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Xiu-Xia Song, Director
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Baishideng Publishing Group Inc
7901 Stoneridge Drive, Suite 501, Pleasanton, CA 94588, USA
Telephone: +1-925-2238242
Fax: +1-925-2238243
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MINIREVIEWS

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

Mohamad H Yousef, Alhareth Al Juboori, Abdulmajeed A Albarrak, Jamal A Ibdah, Veysel Tahan, Division of Gastroenterology and Hepatology, University of Missouri, Columbia, MO 65212, United States

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Correspondence to: Veysel Tahan, MD, FACG, FEBGH, Professor (As) of Medicine, Division of Gastroenterology and Hepatology, University of Missouri, 1 Hospital Dr, Columbia, MO 65212, United States. tahanv@health.missouri.edu
Telephone: +1-573-8846044
Fax: +1-573-8844595

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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

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

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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/m^2 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/m^2, 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/m^2[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 necroinflammaotry 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 varients; 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 contact 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 predicted 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/m^2, 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 no 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 patents[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 advance 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/m^2. 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 improving 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, NALFLD 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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MINIREVIEWS

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

Rushikesh Shah, Cesar Taborda, Saurabh Chawla

Rushikesh Shah, Saurabh Chawla, Division of Digestive Diseases, Department of Internal Medicine, Emory University School of Medicine, Atlanta, GA 30322, United States

Rushikesh Shah, Cesar Taborda, Saurabh Chawla, Department on Medicine, Emory University School of Medicine, Atlanta, GA 30322, United States

Saurabh Chawla, Grady Memorial Hospital, Atlanta, GA 30303, United States

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Correspondence to: Saurabh Chawla, MD, Assistant Professor of Medicine, Division of Digestive Diseases, Department of Internal Medicine, Emory University School of Medicine, Faculty Office Building, 49 Jesse Hill Jr. Drive, Suite 431, Atlanta, GA 30322, United States. saurabh.chawla@emory.edu
Telephone: +1-140-47781684
Fax: +1-140-47781681

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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

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.

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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 ($\alpha 2/\beta S2$). 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].

HEPATOBILIARY 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 Chronic-normal or elevated	Acute-elevated Chronic-normal or elevated	Acute - normal to slightly elevated; Chronic - mostly normal	Based on AASLD guidelines
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-medicated 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 reticuloendothelial 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 then 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 a 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 *et al*[46]	6	1, 3, 5, and 10-yr survival rates were 83.3%, 66.7%, 44.4%, and 44.4%, respectively
Mekeel *et al*[48]	3	Patient and graft survival was 66%
Baichi *et al*[49]	2	100% mortality in post-transplant period due to multiorgan failure
Emre *et al*[50]	1	Failure of graft in 5 mo due to SCD crisis
Greenberg *et al*[51]	1	Successful but follow up only till day 28
Kindscher *et al*[52]	1	Successful with extrahepatic complications
Lang *et al*[53]	1	Successful at 6 mo
Ross *et al*[54]	1	Successful at 22 mo - death due to PE
van den Hazel *et al*[55]	1	Successful at 5.5 yr
Gilli *et al*[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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ORIGINAL ARTICLE

Prospective Study

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

Brian K McFarlin, Andrea L Henning, Erin M Bowman, Melody A Gary, Kimberly M Carbajal

Brian K McFarlin, Andrea L Henning, Erin M Bowman, Melody A Gary, Applied Physiology Laboratory, University of North Texas, Denton, TX 76203, United States

Brian K McFarlin, Andrea L Henning, Kimberly M Carbajal, Department of Biological Sciences, University of North Texas, Denton, TX 76203, United States

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.

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Correspondence to: Brian K McFarlin, PhD, FACSM, FTOS, Associate Professor, Applied Physiology Laboratory, University of North Texas, 1921 West Chestnut Street, PEB Room 209, Denton, TX 76203, United States. brian.mcfarlin@unt.edu
Telephone: +1-940-5653165
Fax: +1-940-5654904

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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-1β (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

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 compose of endosomes 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 that 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.

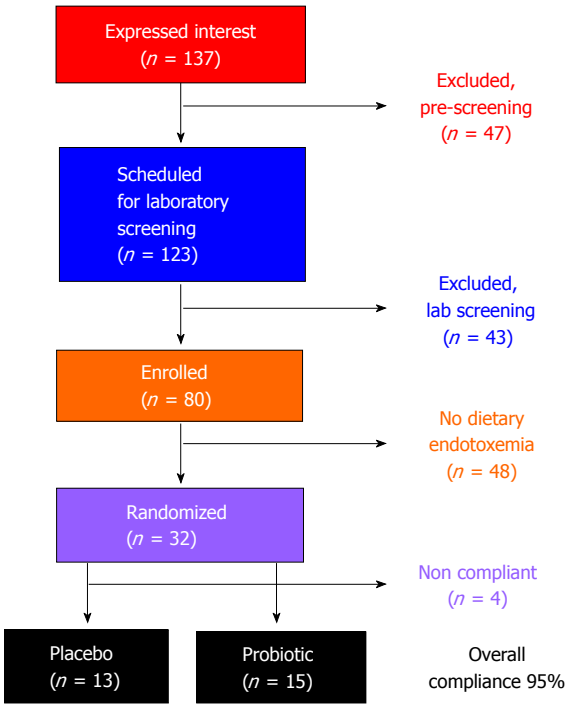

Figure 1 Represents the consort diagram for the study that indicates the number of participants that matriculated through the study. Subjects were carefully screened for exclusion/inclusion criteria and if qualified were enrolled in the study. Consistent with our preliminary data 2 out of every 6 subjects 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 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).

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.

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 ℃ until additional analysis.

Dietary endotoxin measurement

Serum was analyzed for endotoxin concentration using a commercially available kinetic limulus amebocyte lysate (LAL) assay (Lonza; Allendale, NJ, United States). Briefly, serum samples were diluted 1:100 in endotoxin-free water and heated at 70 ℃ 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.*,

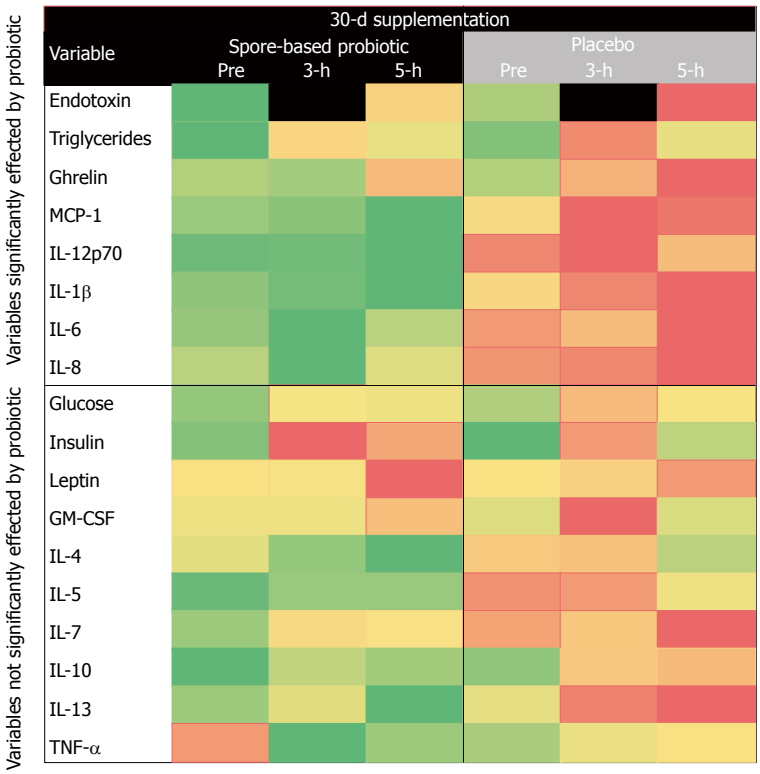

Figure 2 The change score for each variable compared to baseline was calculated and plotted as a heat map for probiotic *vs* placebo. Venous blood samples were collected prior to 3, and 5-h after consumption of a high-fat, high-calorie meal. Serum samples were analyzed using various accepted methods. 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.

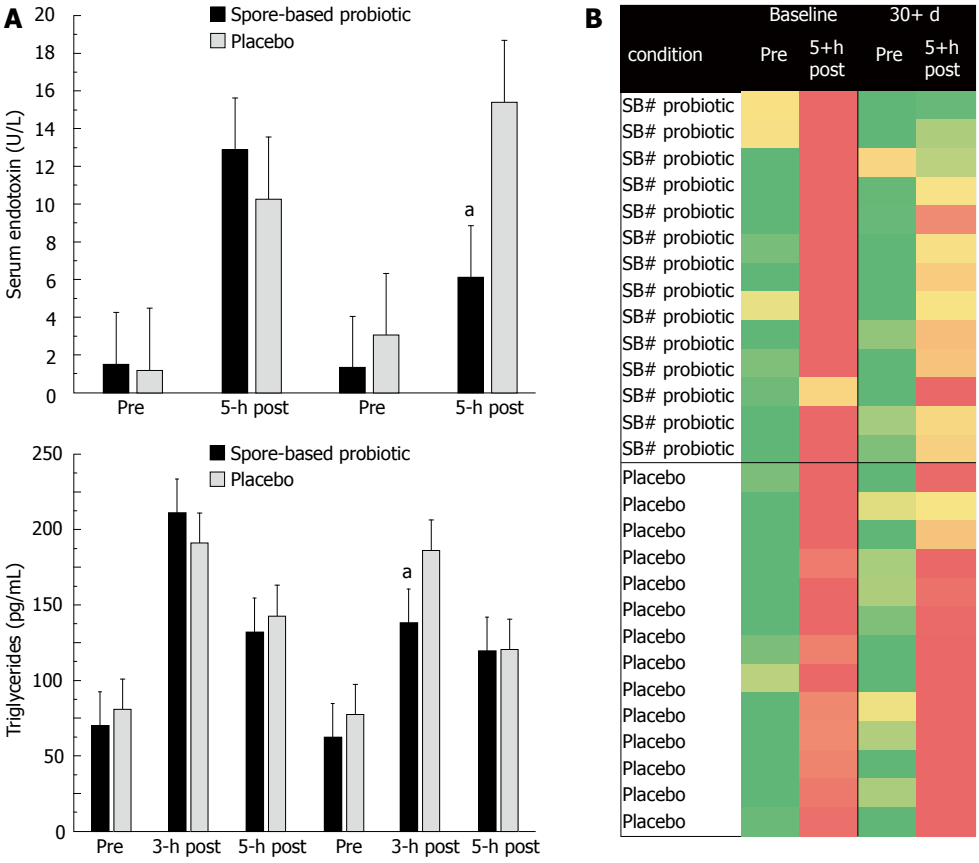

Figure 3 Serum endotoxin (A) and triglyceride (B) response to consumption of a commercially available high-fat, high-calorie pizza meal. [a]$P < 0.05$ indicates significantly less than placebo, less than pre-meal, less than and same time point at baseline. Venous blood samples were collected following an overnight fast and 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 compare 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).

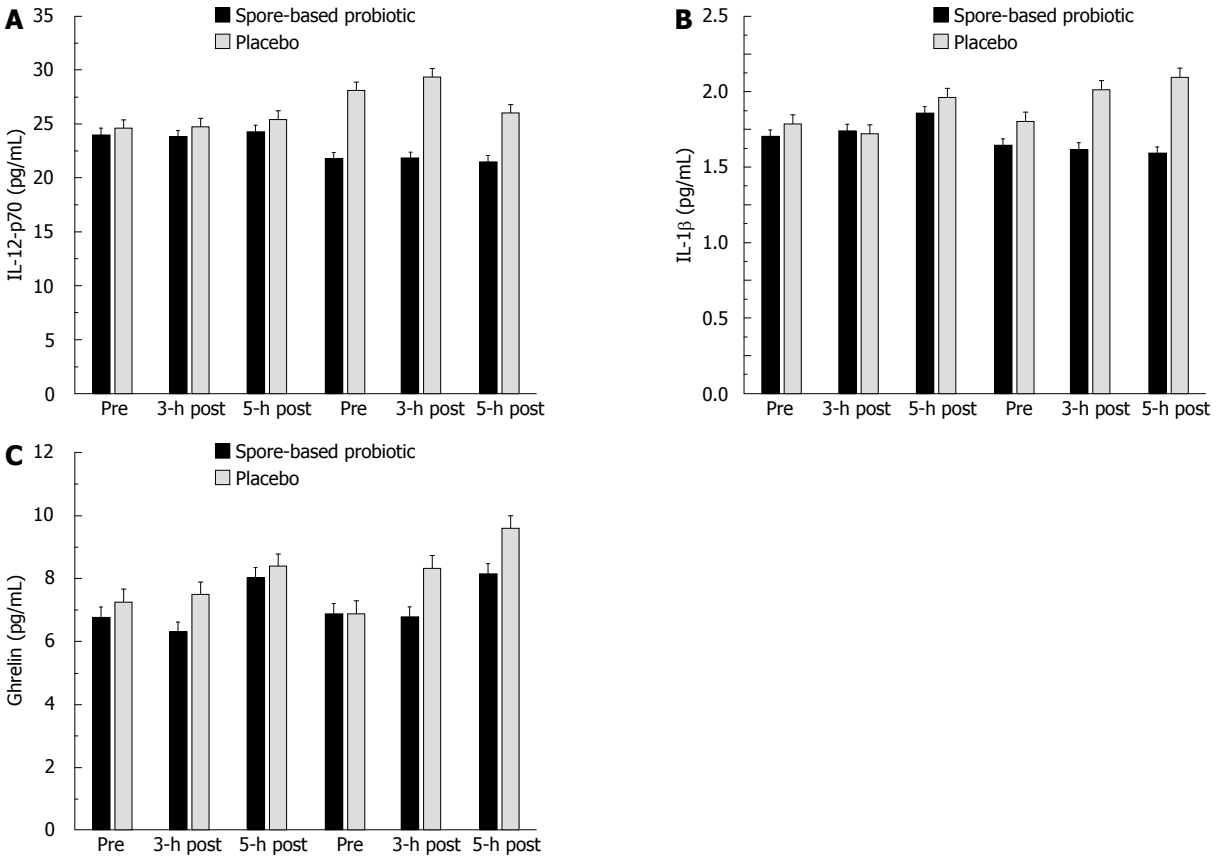

Figure 4 Serum IL-12p70 (A), IL-1β (B), and ghrelin (C) response to consumption of a commercially available high-fat, high-calorie pizza meal. Venous blood samples were collected following an overnight fast and abstention from exercise. Serum samples were analyzed using an automated chemistry analyzer. 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 compare 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 × condition interactions for IL-12p70 (P = 0.017; Figure 4A), IL-1β (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-1β, 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

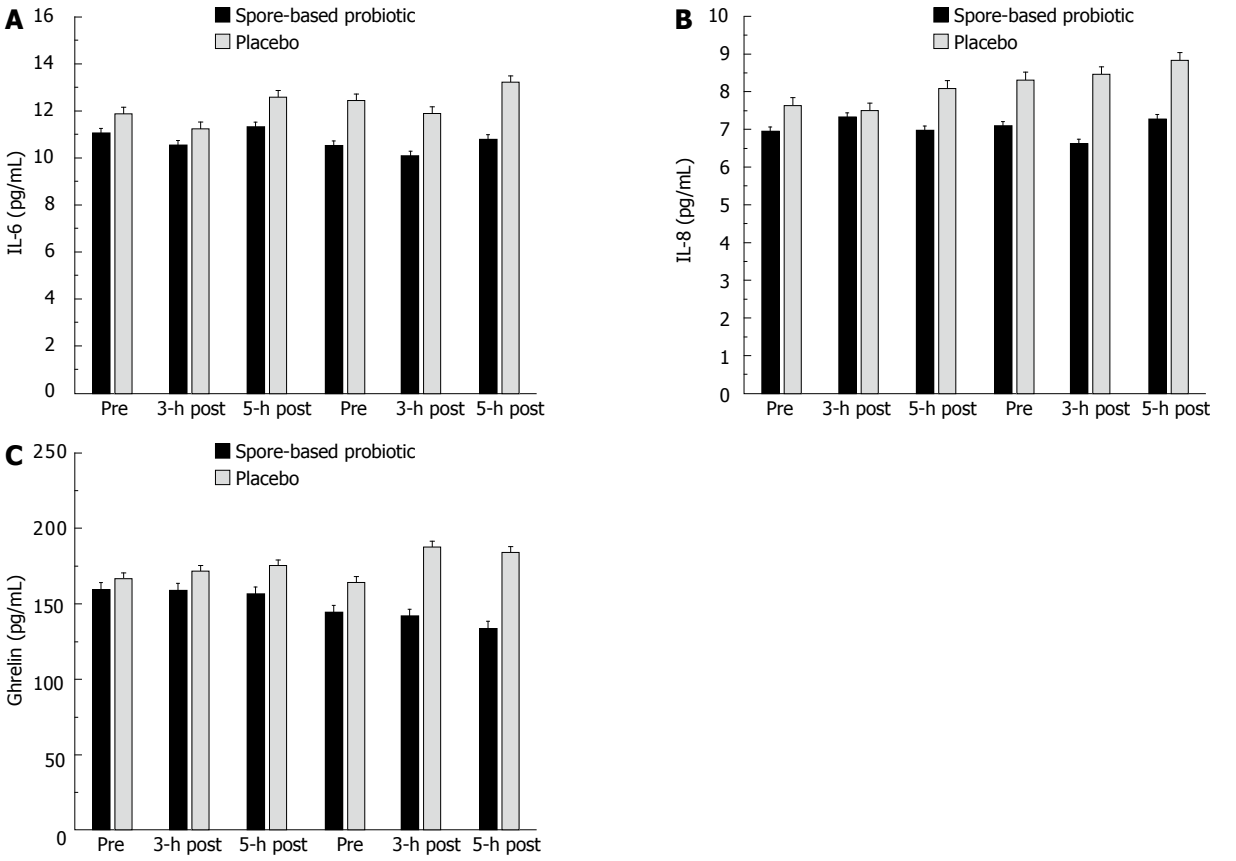

Figure 5 Serum IL-6 (A), IL-8 (B), and MCP-1 (C) response to consumption of a commercially available high-fat, high-calorie pizza meal. Venous blood samples were collected following an overnight fast and abstention from exercise. Serum samples were analyzed using an automated chemistry analyzer. Subjects 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 compare 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 ones' 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 than 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-1β, 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-1β, 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.

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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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ORIGINAL ARTICLE

Prospective Study

Assessment of serum angiogenic factors as a diagnostic aid for small bowel angiodysplasia in patients with obscure gastrointestinal bleeding and anaemia

Grainne Holleran, Mary Hussey, Sinead Smith, Deirdre McNamara

Grainne Holleran, Mary Hussey, Sinead Smith, Deirdre McNamara, Department of Clinical Medicine, Trinity Centre for Health Sciences, Tallaght Hospital, 24 Dublin, Ireland

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Correspondence to: Grainne Holleran, MD, Department of Clinical Medicine, Trinity Centre for Health Sciences, Tallaght Hospital, 24 Dublin, Ireland. hollerag@tcd.ie
Telephone: +353-18963844

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Abstract

AIM

To assess the use of serum levels of angiopoietin-1 (Ang1), Ang2 and tumor necrosis factor-α (TNFα) 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 TNFα 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 TNFα 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 TNFα 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

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 (TNFα) 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 (n = 40)	Abnormal (n = 40)	Normal (n = 40)
Ang1 (pg/mL)			
Median (range)	40976 (974-97511)	44770 (2660-101930)	47639 (17899-95173)
P value *vs* SBA		0.33	0.04
Ang2 (pg/mL)			
Median (range)	3759 (1915-14731)	2261 (842-14000)	2620 (686-8850)
P value *vs* SBA		< 0.004	< 0.003
Ratio of Ang1/Ang2			
Median	11.4	20.2	19
P value *vs* SBA		< 0.006	< 0.001
TNFα (pg/mL)			
Median (range)	5.76 (0.35-40)	9.76 (0.46-58)	10.14 (0.35-38)
P 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 ℃ 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 TNFα 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 TNFα 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 TNFα, 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% Specificity = 50%
False negative n = 7	False negative n = 40	Positive predictive value = 45% Negative predictive value = 85%

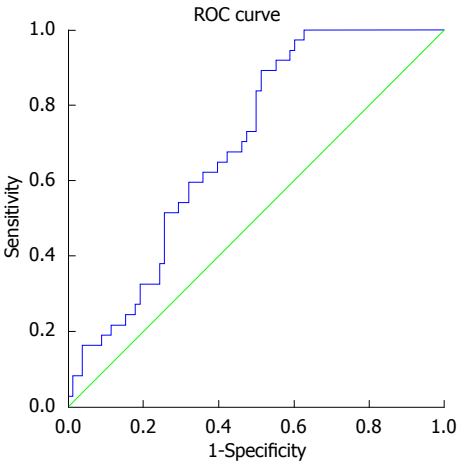

Figure 1 Receiver operator characteristic curve for serum Ang2, area under the curve = 0.695.

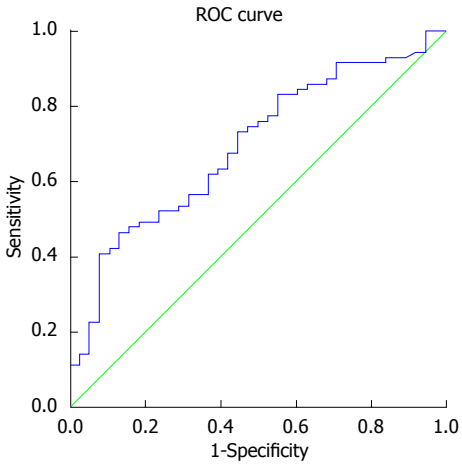

Figure 2 Receiver operator characteristic curve for the ratio of serum Ang1/Ang2, area under the curve = 0.692.

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 (TNFα). 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 f 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. TNFα 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 patho-physiology 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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ORIGINAL ARTICLE

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

Ashish Kumar Jha, Sharad Kumar Jha, Amarendra Kumar, Vishwa Mohan Dayal, Sanjeev Kumar Jha, Department of Gastroenterology, Indira Gandhi Institute of Medical Sciences, Sheikhpura, Patna 800014, India

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Correspondence to: Ashish Kumar Jha, MD, DM, Assistant Professor, Department of Gastroenterology, Indira Gandhi Institute of Medical Sciences, Sheikhpura, Patna 800014, India. ashishjhabn@yahoo.co.in
Telephone: +91-612-2297631
Fax: +91-612-2297225

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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

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.

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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 are 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 decompansated 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 Turcott Pugh (CTP) score $\geqslant$ 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 utrasonography 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-Meir 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 phosphorus were higher in

Table 1 Baseline characteristics of patients in treatment (n = 51) and control group (n = 50) n (%)

Parameters	Control groups (n = 50)	Treatment groups (n = 51)	P-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 ($mm^3/\mu L$)	10902 (9481-12322)	8407 (7238-9576)	< 0.05
Mean (CI) Ascitic fluid TLC ($mm^3/\mu L$)	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	3rd month	6th 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)

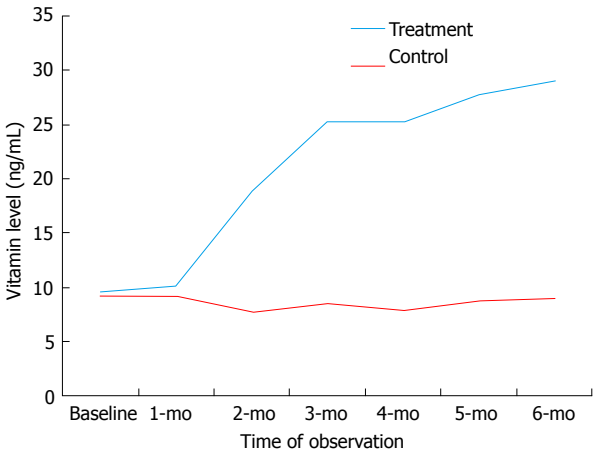

Figure 1 Comparison of vitamin D level between treatment and control group.

treatment group. Over the period of time the VD levels was 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
6th 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
6th 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 $\geqslant$ 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 *et al*[27], 2016	Compensated CLD (*n* = 207)	80% overall; 35% < 10 ng/mL; 45% < 20 ng/mL	VDD (< 10 ng/mL) in advanced *vs* no advanced fibrosis: 53% *vs* 24% ($P < 0.05$)
Gevora *et al*[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 *et al*[8], 2013	ALD (*n* = 324)	59% < 10 ng/mL	VDD are significantly associated with increased liver damage and mortality
Kitson *et al*[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 *et al*[19], 2010	CLD (*n* = 113)	92% < 32 ng/mL	VDD in cirrhotics *vs* noncirrhotics: 30% *vs* 14% ($P = 0.05$)
Costa Silva *et al*[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 *et al*[30], 2014	ALD (*n* = 30)	67% < 50 nmol/L	Highest prevalence of VDD were seen in CTP-C patients ($P < 0.05$)
Corey *et al*[31], 2014	ESLD (*n* = 158)	67% < 25 ng/mL	VDD is common among patients with ESLD awaiting LT
Putz-Bankuti *et al*[9], 2012	Cirrhosis (*n* = 75)	53% < 20 ng/mL	VD levels are inversely correlated with MELD and CTP scores ($P < 0.05$)
Malham *et al*[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 *et al*[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 *et al*[10], 2014	Cirrhosis (*n* = 88)	57% < 10 ng/mL	Severe VDD is a predictor of infection [OR = 5.44 (1.35-21.97), $P < 0.05$]
Stokes *et al*[18], 2014	Cirrhosis (*n* = 65)	86% < 20 ng/mL	VD levels is an independent predictors of survival [OR = 6.3 (1.2-31.2); $P < 0.05$]
Fernández Fernández *et al*[13], 2016	CLD (*n* = 94)	87% < 30 ng/mL or < 20 ng/mL	VD supplementation significantly improves CTP score
Zhang *et al*[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 *et al*[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 peritonitits (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 decompansation 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 *et al*[22], 2015	CHB (*n* = 426)	82% < 32 ng/mL	VDD is associated with adverse clinical outcomes
Bril *et al*[33], 2015	NASH (*n* = 239)	31% < 30 ng/mL; 47% < 20 ng/mL	VD level is not associated the severity of NASH
Finkelmeier *et al*[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 *et al*[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 *et al*[37], 2013	HCV-HIV coinfection (*n* = 116)	41% < 15 ng/mL	VDD is not associated with significant liver fibrosis (METAVIR ≥ 2)
Terrier *et al*[38], 2011	HIV-HCV coinfection (*n* = 189)	85% ≤ 30 ng/mL	Low VD level correlate with severe liver fibrosis
Petta *et al*[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 *et al*[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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CASE REPORT

Multiple endocrine neoplasia 2B: Differential increase in enteric nerve subgroups in muscle and mucosa

John M Hutson, Pam J Farmer, Cristal J Peck, Chung W Chow, Bridget R Southwell

John M Hutson, Pam J Farmer, Cristal J Peck, Bridget R Southwell, Surgical Research/Gastrointestinal Motility Laboratory, Murdoch Children's Research Institute, Parkville, Victoria 3052, Australia

John M Hutson, Chung W Chow, Bridget R Southwell, Departments of Urology and Anatomical Pathology, The Royal Children's Hospital, Parkville, Victoria 3052, Australia

John M Hutson, Bridget R Southwell, Department of Paediatrics, University of Melbourne, Melbourne 3010, Australia

Author contributions: Hutson JM surgeon managing patient and wrote manuscript; Farmer PJ and Peck CJ performed histology and immunohistochemistry and generated images; Chow CW provided pathological diagnosis; Southwell BR supervised study and revised manuscript.

Institutional review board statement: The study was performed under ethics approval to do further research on samples collected for diagnositc 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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Correspondence to: Dr. Bridget R Southwell, Surgical Research/Gastrointestinal Motility Laboratory, Murdoch Childrens Research Institute, Royal Children's Hospital, Flemington Road, Parkville, Victoria 3052, Australia. bridget.southwell@mcri.edu.au
Telephone: +1-61-393455069
Fax: +1-61-393457997

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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

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 phaeochromocytoma[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 ganlioneuromatosis 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

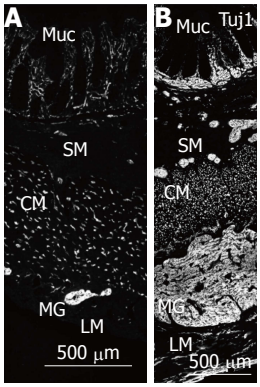

Figure 1 Nerve fibres in normal colon and MEN2B colon. Tuj1 immunoreactivity showing nerve fibres in full thickness biopsy of the transverse colon cut with circular muscle in cross section. A: Control patient (4-year-old, HSCR normal margin) showing multiple nerve fibres in the mucosa (Muc) within and 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 mucle; LM: Longitudinal muscle; MG: Myenteric ganglia.

in the muscosa.

Cryostat sections (10 μm thick) were air-dried at 4 ℃ 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

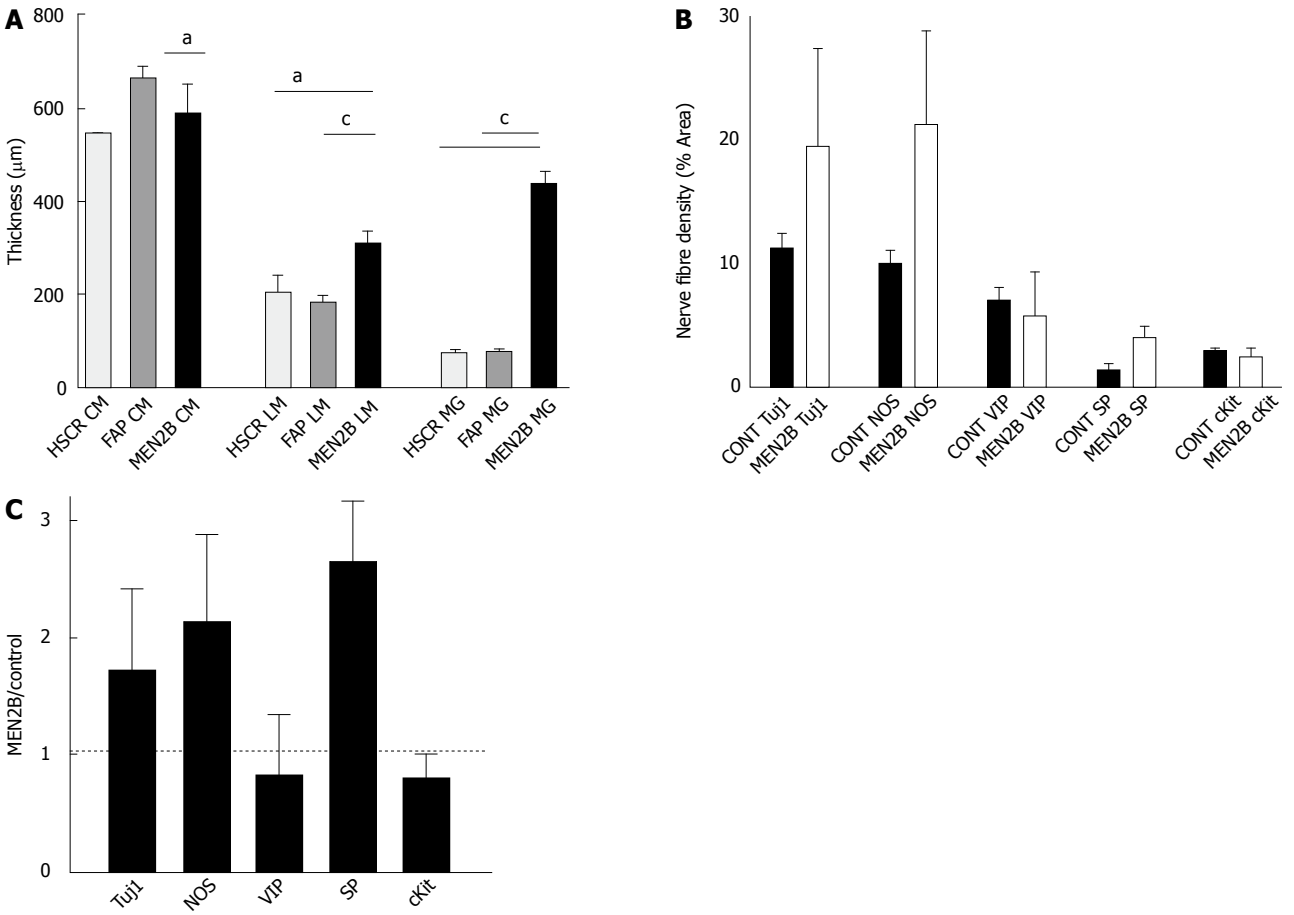

Figure 2 Thickness of muscle layers and ganglia and density of nerve fibres in normal colon and MEN2B colon. A: Comparison of thickness (mm) of muscle and myenteric ganglia in colon from Control (HSCR and FAP) and MEN2B patient. Graph shows mean and SEM. HSCR n = 6, FAP n = 12, MEN2B n = 24, $^{a}P < 0.05$, $^{c}P < 0.001$; 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 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).

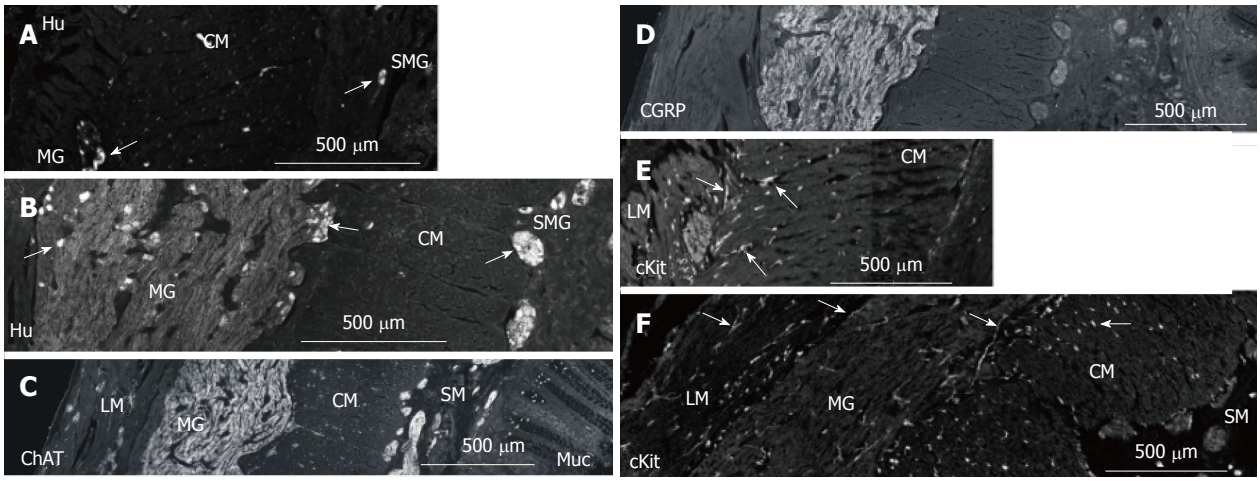

Figure 3 Nerve cells in myenteric and submucosal ganglia in normal colon and MEN2B colon. A: Hu immunoreactivity showing nerve cell bodies in transverse colon cut with circular muscle in cross section. Control patient (HSCR) showing myenteric ganglia (MG) neurons and submucosal ganglia; B: MEN2B patient 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 acetyle transferase (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.

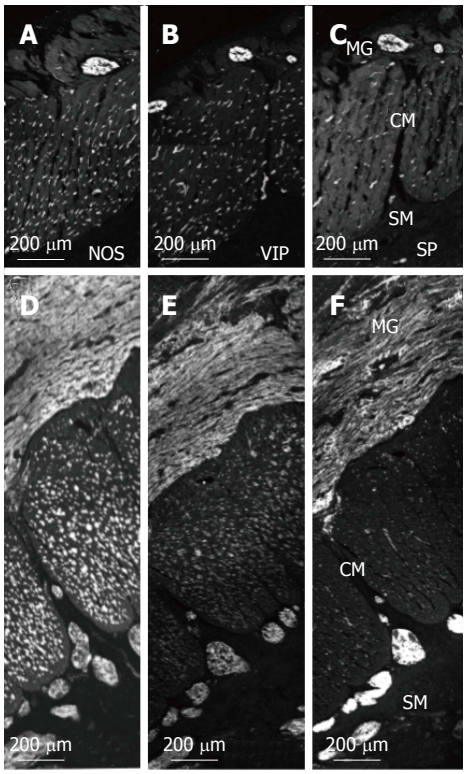

Figure 4 NOS, VIP and SP nerve fibres in myenteric and submucosal ganglia and circular muscle in normal colon and MEN2B colon. A: NOS-; B: VIP-; C: SP-immunoreactivity in Control (HSCR) patient; D: NOS-; E: VIP-; F: SP-immunoreactivity in MEN2B patient. Note all images are at the same magnification. 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.

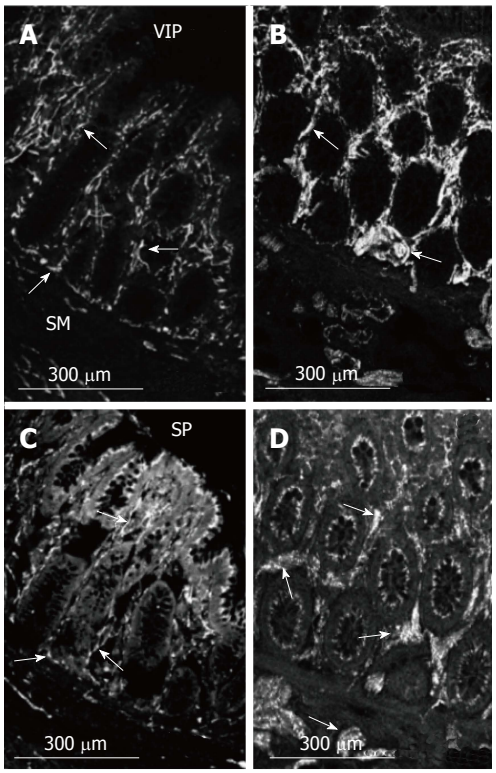

Figure 5 VIP and SP nerve fibres in the mucosa in normal colon and MEN2B colon. VIP-immunoreactivity in (A) Control (HSCR) and (B) MEN2B patient. Crypts in transverse colon. VIP immunoreactive nerve fibres (arrows) are plentiful at the base and within the arms of the crypts in the control. There is a 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.

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 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

Thryoidectomy 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 phaeochromocytoma 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 secretomotory 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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