ± 1 ^b	10 ± 3 ^b	8 ± 1 ^b
Heart	40 ± 1	40 ± 1	42 ± 1	41 ± 1	42 ± 1	40 ± 2	43 ± 3	42 ± 1	40 ± 1	40 ± 3	42 ± 1
Lung	64 ± 3	74 ± 8	67 ± 3	69 ± 2	71 ± 4	77 ± 4	71 ± 6	72 ± 5	78 ± 1	77 ± 5	72 ± 5
Liver	374 ± 16	361 ± 13	372 ± 10	358 ± 11	380 ± 8	360 ± 14	383 ± 21	384 ± 9	380 ± 12	416 ± 16 ^{a,d}	384 ± 9
Stomach	58 ± 4	62 ± 2	64 ± 2	52 ± 5	57 ± 4	58 ± 5	63 ± 5	60 ± 4	55 ± 5	52 ± 5	60 ± 4
Caecum	40 ± 2	35 ± 4	38 ± 2	37 ± 1	38 ± 2	55 ± 5 ^b	48 ± 4 ^a	46 ± 4 ^a	57 ± 2 ^b	51 ± 5 ^a	46 ± 4 ^a
Spleen	23 ± 1	27 ± 2	24 ± 1	26 ± 2	25 ± 2	18 ± 1 ^a	18 ± 1 ^a	17 ± 1 ^b	17 ± 1 ^b	18 ± 1 ^a	17 ± 1 ^c
Left kidney	41 ± 2	43 ± 2	42 ± 1	40 ± 1	40 ± 1	40 ± 1	43 ± 2	41 ± 1	42 ± 1	43 ± 1	41 ± 1
Right kidney	42 ± 2	44 ± 2	43 ± 1	41 ± 1	42 ± 1	44 ± 1	43 ± 1	44 ± 1	42 ± 1	43 ± 1	44 ± 1
Adrenal glands	3 ± 1	3 ± 1	3 ± 1	2 ± 1	3 ± 1	3 ± 1	8 ± 1	3 ± 5	7 ± 4	3 ± 1	3 ± 5

Organ weights are expressed as % (wtg/gbw). Data are expressed as means ± SEM. ^a*P* < 0.05 vs saline + PBS, ^b*P* < 0.01 vs saline + PBS, ^c*P* < 0.05 vs 5-FU + PBS, ^d*P* < 0.01 vs 5-FU + PBS. All values are 10⁻². 5-FU: 5-fluorouracil; PBS: Phosphate-buffered saline; AHE: Almond hull extract; ABW: Almond blanched water.

Table 3 Effects of almond extracts on gastrointestinal organ weights and lengths of female Dark Agouti rats 72 h after 5-fluorouracil or saline injection

	Sal + PBS	Sal + AHE1	Sal + AHE2	Sal + ABW1	Sal + ABW2	5-FU + PBS	5-FU + AHE1	5-FU + AHE2	5-FU + ABW1	5-FU + ABW2
Duodenum										
Weight (10 ⁻³)	31 ± 1	31 ± 2	33 ± 3	31 ± 2	30 ± 1	31 ± 3	28 ± 1	31 ± 2	32 ± 1	30 ± 2
Length	6.2 ± 0.2	6.0 ± 0.2	6.1 ± 0.2	6.3 ± 0.1	6.1 ± 0.1	5.9 ± 0.3	5.9 ± 0.2	5.9 ± 0.1	5.7 ± 0.1	6.0 ± 0.3
Jejunum + ileum										
Weight (10 ⁻³)	230 ± 5	226 ± 8	221 ± 6	222 ± 7	229 ± 7	210 ± 11 ^a	208 ± 10 ^b	203 ± 6 ^b	212 ± 11 ^a	209 ± 9 ^a
Length	75.1 ± 1.3	74.8 ± 1.4	75.8 ± 1.2	74.5 ± 0.9	75.4 ± 1.0	71.3 ± 0.9 ^a	70 ± 1.5 ^b	70.1 ± 1.9 ^b	71.5 ± 0.7 ^a	71.6 ± 1.0 ^a
Colon										
Weight	58 ± 1	59 ± 2	58 ± 2	58 ± 2	60 ± 2	66 ± 2	64 ± 3	68 ± 4 ^a	73 ± 4 ^b	69 ± 4 ^b
Length	13.0 ± 0.6	13.1 ± 0.5	13.0 ± 0.4	12.8 ± 0.2	13.0 ± 0.2	11.5 ± 0.3 ^a	11.3 ± 0.6 ^a	11.5 ± 0.4 ^a	11.0 ± 0.4 ^b	10.9 ± 0.8 ^b

Organ weights are expressed as % (wtg/gbw) and lengths are expressed in cm. Data are expressed as means ± SEM. ^a*P* < 0.05 vs saline + PBS, ^b*P* < 0.01 vs saline + PBS. 5-FU: 5-fluorouracil; PBS: Phosphate-buffered saline; AHE: Almond hull extract; ABW: Almond blanched water.

effects on MPO activity in healthy animals.

Histological severity score

Mucositis caused characteristic histological changes in the mucosal architecture of the jejunum and ileum (Figure 3). Chemotherapy administration significantly increased disease severity score in the proximal jejunum and ileum in all treated animals (Figure 4) in comparison with PBS- treated saline control animals (*P* < 0.001 for all). Almond extract treatments failed to normalize histological severity score (Figure 3). Presence of mucositis with or without almond extracts led to increases in all individual severity scoring parameters (Table 4). Scores for these parameters were not significantly different between almond extract-treated and 5-FU control groups.

Villus height and crypt depth measurements

5-FU injection caused a shortening of the villi in the jejunum (34%, *P* < 0.001) and ileum (40%, *P* = 0.01) compared to saline controls (Figure 5). However, there was no general reduction in crypt depth in the jejunum although this was noted in the ileum. Saline + AHE2 also caused a reduction in ileal crypt depth in healthy animals in comparison with saline injected control animals (15%, *P* = 0.02). However, AHE2 caused neither beneficial nor

adverse effect on ileal crypt depth when administered with 5-FU.

DISCUSSION

The present study describes the first investigation into the effect of selected almond production by-products on chemotherapy-induced mucositis. Whilst the data do not identify any beneficial effects of the extracts at the doses selected, they do confirm the safety of the agents in an *in-vivo* model. This is an important finding given the controversy surrounding the use of bitter almonds as chemotherapeutic agents identified in a recent Cochrane review^[17], and the common confusion between the properties of bitter and sweet almonds. Importantly, investigations conducted as part of this work have identified burrowing behaviour as a promising method of determination of animal affective state. To our knowledge this is the first example of a measure of affective state being successfully used in an animal model of chemotherapy-induced toxicity.

The chemotherapeutic agent 5-FU typically causes greater damage to the small intestine than the more distal regions of the gut. This is presumably due to the increased cell-turnover rate which is a characteristic

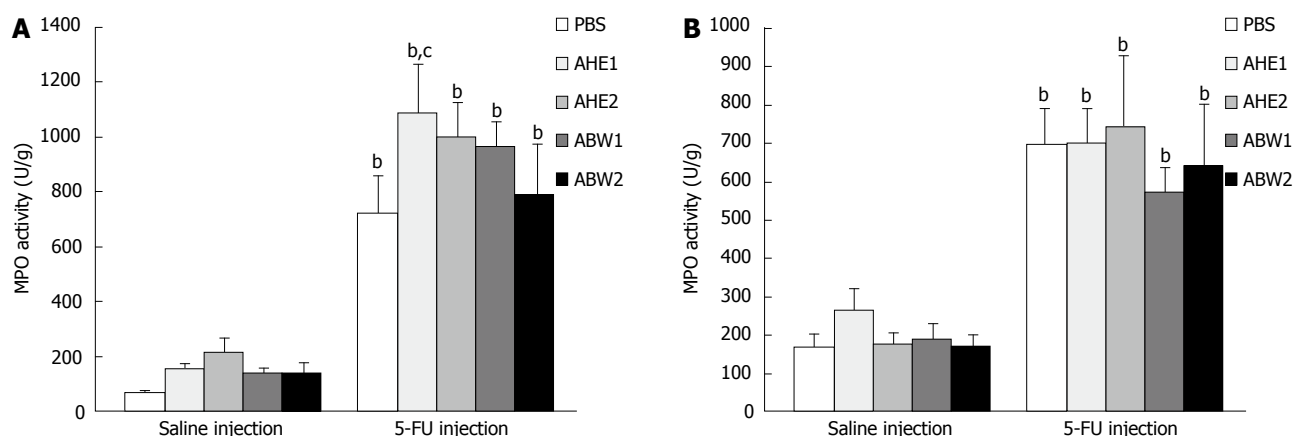


Figure 2 Effect of almond extracts on myeloperoxidase activity in the jejunum (A) and ileum (B) 72 h after either saline or 5-fluorouracil injection. Data are expressed as mean (MPO units/g tissue) $\pm$ SEM. ^a $P < 0.05$ vs saline + PBS, ^b $P < 0.01$ vs saline + PBS, ^c $P < 0.05$ vs 5-FU + PBS, ^d $P < 0.01$ vs 5-FU + PBS. 5-FU: 5-fluorouracil; PBS: Phosphate-buffered saline; AHE: Almond hull extract; ABW: Almond blanched water; MPO: Myeloperoxidase.

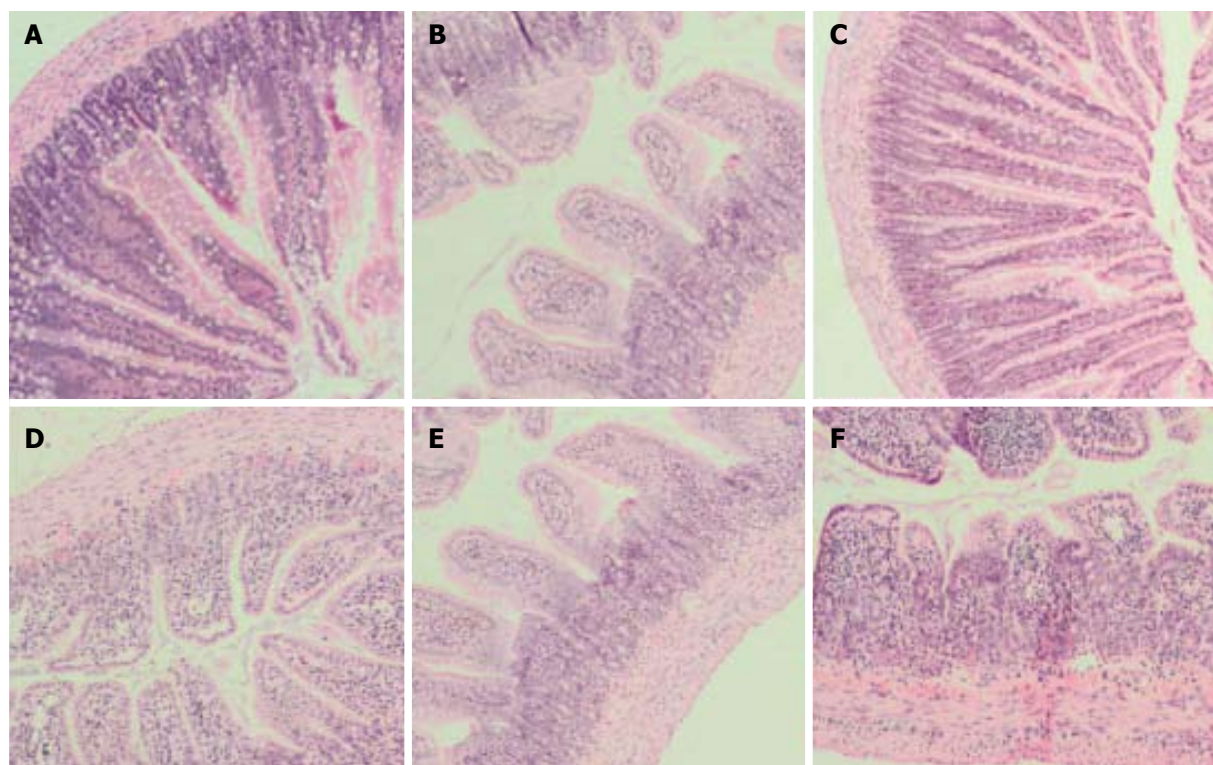


Figure 3 Representative photomicrographs ($\times 100$) of the proximal jejunum and distal ileum sections stained with haematoxylin and eosin. A: Saline/PBS jejunum; B: 5-FU/PBS jejunum; C: Saline/PBS ileum; D: 5-FU/PBS ileum; E: 5-FU/AHE2 jejunum; F: 5-FU/AHE1 ileum. Note: Blunting of villi, reduced goblet cell numbers, edema, mixed inflammatory infiltrate and loss of epithelial structure following 5-FU treatment. 5-FU: 5-fluorouracil; PBS: Phosphate-buffered saline; AHE: Almond hull extract; ABW: Almond blanched water.

feature of the small intestine^[3]. The main histological findings of villus shortening and crypt disruption caused by 5-FU administration were typical, and in concordance with other studies^[27-29].

The presence of crypt lengthening in response to chemotherapeutic agents has been an inconsistent finding in animal studies. A number of studies have reported a decrease in crypt depth in both jejunum and ileum following 5-FU injection^[19,30,31], yet another failed to demonstrate any change in crypt depth^[32]. Conversely,

an increase in crypt length following 5-FU administration has been demonstrated in rodents^[28]. Knowledge of the pathogenesis of mucositis suggests that crypt shortening is the most likely response to chemotherapy agents; due to crypt hypoplasia as a result of clonogenic cell death, or numerous signalling events^[33]. In the current study, crypt shortening was observed consistently in the ileum but not in the jejunum. Nevertheless, there was clear crypt cell disruption and a reduced number of cells on histological assessment of jejunal tissue. This apparent

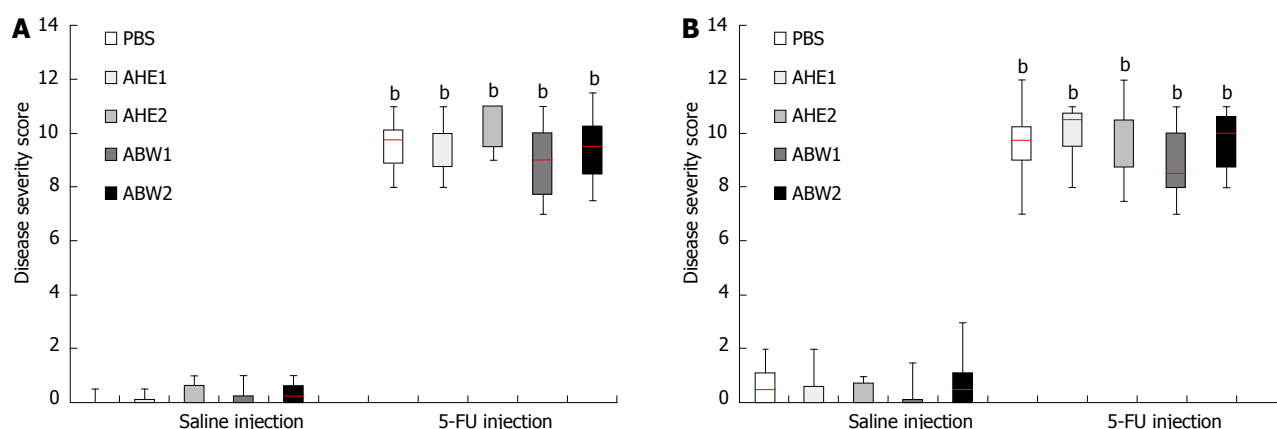


Figure 4 Effect of almond extracts on disease severity score in the jejunum (A) and ileum (B) 72 h after either saline or 5-fluorouracil injection. The severity scores were rated based on 8 parameters on different layers of intestinal tissues. The box plots represent the first and third quartiles, horizontal lines represent the median disease severity score and the whisker ends represent the maximum and minimum score. ^b $P < 0.01$ vs saline + PBS. 5-FU: 5-fluorouracil; PBS: Phosphate-buffered saline; AHE: Almond hull extract; ABW: Almond blanched water.

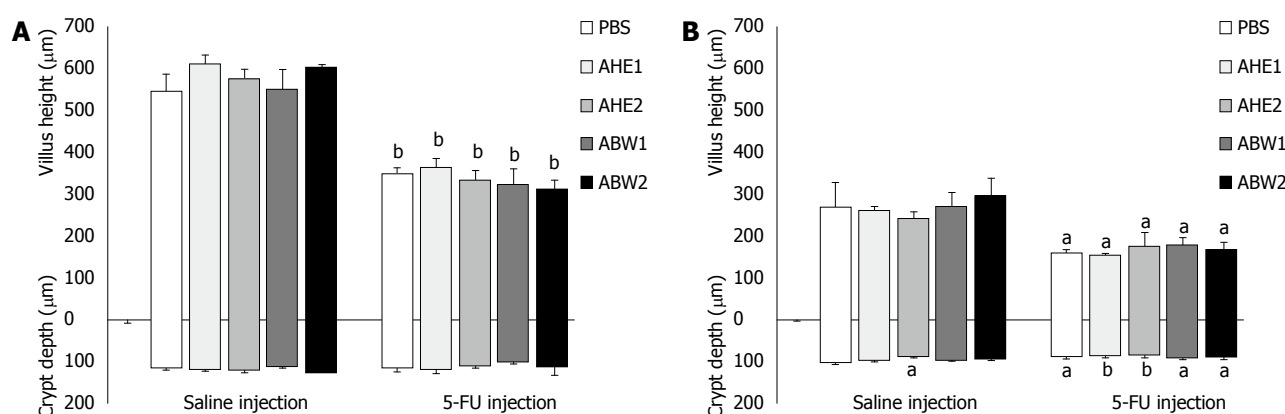


Figure 5 Effect of almond extracts on villus height and crypt depth in the jejunum (A) and ileum (B) 72 h after either saline or 5-fluorouracil injection. Data are expressed as mean $\pm$ SEM. ^a $P < 0.05$ vs saline + PBS, ^b $P < 0.01$ vs saline + PBS. 5-FU: 5-fluorouracil; PBS: Phosphate-buffered saline; AHE: Almond hull extract; ABW: Almond blanched water.

inconsistency supports the proposal of Mashtoub *et al* 2013 for studies to evaluate crypt proliferation and apoptosis using techniques such as the proliferative cell nuclear antigen, and TUNEL assay^[30]. Whilst in the current study, almond extracts had no beneficial effect on histological parameters, the study time-course was short. Gut damage is typically maximal in rats 3 d after 5-FU administration with regeneration and repair ensuing^[30]. Bioactive factors may not prevent this early gut damage but still be beneficial by aiding healing, for example by preserving crypt stem cell microcolonies. Evaluation of such a protective effect would need a longer study and specific evaluation of crypt cell numbers and organisation.

At a biochemical level myeloperoxidase activity can chart the inflammatory process occurring in the chemotherapy-damaged gut. Myeloperoxidase is the most common proinflammatory enzyme and is stored in the azurophilic granules of neutrophilic granulocytes^[34]. Therefore, measurement of the enzyme levels provides a reliable marker of neutrophil infiltration and hence acute inflammation^[25]. Given the intestinal damage caused by

chemotherapeutic agents an increase in MPO levels as a result of concomitant inflammation would be expected. Indeed a large number of animal-based investigations into mucositis support this hypothesis^[19,35-37]. However, Keefe *et al*^[38] 2000 suggest that the role of inflammation in mucositis progression is far from established.

Nevertheless, in the current study, an increase in MPO activity was established in both the jejunum and ileum in the 5-FU treated groups implying that inflammation was present. The administered almond extracts failed to moderate this activity and AHE1 actually elevated MPO activity in comparison with chemotherapy controls. This implies an immune-stimulatory effect of AHE 1. One mechanism of this pro-inflammatory activity could be the presence of omega-6 polyunsaturated fatty acid (ω -6 PUFA), linoleic acid, at greater levels in this extract^[39]. Indeed, sunflower oil with a highly imbalanced ω -6 PUFA to ω -3 PUFA ratio resulted in a pro-inflammatory response in mice^[40]. Furthermore, in previous studies a greater intake of ω -6 PUFA increased cytokine-induced inflammation *via* Prostaglandin E₂ production^[41]. However, given that in the current study a similar elevation was

Table 4 Severity scoring parameters for jejunum and ileum

		Villus blunting	Crypt distortion	Reduction in goblet cell numbers	Dilation of lymphatics	Thickening of submucosa	Thickening of muscularis externa	Enterocyte disruption	Lymphocytic and PMN infiltration
Sal + PBS	J	0	0	0	0	0.06 ± 0.06	0	0	0
	I	0.19 ± 0.12	0	0	0	0.31 ± 0.15	0.19 ± 0.12	0	0
Sal + AHE1	J	0	0	0	0	0.13 ± 0.08	0	0	0
	I	0	0	0	0	0.31 ± 0.15	0.13 ± 0.12	0	0
Sal + AHE2	J	0	0	0	0	0.21 ± 0.14	0	0	0
	I	0.07 ± 0.07	0	0.14 ± 0.13	0	0.07 ± 0.07	0.07 ± 0.07	0	0
Sal + ABW1	J	0.13 ± 0.12	0	0	0	0.13 ± 0.12	0	0	0
	I	0	0.06 ± 0.06	0	0	0.13 ± 0.12	0.06 ± 0.06	0	0
Sal + ABW2	J	0	0	0	0	0.25 ± 0.13	0.13 ± 0.08	0	0
	I	0.13 ± 0.12	0	0.12 ± 0.12	0	0.38 ± 0.15	0.19 ± 0.12	0	0
5-FU + PBS	J	1.63 ± 0.15 ^b	1.38 ± 0.17 ^b	1.81 ± 0.12 ^b	1 ± 0 ^b	0.88 ± 0.12 ^b	0.88 ± 0.12 ^b	1 ± 0 ^b	1 ± 0 ^b
	I	1.56 ± 0.24 ^b	1.62 ± 0.17 ^b	1.89 ± 0.12 ^b	1 ± 0 ^b	0.88 ± 0.21 ^b	0.63 ± 0.17 ^a	1 ± 0.18 ^b	1 ± 0 ^b
5-FU + AHE1	J	1.71 ± 0.17 ^b	1.43 ± 0.19 ^b	1.86 ± 0.13 ^b	1 ± 0 ^b	0.79 ± 0.14 ^b	0.71 ± 0.14 ^b	1 ± 0 ^b	1 ± 0 ^b
	I	1.64 ± 0.17 ^b	1.57 ± 0.21 ^b	1.79 ± 0.2 ^b	1 ± 0 ^b	1 ± 0 ^b	1 ± 0 ^b	1 ± 0 ^b	1 ± 0 ^b
5-FU + AHE2	J	1.79 ± 0.14 ^b	1.71 ± 0.17 ^b	2 ± 0 ^b	0.93 ± 0.07 ^b	1 ± 0 ^b	0.93 ± 0.07 ^b	1 ± 0 ^b	1 ± 0 ^b
	I	1.86 ± 0.13 ^b	1.57 ± 0.19 ^b	1.93 ± 0.07 ^b	1 ± 0 ^b	0.71 ± 0.2 ^b	0.64 ± 0.22 ^a	1 ± 0 ^b	1 ± 0 ^b
5-FU + ABW1	J	1.44 ± 0.16 ^b	1.25 ± 0.15 ^b	1.63 ± 0.17 ^b	1 ± 0 ^b	0.94 ± 0.19 ^b	0.88 ± 0.19 ^b	0.88 ± 0.12 ^b	0.88 ± 0.12 ^a
	I	1.25 ± 0.15 ^b	1.25 ± 0.15 ^b	1.63 ± 0.17 ^b	1 ± 0 ^b	0.88 ± 0.12 ^b	0.88 ± 0.12 ^b	1 ± 0 ^b	1 ± 0 ^b
5-FU + ABW2	J	1.75 ± 0.13 ^b	1.19 ± 0.12 ^b	1.94 ± 0.06 ^b	1 ± 0 ^b	0.88 ± 0.15 ^b	0.63 ± 0.17 ^b	1.13 ± 0.12 ^b	1 ± 0 ^b
	I	1.75 ± 0.15 ^b	1.56 ± 0.16 ^b	1.75 ± 0.15 ^b	1 ± 0 ^b	0.94 ± 0.06 ^b	0.69 ± 0.15 ^a	1 ± 0 ^b	1 ± 0 ^b

Data are expressed as means ± SEM. ^a*P* < 0.05 *vs* saline + PBS, ^b*P* < 0.01 *vs* saline + PBS. 5-FU: 5-fluorouracil; PBS: Phosphate-buffered saline; AHE: Almond hull extract; ABW: Almond blanched water.

not observed in the ileum, and that the histological data did not demonstrate any detrimental effects of AHE1 on mucosal structure, this finding is unlikely to be clinically significant. The pro-inflammatory nature of almond hull extracts should however be evaluated further if future investigations into this product are performed, especially since AHE2 also caused a reduction in crypt depth in healthy animals.

Patient symptoms arising as a result of the barrage of pathophysiological changes occurring in the alimentary tract in mucositis typically include reduced food consumption, and hence body weight decrease^[29,31]. Clinical features in the current study were classic although we observed increased urine output in the absence of polydipsia. This has not been previously reported in mucositis studies. This effect was common to all chemotherapy-treated animals and was not therefore linked to extract treatment. This observation could have resulted from a generalised stress response of psychogenic origin, or as a result of kidney changes due to drug metabolism^[42]. However, there was considerable inter-individual variation in fluid intake and urine output, and recording of these parameters may have been subject to error due to excessive leakage of bottles or contaminants in the urine collection chambers. Furthermore, there were no generalised increases in liver or kidney weights to suggest organ pathology except in the group receiving ABW2 which demonstrated an increase in liver weight. However, it would be valuable to perform confirmatory serum biochemistry tests in future studies.

Burrowing activity has been described as a “luxury” behavior since it is non-essential and therefore provides an indication of positive well-being^[43]. This category of behaviors is usually the first to be reduced in the event

of environmental challenge^[44]. Furthermore, the results from a variety of studies have concluded that such complex behaviors may mirror “activities of daily living (ADL)” in humans. Such activities, which include eating, bathing and dressing^[45], are often negatively affected by an array of clinical conditions^[46,47]. Previous literature using the rat model has demonstrated that burrowing activity or propensity to perform the behavior is reduced in painful conditions^[48,49] and as a result of sickness behavior due to cytokine release^[50]; both conditions in existence in mucositis. Few studies have evaluated burrowing behavior in gastro-intestinal disease models although Jirkof *et al.*^[51] 2013 successfully demonstrated a decrease in burrowing behavior in a mouse colitis model. The finding of a change in propensity to burrow following 5-FU injection in the current study is significant since it identifies burrowing behavior as a possible tool to evaluate rodent affective state. This is of particular importance in this disease model since mucositis is a self-limiting condition, and the key treatment outcome is to improve patient well-being. Measurement of burrowing may therefore provide a simple method of improving the translational validity of this animal model by providing an objective way to assess the subjective state of how the patient “feels”. Therapeutic success would then be evidenced by normalization of burrowing activity to baseline levels.

The current study refined the practical technique for burrowing behavior assessment utilized in Whittaker *et al.*^[23] 2015. In that study, burrowing behavior both before and after administration of chemotherapy was determined to have an inherently large inter and intra-individual variability. This impacted on interpretation of the results. It was also clear that there was a learning

phase to the behavior such that propensity to burrow increased with experience. To counteract these issues the current study introduced a training phase where the technique of social facilitation was used^[52]; pairs of rats were introduced to the test cage on a number of occasions prior to recording of burrowing values. Furthermore, animals were selected into their treatment groups to create similar baseline burrowing group means as described by Deacon 2006^[24]. Future studies should build on the results from the current study by incorporation of a positive control group utilizing a drug which provides known symptomatic relief against mucositis.

Interest in almond extracts as potential therapeutic candidates for mucositis is primarily based on their high anti-oxidant status^[53], since other potent anti-oxidants such as grapeseed extract have reduced intestinal damage in animal models of mucositis^[19,54]. Additionally, almond consumption has been associated with lower levels of C-reactive protein, interleukin-6 and E-selectin which represent biomarkers and an adhesion molecule involved in the inflammatory response^[53]. These data imply an inflammatory action of almonds which would be expected to reduce the severity of mucositis symptoms. However, in the current study despite the extracts showing moderate anti-oxidant activity as determined by the L-ascorbic acid equivalent test^[55], they failed to evoke measurable effects on the outcomes evaluated. It is possible that the dosages evaluated were too low to cause an effect or that bioactive components are degraded in an *in-vivo* model system. Alternatively, the oral gavage administration method for the extracts may have reduced their bioavailability in comparison to dietary exposure. Dietary exposure allows the chemical to interact with the mucosal surfaces in the oral cavity. Chemicals absorbed *via* this route avoid a first-pass metabolism by the gut wall and the liver. This avoidance of first-pass metabolism leads to a comparatively higher bioavailability^[56]. Furthermore, oral gavage dosing-induced stress can lead to increased heart rate, blood pressure and activation of the hypothalamic-pituitary axis. These changes have the potential to confound results. However, it has been demonstrated that these stress effects are generally short-lived or inconsistent in rats in comparison to mice; with blood pressure and heart rate generally returning to normal an hour after gavage^[57], and corticosterone rises being inconsistently seen^[58,59]. Stress-induced effects were therefore unlikely to have caused a significant confounding effect on the results of this study, but bioavailability may have been influenced by the administration method. Future studies could identify methods to protect bioactive components of almond extracts from degradation, for instance microencapsulation, or could investigate use of different administration routes which are not subject to first-pass metabolism.

In conclusion, this study provides the first investigation into the use of by-products of the almond production process as a potential therapeutic for chemotherapy-

induced mucositis. No definitive evidence was found for the extracts producing a beneficial effect at the dosages employed. Importantly, this study is the first to identify a measure of affective state in an animal model of mucositis. This finding has the potential to improve assessment of therapeutic effect in future animal studies of mucositis.

COMMENTS

Background

As cancer incidence rises and with it the incidence of chemotherapy-induced toxicity, the need to identify novel treatment approaches for chemotherapy-induced mucositis grows. There has been mounting interest in the use of plant-based nutraceuticals as therapeutics in cancer and to relieve symptoms resulting from chemotherapy treatment. Based on pre-clinical literature demonstrating anti-cancer properties of fruits from the *Prunus* genus, and the moderate procyanidin content of almonds, almond extract was investigated to determine whether it reduced mucositis severity in rats. Furthermore, they employed burrowing as a novel behavioural measure of affective state in order to validate this technique in a chemotherapy-induced mucositis model.

Research frontiers

To the knowledge, this is the first study to successfully employ a behavioural measure of affective state (as opposed to pain alone) in an animal model of intestinal mucositis. This has potential to improve the translational validity of animal models used in this area of research.

Innovations and breakthroughs

This is the first study to successfully use a behavioural measure of affective state, in a chemotherapy-induced mucositis model.

Applications

The finding that burrowing behaviour was decreased in animals with mucositis provides researchers using this animal model with a simple method of identifying affective state which may be replicated in the human patient. This is important given the self-limiting nature of the mucositis condition where the primary therapeutic goal is reduction of pain and discomfort, and improvement of quality of life. To date, animal studies of mucositis have failed to measure this primary outcome. Future studies should build on the results from the current study by incorporation of a positive control group utilizing a drug which provides known symptomatic relief against mucositis.

Terminology

5-fluorouracil is a widely utilised chemotherapy drug used to treat a number of cancer forms including colo-rectal cancer, breast cancer and cervical cancer amongst others. It may be administered alone or in combination with other chemotherapy drugs, such as methotrexate.

Peer-review

This interesting study shows that the severity of mucositis caused by 5-fluorouracil in rats was not ameliorated oral intake of almond extracts that contain phenolics and have anti-oxidant activity. A second aspect of the work is the use of burrowing behaviour by the rats as a monitor of animal well-being and severity of mucositis.

ACKNOWLEDGEMENTS

Grateful thanks are extended to Ms K. Lymn for her assistance with the animal study.

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