TABLE OF CONTENTS

Editorial
1. Endoscopy: From Diagnosis to Therapeutics
   – Ariel A. Benson and Mizrahi Meir’
   e1-e3

Research
2. An Observational Chart Review on the Efficacy of Subcutaneous
   Methotrexate in Mild to Moderate Ulcerative Colitis and the Description
   of Occurrence of Adenomatous Polyps in Afflicted Patients
   – Raymond Soriano’, Kristina Blake and Monica Gonzalez
   23-29

Review
3. Non-alcoholic Fatty Liver Disease and the Gut Microbiota: Exploring
   the Connection
   – Edward C. Oldfield IV, Ray Z. Dong and David A. Johnson’
   30-43

Review
4. The Role of Energy Metabolism in Driving Disease Progression
   in Inflammatory, Hypoxic and Angiogenic Microenvironments
   44-58

Short Communication
5. Mucinous Tumour in Ileal Pouch Post Restorative Proctocolectomy
   and Ileal Pouch Anal Anastomosis for Familial Adenomatous Polyposis
   – James Wei Tatt Toh’, Kasim Rahman and Daniel Kozman
   59-60
Endoscopy: From Diagnosis to Therapeutics

Ariel A. Benson1 and Mizrahi Meir2*

1Division of Medicine, Institute of Gastroenterology and Liver Diseases, Hadassah-Hebrew University Medical Center, Jerusalem, Israel
2Division of Gastroenterology, Center for Advanced Endoscopy, Beth Israel Deaconess Medical Center and Harvard Medical School, 330 Brookline Ave., Boston, MA 02215, USA

*Corresponding author:
Mizrahi Meir, MD
Division of Gastroenterology
Center for Advanced Endoscopy
Beth Israel Deaconess Medical Center
and Harvard Medical School
330 Brookline Ave., Boston, MA 02215, USA
E-mail: mmizrahi@bidmc.harvard.edu

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Two decades ago, gastroenterologists used endoscopy for the diagnosis of Gastrointestinal (GI) lesions, thereby helping surgeons to localize lesion that necessitated resection and treatment with surgery. Today, the gastroenterology field has advanced beyond the diagnostic era and now, a large number of GI lesions can be treated via endoluminal procedures performed by the gastroenterologist with no need for surgical intervention.

In recent years, the improvement of endoscopic imaging and tools, such as snares, clips and needles which can be delivered through the endoscope channel, have helped to change the field of gastroenterology. This allowed for the development and advancement of Endoscopic Mucosal Resection (EMR) and Endoscopic Submucosal Dissection (ESD). The pioneers of EMR and ESD were from Japan and the first articles describing these techniques were published in the 1990s.1,2

Two classification systems are used for describing GI lesions to be considered for EMR and ESD. One system is the Japanese classification3 and the second is the Paris system, which was proposed in 2002.4 The Japanese classification was originally developed for early gastric cancer management, but it can be applied to lesions throughout the GI tract. Once lesions are classified, EMR can be performed in different GI locations, including the esophagus, stomach, colon and rectum. When lesions are more complex and beyond the mucosa, ESD can be performed with careful dissection.

Endoscopic ultrasound (EUS) may be useful in deciding whether to perform EMR or ESD. EUS can aid in determining the penetration of the tumor to layers beyond the mucosa. EUS can also determine the location of the lesion in proximal parts of the colon. If the lesion cannot be reached by a regular EUS endoscope special high frequency mini-probes may be used through the colonoscope working channel.

Two methods are used in performing EMR. The first method is ‘suck and cut’ and the second method is ‘lift and cut’. Both usually begin with a submucosal injection prior to resection of the lesion the suc cut method may be used also without submucosal injection especially when being performed in the esophagus. The injection is used to expand the submucosa, separating the deeper muscularis propria from the more superficial mucosa and submucosa layers. Several solutions may be used as the injectate. These include normal saline with or without diluted epinephrine, hypertonic saline, dextrose solution, sodium hyaluronate, fibrinogen combination, glycerol, and fructose solutions all of these may be mixture with methylene blue depend on the preference of the endoscopist.5-8 Conio, et al. compared the solutions and showed that the disappearing time of normal saline is approximately 3 minutes with or without epinephrine, while the disappearing times of 50% dextrose and 10% glycerol and hyaluronic acid solutions were 4.7, 4.2 and 22 minutes respectively.9

After injection, one of the two resection methods can be applied. The ‘suck and cut’
method utilizes a transparent cap on the endoscope and requires suctioning of the lesion into the cap. The lesion is then resected by a snare which exits through the working channel into the transparent cap. A variation of this method is the use of bands instead of an injection solution, and this method is best for the treatment of esophageal lesions. Following injection, the ‘lift and cut’ method involves the use of a grasper to pull the lesion from the muscularis propria. The ‘lift and cut’ method was the first EMR technique, but because of complexity, the ‘suck and cut’ method is now used more widely.

When the lesion is thought to be deeper or wider, if it is a submucosal lesion, or if en bloc resection is preferred, ESD may be used for resection. ESD includes the same inject and lift method as EMR, but instead of using a snare, needle knife is used to resect the lesion through dissection. ESD can be performed in different GI locations such as esophagus, stomach, duodenum, colon and rectum.

Once removal of the lesion is complete, tattooing of the procedure area can be considered in order to assist in surveillance. Use of India ink tattooing is recommended, but careful injection is necessary as India ink can result in tissue scarring if injected to the submucosal space. One method to prevent scarring is to first inject normal saline to separate the mucosa and the submucosal layers and then inject the ink.

The long term outcomes of both EMR and ESD have been positive. Merkow, et al. compared the outcomes of patients with early esophageal cancer treated by EMR or surgery. There was a higher 30 day mortality rate in the surgical group and 5 year survival was 77% and 88% in the EMR and surgical groups respectively. EMR for gastric lesions (when the margins of the lesion are clear with no dysplasia) has also had favorable outcomes. The outcomes of colonic EMR for early colon cancer are similar to the results in esophageal and gastric EMR. Bledeso, et al. reported the results of a meta-analysis of colonic EMR showing that the recurrence rate was 15% and that piecemeal resection had a higher recurrence rate as compared to en bloc resection (20% versus 3% respectively). Ikematsu, et al. reported long term outcomes after resection of submucosal invasive colorectal cancer by ESD or surgery. In patients with low risk lesions (lesion entirely resected, well to moderately differentiated adenocarcinoma, no vascular invasion and submucosal invasion <1 mm), there were recurrence rates of 0% and 6% for rectal and colonic lesions, while in patients with high risk lesions, the recurrence rates were 1.4% and 16% for rectal and colonic lesions respectively.

Both EMR and ESD can lead to adverse events and in order to lower complication rates the procedures should be performed by experienced endoscopists. Adverse events, including perforations, strictures, and immediate or late bleeding during esophageal EMR were reported to be present in 0% to 13% of cases. Gastric EMR adverse events have been reported to range from 1% to 5%. In colonic EMR, bleeding events may occur in as many as 24% of cases. Colonic ESD may be complicated by perforation in up to 10% of cases as reported by Takegami Y, et al. but in most cases the perforation may be treated endoscopically without the need for surgical intervention.

In conclusion, EMR and ESD will continue to be performed worldwide, likely with increased frequency, during the next several years. Nonetheless, in order to avoid and reduce complications, it is recommended that EMR and ESD be performed at high volume centers and by experienced endoscopists.

**CONFLICTS OF INTEREST:** None.

**REFERENCES**

9. Conio M, Rajan E, Sorbi D, et al. Comparative performance in the porcine esophagus of different solutions used for submu-


An Observational Chart Review on the Efficacy of Subcutaneous Methotrexate in Mild to Moderate Ulcerative Colitis and the Description of Occurrence of Adenomatous Polyps in Afflicted Patients

Raymond Soriano*, Kristina Blake and Monica Gonzalez

1Department of History, Wells College, 170 Main St, Aurora, NY 13026, USA
2Department of Biology, Wells College, 170 Main St, Aurora, NY 13026, USA
3South Texas Research Alliance, LLC, 2344 Laguna Del Mar Ct # 204, Laredo, TX 78041, USA

ABSTRACT

Ulcerative Colitis (UC) is a type of Inflammatory Bowel Disease (IBD) that affects the large intestine and produces mainly symptoms of abdominal pain and bloody stools. Chart reviews of patients from July 2011 to July 2012 with mild to moderate UC enrolled in a community-based NIH trial on the efficacy of 8 weeks of 12.5 mg once daily subcutaneous methotrexate demonstrated no significant improvement of abdominal pain and bloody stools. In their patient diaries and IBD Questionnaire, all 9 patients reported a sense of heaviness or abdominal fullness, bloating or cramps. The patients experienced decreased energy levels and depressed feelings with anxiety and decreased sleep at night. Overall, the majority of them reported a decreased quality of life despite the 8 week trial of MTX. We also noted in the chart review the incidental finding of histopathologically-confirmed distal adenomatous polyps in all 9 patients for which we postulate the following: 1) that distal adenomatous polyps may be a risk factor in UC, 2) that surgical removal or polypectomy for these polyps, if diagnosed earlier especially in those younger than 40 years of age, may delay progression or prevent development of UC, 3) the presence of polyps in all 9 patients could have prevented the desired therapeutic response from methotrexate, and 4) that the presence of the polyps may indicate that low dose methotrexate is not an effective treatment for UC. The presence of intestinal polyps realigns the structural integrity and dynamics of the movement of the intestinal walls and valves, causing affected patients to frequently report feelings of discomfort and lethargy. It is important to understand that further diagnosis and therapies for UC and IBD are accompanied with ethical questions, such as treating affected patients with cytotoxic medications and performing colonoscopies under the standard age care of 50 years. Further research needs to address combination therapies and other risk factors such diets and preservatives to determine the full extent of this potential ground-breaking science.

KEYWORDS AND ABBREVIATIONS: Ulcerative Colitis (UC); Inflammatory Bowel Disease (IBD); Methotrexate (MTX); Subcutaneous (SC); Mayo Disease Activity Index (Mayo DAI); Inflammatory Bowel Disease Questionnaire (IBDQ).

INTRODUCTION

Inflammatory Bowel Disease (IBD) is a chronic inflammatory disease primarily of the large intestine. It is a disease that affects mostly Caucasians in western countries and those
of Jewish ancestry.\textsuperscript{12} IBD consists of two major subsets of unknown etiology: Crohn’s Disease and Ulcerative Colitis (UC). In both diseases, the inflammation of the large intestine, or colon, is a result of the complex interaction of genetic predisposition, immune dysfunction and environmental triggers.\textsuperscript{1,13} The environmental trigger (food, bacteria, or any intestinal material) may directly cause the inflammation or stimulate the immune system to go unregulated e.g. “turned on” for chronic periods of time. This cascade of events damages the intestines and inflicts severe, adverse biological consequences on the human body.

In Ulcerative Colitis, the mucosal lining of the colon has marked erythema, edema, granularity, abnormal vascular patterns with varying depth and severity of ulcerations. Major gastrointestinal symptoms include crampy abdominal pain, persistent diarrhea and bloody stools. There are also associated extraintestinal symptoms. These symptoms can go for long periods of time and can be unpredictable, so-called flare-ups. In a Norwegian Study,\textsuperscript{4} it was found that there is an incidence of 32\% of rectal involvement (proctitis), 33\% left-sided colitis, and 35\% extensive or pancolitis. It is believed that 90\% of cases will experience a relapsing course of which approximately 10\% may require surgery.\textsuperscript{4,44} However the American Society of Colon and Rectal Surgeons estimates that 38\% will require surgery within 13 years of diagnosis.\textsuperscript{5} The majority of patients will be on lifetime medical treatment.

The severity (mild, severe, or fulminating) of disease in afflicted UC patients can be assessed by the Mayo Score and the Disease Activity Index\textsuperscript{8} which is based on the following parameters: stool frequency, rectal bleeding, mucosal appearance at colonoscopy and physician rating of disease activity. The extent of the disease determines mode of therapy with distal disease requiring topical therapy and more extensive disease requiring a combination of oral and topical medications. It is the goal of medical treatment to induce and maintain remission in mild to moderate disease and prevent surgery in severe cases. Surgery can be curative in severe cases when complications are lifethreatening (massive bleeding, perforation, and infection) with increased risk of developing colon cancer.\textsuperscript{9}

The standard medical treatment of UC consists of a combination of oral and rectal (enema, suppositories) aminosalicylates and corticosteroids (oral, intravenous, rectal).\textsuperscript{1,2,8,10-16} Immunosuppressants (6-mercaptopurine, azathioprine, methotrexate, cyclosporine, tacrolimus, and infliximab) have been used in steroid-dependent or steroid-refractory cases.\textsuperscript{1,17-19}

Methotrexate (MTX) is an antimetabolite used in the treatment of certain neoplastic diseases, severe psoriasis, and adult rheumatoid arthritis.\textsuperscript{26-28} It is classified as antineoplastic and anti-metabolite. As an anti-metabolite it inhibits folic acid reductase which is responsible for the conversion of folic acid to tetrahydrofolic acid which is important in DNA synthesis. It selectively affects the most rapidly dividing cells.\textsuperscript{22-25} Both mechanisms of action lead to cell death. Side effects of low-dose MTX therapy include: anorexia, nausea, stomatitis or diarrhea. Adverse events include bone marrow suppression, liver toxicity, and on rare occasions, opportunistic infections.\textsuperscript{3,14,26-27}

Chemically methotrexate is N-[4-[[2,4-diamino-6-pteridinyl]methyl]-methylamino]benzoyl]-Glutamic acid. The structural formula is:

Molecular weight: 454.45 \( C_{20}H_{22}N_8O_5 \)

MTX has been found to induce and maintain remission in Crohn’s disease.\textsuperscript{26,28,29} However there have been limited studies of its application in UC. The oral dose of 20 mg/week has been found to be well-tolerated and moderately effective.\textsuperscript{30} In uncontrolled studies, the parenteral intramuscular dose of 12.5 mg has been found to be effective.\textsuperscript{27,28,31} It has been described that the Subcutaneous (SC) and intramuscular administration produces similar bioavailability of the drug but the SC is better tolerated.\textsuperscript{30,29,32-34} In a prospective double-blind controlled trial using SC methotrexate in rheumatoid arthritis a higher response rate is produced.\textsuperscript{31,33} There are limited studies comparing oral versus parenteral efficacy of MTX in IBD. However it has been established that in Rheumatoid Arthritis (RA) and IBD, there is limited value in monitoring drug levels as they do not correlate with efficacy.\textsuperscript{35-39} Currently, there is an on-going MERIT-UC NIH funded multi-center prospective placebo controlled study investigating the safety and efficacy of 25 mg MTX applied subcutaneously once weekly in patients with active UC, who are either steroid dependent or are intolerant or not responding to aminosalyslate therapy or have not responded or lost response to infliximab.\textsuperscript{30} Preliminary data show 46\% clinical response after 4 weeks of subcutaneous MTX following failure of a 12 week course of steroids.\textsuperscript{20}

\textbf{METHODOLOGY}

\textbf{Purpose of Study}

A multi-center, community-based, double blind,\textsuperscript{40} NIH trial on the efficacy and safety of 12.5 mg subcutaneous Methotrexate (MTX) in mild to moderate ulcerative colitis or proctitis from July 2011 to July 2012.

The aims of the trial were: 1) To evaluate the safety and tolerability of 12.5 mg MTX applied SC once weekly over a time period of 8 weeks, 2) To objectively evaluate the relapse-free survival of MTX maintenance therapy after 8 weeks of therapy through colonoscopy or proctosigmoidoscopy, and 3) To evaluate the efficacy of MTX over a time period after 8 weeks of therapy through patient diary of presence or absence of abdominal pain and bloody stools, and response to quality of life issues in the 32-question Inflammatory Bowel Disease Questionnaire.
Materials and Methods

A review of clinic notes of patients included in the phase IIIa study trial of open-label use of 12.5 mg of subcutaneous methotrexate in mild to moderate ulcerative colitis as assessed by the Mayo Disease Activity Index (Mayo DAI) (Table 1). Table 2 and Table 3 lists the parameters used in patient selection. A protocol guideline for allowed medications and procedures and prohibited chemotherapeutic agents was followed (Table 4).

Methotrexate 12.5 mg was administered subcutaneously for 8 weeks. At the end of the study period, the patients were administered the Inflammatory Bowel Disease Questionnaire (IBDQ; McMaster University, Hamilton, ON, Canada 2010). This 32-item questionnaire is a reliable and validated tool widely used to assess the general well-being of patients in the recent 2 weeks. It is scored in four domains: bowel symptoms, emotional health, systemic systems, and social function.

<table>
<thead>
<tr>
<th>Stool Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>0= normal no. of stools for this patient</td>
</tr>
<tr>
<td>1= 1-2 stools/day more than normal</td>
</tr>
<tr>
<td>2= 3-4 stools/day more than normal</td>
</tr>
<tr>
<td>3= &gt;=5 stools/day more than normal</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Rectal bleeding*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 = None</td>
</tr>
<tr>
<td>1 = Streaks of blood with stool less than half the time</td>
</tr>
<tr>
<td>2 = Obvious blood with stool half of the time or more</td>
</tr>
<tr>
<td>3 = Passing blood alone</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mucosal appearance at endoscopy</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 = Normal or inactive disease</td>
</tr>
<tr>
<td>1 = Mild disease (erythema, decreased vascular pattern, mild friability)</td>
</tr>
<tr>
<td>2 = Moderate disease (marked erythema, absent vascular pattern, friability, erosions)</td>
</tr>
<tr>
<td>3 = Severe disease (spontaneous bleeding, ulceration)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Physician rating of disease activity: takes into consideration the patient’s abdominal discomfort, general sense of well-being, and functional status</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 = Normal</td>
</tr>
<tr>
<td>1 = Mild</td>
</tr>
<tr>
<td>2 = Moderate</td>
</tr>
<tr>
<td>3 = Severe</td>
</tr>
</tbody>
</table>

Table 1: Mayo Disease Activity Index (DAI).

RESULTS

Of the 9 subjects reviewed in this observational chart review, 5 were under 40 years of age. After 8 weeks of subcutaneous therapy of 12.5 mg of MTX, bloody stools were evident in 9 of the 9 patients (100%). Many instances of abdominal pain were also noted by the patients as well. Some of them mentioned having suffered leg cramping, especially during the middle of the night. Individually, many of them complained of being unable to get a good night’s rest, and as a result, demonstrating a lack of energy throughout the day. However, even if the former was not the case, some of these individuals indicated in their diaries that they experienced lethargy, perhaps due to the pressure of the colonic disease affecting their ability to properly perform the tasks that they would normally do. Another prominent symptom common among these patients is the negativity of their emotional and psychological state. Subjects were given a set of standardized questionnaire, called the IBDQ that characterized and assessed their emotional state and well being. In their responses, these subjects often noted feelings of depressed mood and uneasiness indicating that the abdominal condition may have inflicted a heavy toll on their mental and physical state.

An interesting finding during colonoscopy was the presence of histopathologically-confirmed distal adenomatous polyps in all 9 patients. Adenomatous colonic polyps are rare in ulcerative colitis. In this 2004 study reviewing 150 patients, only 6 (4%) had adenomatous polyps. The authors postulate that the decreased prevalence of adenomatous polyps in UC may be possibly due to drug treatments undergone by the patients. Dixon et al. in 2006 reviewed 80 ulcerative colitis patients and found distal adenomatous polyps in only 3 patients ages 55-64 years. Neither did they document polyps in those with Crohn’s disease or indeterminate colitis. Our findings of distal polyps in 9 out of 9 patients (100%) do not support these prior 2 studies.

<table>
<thead>
<tr>
<th>Table 2: Inclusion Criteria and Exclusion Criteria.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1- male or non-pregnant females, 18 years old and older with the diagnosis of mild to moderate ulcerative colitis confirmed by colonoscopy.</td>
</tr>
<tr>
<td>2- Mild to Moderate UC as graded as a Mayo DAI total score between 5 and 10.</td>
</tr>
<tr>
<td>3- score of 2 or more for the rectal bleeding subscore of the Mayo DAI</td>
</tr>
<tr>
<td>4- score of 2 or more for the findings of the flexible sigmoidoscopy or colonoscopy</td>
</tr>
<tr>
<td>5- failure of prior therapies for ulcerative colitis</td>
</tr>
<tr>
<td>6- absence of other malignancy or cancer</td>
</tr>
<tr>
<td>7- absence of intestinal infections and prior surgeries such as intestinal resections and partial intestinal removal procedures</td>
</tr>
<tr>
<td>8- Any clinically significant condition or disease that in the opinion of the investigator would interfere in patient safety</td>
</tr>
<tr>
<td>9- signed informed consent to participate in this study</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 3: Exclusion Criteria.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1- presence of other digestive diseases and malignancies</td>
</tr>
<tr>
<td>2- chronic use of aminosalicylates</td>
</tr>
<tr>
<td>3- significant use of corticosteroids, immunosuppressants or biologic agents prior to the study</td>
</tr>
<tr>
<td>4- known contraindications to analgesia, flexible proctosigmoidoscopy or colonoscopy</td>
</tr>
<tr>
<td>5- abnormal screening laboratory values (complete blood count or CBC and serum chemistry with liver profile)</td>
</tr>
<tr>
<td>6- presence of other clinical significant medical and or psychological illnesses precluding participation</td>
</tr>
<tr>
<td>7- participation in other observational studies</td>
</tr>
<tr>
<td>8- unable or unwilling to complete the follow-up evaluation required in this study</td>
</tr>
</tbody>
</table>
representative of Hispanic population.

This could hypothesize that individuals of this ethnic heritage do exhibit immunity to IBD/UC. Therefore, it would remain to conclusion, since it has yet to be tested from a larger sample size to be seen whether or not this would legitimately result in a valid conclusion, since it has yet to be tested from a larger sample size representative of a Hispanic population.

CONCLUSION

Although methotrexate is a specific drug considered to be effective in Inflammatory Bowel Disease specifically in Crohn’s disease, its effectiveness given as a low dose (12.5 mg) subcutaneously for 8 weeks in mild to moderate Ulcerative Colitis may be limited. However, its therapeutic effect may be altered by the dosage, mode of administration and length of treatment. In particular, if required to be administered through the SC method may require a higher dosage. For this particular study and experiment, the sample size was chosen from a predominantly hispanic community with low prevalence of IBD. This could hypothesize that individuals of this ethnic heritage do exhibit immunity to IBD/UC. Therefore, it would remain to be seen whether or not this would legitimately result in a valid conclusion, since it has yet to be tested from a larger sample size representative of a Hispanic population.

DISCUSSION

Methotrexate plays an important role in the blockage of lymphocytes, proliferation of inflammatory precursors, reduction of neutrophil chemotaxis and adherence, and decrease of serum immunoglobulins.6,23 These biological phenomena serve as the basis of its usage as an immunomodulator in inflammatory bowel disease. Methotrexate bioavailability is affected adversely if high doses are absorbed all at once, thus affecting the rate of response by the patient receiving the drug.27 This drug must be utilized by controlled entry in order to efficiently ensure the clinical benefits for patients with IBD or UC.27

The administration of low dose Methotrexate has been effectively used in the treatment of inflammatory diseases such as Crohn’s and more recently has been administered in ulcerative colitis. Roughly 12 observational trials were reviewed and data was analyzed.27 Results showed that at a 12.5 mg oral dose no results were observed; however in an uncontrolled analysis and at a dose between 20-25 mg a clinical response was observed in 30%-80% of patients.27

In this observational chart review of patients with mild to moderate UC given low dose subcutaneous methotrexate (12.5 mg) there was no significant improvement of abdominal pain and bloody stools. Moreover, in the review of all the patient diaries, all 9 of them experienced a sense of heaviness or abdominal fullness, bloating or cramps. The patients also reported with decreased energy levels and depressed feelings with anxiety and decreased sleep at night. Overall, the majority of them reported a decreased quality of life despite the 8 week trial of MTX.

Although distal adenomatous polyps are rare in ulcerative colitis, it has been described that ulcerative colitis is a risk factor for the development of polyps.43 In this observational chart review of 9 patients, the incidental finding of distal adenomatous polyps in all 9 patients leads us to postulate the following: 1) that distal adenomatous polyps may be a risk factor in UC, 2) that surgical removal or polypectomy for these polyps, if diagnosed earlier especially in those younger than 40 years of age, may delay progression or prevent development of UC, 3) the presence of polyps in all 9 patients could have prevented the desired therapeutic response from methotrexate. However another pos-

### Table 3: Patient Characteristics and Findings after 8 week administration of SC MTX.*

<table>
<thead>
<tr>
<th>Subject</th>
<th>Pt 1</th>
<th>Pt 2</th>
<th>Pt 3*</th>
<th>Pt 4</th>
<th>Pt 5</th>
<th>Pt 6</th>
<th>Pt 7</th>
<th>Pt 8</th>
<th>Pt 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bloody stool</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Abdominal Pain</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Colonoscopy Distal Polyps</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Age &lt; 40 yrs</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Prior Therapies</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Mayo DAI score</td>
<td>8</td>
<td>8</td>
<td>1</td>
<td>5</td>
<td>9</td>
<td>8</td>
<td>9</td>
<td>8</td>
<td>10</td>
</tr>
</tbody>
</table>

*These were the only enrolled patients in the 12-month study from July 2011 to July 2012.

*Patient 3 did not complete 8 week course because of low Mayo score but had a mild UC classification per colonoscopy.

Table 3: Patient Characteristics and Findings after 8 week administration of SC MTX.*

1. Allowed Concomitant Medications:
   Medications taken 90 days prior to and during the study were documented such as intravenous fluids, herbal products, vitamins and any over the counter medications

2. Prohibited Medications:
   Aminosalycilates, corticosteroids, immunosuppressants, biologic response modifiers, change in use of nicotine products, probiotic supplements

3. Allowed Adjunctive Therapy or Procedures:
   Procedures such as psychotherapy, surgery, dental work, acupuncture, physiotherapy, chiropracty, osteopathy or massage therapy for other illnesses were documented in the chart including diagnostic tests done (such as chest x-rays or electrocardiogram ) for other diseases outside the study.

Table 4: Protocol Guideline on Use of Other Medications, Therapies and Procedures.
sible explanation would be that the polyps are a manifestation of the ineffectivity of low dose SC MTX in UC. As Kitiyakara suggested in 2004, the decreased prevalence of adenomatous polyps in UC may be due to the therapeutic response.41

However, the presence of these polyps in these younger patients described may be a cause for concern as the standard care for colorectal cancer screening as presented by the U. S. Preventive Services Task Force (USPSTF) is age 50.9 Although only a small percentage of adenomatous polyps become cancerous, almost all malignant polyps are adenomatous.40 As such, the possibility of development of malignant tumors in these patients increases.

Although there is no data or evidence regarding whether or not patient diet was a factor in terms of the biological abnormalities, it could possibly be that artificially added preservatives could be a factor in disease activity. There are not enough studies regarding foreign substances and unconventional body chemicals affecting UC response to medical treatment. Dietary recall should have been included in the questionnaire or patient diaries.

The extent of distribution of the inflammation in UC determines treatment. In most cases of UC, approximately 25-75% suffer inflammation within the rectosigmoid region, proctosigmoiditis.1,27 Other types of inflammation, such as left-sided colitis, backwash ileitis, and extensive colitis, are relatively less severe compared to proctosigmoiditis. Treatment of the latter three types of inflammations would generally require therapy with oral or intravenous aminosalicylates, which would suffice for the abatement of the effects of the inflammation around the lower intestinal area.44

Overall, the use of low dose subcutaneous methotrexate in mild to moderate ulcerative colitis has been found to have limited therapeutic value in inducing remission in the 9 patients reviewed. The findings support previous recommendations that further research is needed incorporating higher dosages of MTX given parenterally.27

FURTHER RESEARCH

The use of MTX subsequent to or in conjunction with other immunosuppressants and or biologic therapies may be worth investigating. Also, in order to fully understand and formulate a procedure to investigate the true nature of the IBD UC with regards to the particular presence of intestinal polyps, it may be necessary to determine the primary cause of the appearance of polyps. Specifically, data must be accounted regarding the biochemical content of the food intake during the time spanning the initial mention of the symptoms to the time of the colonoscopy. This will allow conclusions to be made based on any relationships observed between the amount of preservatives, type of food consumed, and the frequency of the symptoms. Also, the idea in which very young patients, the majority of them under the age of 50, experience signs of IBD UC, warrants the need to implement clinical studies that would investigate the pros and cons of performing earlier colonoscopy screening than the recommended age of 50. In particular, a comparative study, consisting of a control group that represents the standard care age group of 50-yr. old patients, and a younger group with several risk factors may provide more information on the incidence of polyps, UC and colorectal cancer. First and foremost, investigation of adenomatous polyps in UC involving a larger sample size in a community where IBD, specifically UC, is of high prevalence would be an interesting study. This will ultimately determine if it is a beneficial, short term approach to the prevention of ulcerative colitis and or colorectal cancer. It remains to be seen if the premature treatment either subsidizes the effects of polyp growth, or causes chronic adverse events that are essentially unrelated to IBD UC inflammation. The financial costs and code of ethics would also be a major factor in this potential approach, especially if it were to yield positive results.

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266.


Non-alcoholic Fatty Liver Disease and the Gut Microbiota: Exploring the Connection

Edward C. Oldfield IV, Ray Z. Dong and David A. Johnson

Eastern Virginia Medical School, 885 Kempsville Road, Suite 114, Norfolk, VA 23502, USA

ABSTRACT

As the gut microbiota continues to be implicated in an increasing number of disease processes, a plethora of new literature surrounding its complexity and role in the maintenance of intestinal homeostasis has become available. Non-alcoholic fatty liver disease (NAFLD) has become the most common nonviral liver disease worldwide and a number of predisposing risk factors for NAFLD have been identified, including obesity and insulin resistance. Recent evidence supports a role for the gut microbiota in the pathogenesis of these risk factors and NAFLD, itself. Additionally, changes in the gut microbiota can lead to activation of immune responses that have the potential to promote progression of NAFLD to the more severe Non-alcoholic steatohepatitis (NASH). Furthermore, the gut microbiota may serve as a potential target for therapeutic options to treat NAFLD. This review seeks to explain the role of the gut microbiota in the pathogenesis of NAFLD and its risk factors, while also discussing potential future treatment options directed at correcting imbalances with in the gut microbiota.

KEYWORDS: Non-alcoholic fatty liver disease; Microbiota; Insulin resistance; Metabolic syndrome; Steatohepatitis; Inflammosomes.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a spectrum of liver disease defined as the presence of lipids in >5% of hepatocytes or a lipid content >5% of liver weight in the absence of significant alcohol intake (>20 g of alcohol/day), hepatic viral infections or the use of potentially hepatotoxic medications. Worldwide NAFLD has become the most common nonviral liver disease, affecting over one billion individuals with an estimated prevalence of 6-30% in the general population, in part due to the increasing incidence of obesity and as well due to related other metabolic risk factors. Currently, NAFLD related chronic liver disease is the 3rd leading indication for liver transplantation in the U.S. and is expected to be the leading cause in 2020. Steatosis in NAFLD can progress to non-alcoholic steatohepatitis (NASH) with fibrosis. This may further be subject to progressive changes in inflammation and fibrosis that can lead to liver cirrhosis, end stage liver disease, and also an increased risk for Hepatocellular carcinoma (HCC). The initial diagnosis of NAFLD is often suggested incidentally during abdominal ultrasonography, as most patients with NAFLD are asymptomatic.3 Predisposing factors for the development of NAFLD include those of the metabolic syndrome: abdominal obesity, hypertriglyceridemia, low HDL, hypertension, and insulin resistance.

With >10^14 different microorganisms, the gut microbiota is considered as a major metabolic internal organ, intimately involved in molecular “cross-talk” with the intestinal epithelium and affecting the intestinal barrier function. Recent attention has focused around the gut microbiota not only as part of the disease process, but also as a potential target for treatment. The focus of this article is to explore the link between the human gut microbiota and NAFLD, as disruption of the gut microbiota may predispose patients to developing NAFLD.

Beginning with a review of the relevant pathophysiology, this article will address the
role of the liver and gut microbiota in both metabolic and immune regulation. Further discussion of specific alterations in the gut microbiota in direct relation to each of the major risk factors for NAFLD will follow. Lastly, a review of the therapeutic options functioning to modify the gut microbiota will be addressed.

**PATHOPHYSIOLOGY**

In order to understand the pathogenesis of NAFLD, it is essential to have a basic understanding of hepatic function and its relationship to the predisposing risk factors for NAFLD. The liver is the main warehouse for various lipids, including triglycerides, Free Fatty Acids (FFA), diaclyglycerol, free cholesterol, cholesterol esters, ceramides, and phospholipids. The hallmark pathogenesis of NAFLD is the presence of ectopic fat within hepatocytes, which results from an imbalance in the levels of lipogenesis and lipolysis. Triglycerides are synthesized from FFAs that accumulate within the liver; therefore, the concentration of FFAs functions as a regulator of lipogenesis. Importantly, the hepatic uptake of FFAs is unregulated and is directly proportional to the level of Nonesterified fatty acids (NEFAs), which accounts for 60% of FFAs accumulation within the liver, primarily from lipolysis in adipose tissue. Other sources of FFAs include de novo lipogenesis (25%) and dietary fatty acids (15%) in the form of chylomicrons lipoproteins. After FFAs are taken up by the liver, they have three potential fates: oxidation within mitochondria, VLDL (Very low-density lipoprotein) assembly and export, or triglyceride synthesis and storage as lipid droplets. Over time, an abundance of triglycerides accumulates and leads to increased adipose tissue storage of lipid droplets, promoting the progression towards NAFLD.

Another critical factor in the pathogenesis of NAFLD is the interactions between the specific risk factors for NAFLD. The result is a complex pathway that leads to a cyclic pattern of inflammation and injury. To start, high fat diet and obesity lead to increased peripheral adipose tissue, which initiates Insulin Resistance (IR). The excessive accumulation of fat in adipocytes promotes an increase in oxidative stress and low grade inflammatory state through the release of inflammatory markers, including Interleukin-6 (IL-6) and Monocyte Chemotactic Protein-1 (MCP-1). Subsequently, the activation of macrophages and lymphocytes promotes further release of proinflammatory cytokines associated with insulin resistance, namely Tumor Necrosis Factor-α (TNF-α) and Interferon-γ (INF-γ), promoting a continuation of the cycle.

Progression from NAFLD to NASH, occurs in roughly 20% of cases, and is characterized by the hallmark lobular chronic inflammatory infiltrate without any secondary causes of hepatic fat accumulation, e.g., significant alcohol consumption, use of steatogenic medication or hereditary disorders. Injury and inflammation are thought to be the major factors that lead NAFLD progression to NASH and fibrogenesis. One potential explanation for the progression to NASH is lipotoxicity, a process in which increased oxidative stress secondary to accumulation of lipids overwhelms the hepatic function of metabolism. Lipotoxicity also leads to impaired autophagy, causes cell damage and cell death, and induces an inflammatory and wound healing response that can lead to fibrogenesis. Additionally, a variety of bacterial products can activate various immune responses, further promoting inflammation through the expression of proinflammatory cytokines. These immune responses will be analyzed and discussed more thoroughly in a later section.

**ROLE OF MICROBIOTA IN NAFLD RISK FACTORS**

Although a number of genetic and environmental factors have been linked in the pathogenesis of NAFLD, obesity, insulin resistance, and immune responses are the more dominant risk. First, obesity, in particular central obesity, is highly predictive of hepatic steatosis and disease progression. In overweight (BMI >25) patients, the prevalence of steatosis is at least two times more frequent than in lean subjects, directly proportional to elevated Body Mass Index (BMI). In extreme obesity (BMI >40), most patients have NAFLD steatosis, and more than one third have NASH. Secondly, insulin resistance plays a huge role in developing NAFLD evidenced by a 5-9 fold increased risk for...
NAFLD in patients with Type 2 Diabetes Mellitus (T2DM) as compared to the general population; further, two thirds of these patients with T2DM develop NAFLD.\textsuperscript{14,15} Third, the immune system regulates inflammatory responses to a variety of bacterial products that can be altered in NAFLD. This section seeks to more closely explore the relationship between each of these risk factors and their association with changes in the gut microbiota.

**Obesity**

The gut microbiota has been recently linked to the pathogenesis of obesity through a number of pathways.\textsuperscript{16} In particular, modification of appetite and alteration of de novo lipogenesis appear to be essential mechanisms by which the gut microbiota maximizes hepatic triglyceride content.\textsuperscript{5,11} Evidence for these mechanisms comes from animal studies where Germ-free (GF) animals, born and raised in a sterile environment lacking gut flora, were resistant to the development of obesity when fed a high-fat, high-sugar diet; however, after introducing gut flora to these GF mice, there was an increase in energy harvested from the diet with increased intestinal monosaccharide uptake. Additionally, these mice had increased weight and body fat content, with increased hepatic lipogenesis and fat deposition, which eventually led to the development of insulin resistance.\textsuperscript{11,16,17}

Within the gut microbiota two predominate species of bacteria, *Firmicutes* and *Bacteroidetes*, have been influential in the development of metabolic syndrome.\textsuperscript{11} The balance of these two bacteria is dysregulated in patients with metabolic syndrome and obesity, evidenced by multiple studies showing an excess of *Firmicutes* and reduction of *Bacteroidetes* compared to lean counterparts.\textsuperscript{11,16,18} In these studies, more *Firmicutes* resulted in increased fermentation end products such as Short-chain fatty acids (SCFAs). These SCFAs, in turn, play a major role in appetite regulation by not only diffusing passively into circulation, but also by acting as signaling molecules.\textsuperscript{11,19} Certain SCFAs, such as propionate and acetate, can bind to G protein-coupled receptors (GPCRs) to induce release of Peptide YY (PYY).\textsuperscript{20} PYY is an enteronecocrine cell-derived hormone that normally inhibits gut motility and increases nutrient absorption, so abundant SCFAs increase calorie absorption by stimulating PYY, leading to obesity. Furthermore, excess SCFAs will also be converted into triglycerides in the liver, which can cause hepatic steatosis.\textsuperscript{21} These studies give us insight that further therapeutic approaches to obesity could target these specific gut flora.\textsuperscript{21}

These “typical” changes in the obese human gut microbiota, however, have not been found by all investigators. Schwierz et al. reported lower ratios of *Firmicutes* to *Bacteroidetes* in obese human adults compared to lean controls;\textsuperscript{22} however, significant diet-dependent reductions in a group of butyrate-producing *Firmicutes* were found.\textsuperscript{23} In 2011, Arumugam et al. studied the phylogenetic composition of 39 fecal samples from individuals representing 6 nationalities and found that there was no correlation between body mass index and the *Firmicutes/Bacteroidetes* ratio.\textsuperscript{24} On the other hand, the identification of three metagenomic-derived functional biomarkers that strongly correlate with Body Mass Index (BMI), suggests that differences at the phylum level are probably less important than metagenomic-based functional aspects.\textsuperscript{20,24}

Besides the gut flora changes and metagenomic biomarkers, there are also a few studies targeting how the gut microbiota puts patients at risk for obesity on a molecular level. Bäckhead et al. showed that Fasting-induced adipocyte factor (*Fiaf*), a member of the angiopoietin-like family of proteins, is suppressed in the intestinal epithelium by the microbiota.\textsuperscript{25} This suppression leads to increased Lipoprotein lipase (LPL), a key regulator of fatty acids, which results in increased cellular uptake of fatty acids and adipocyte triglyceride accumulation. Further investigation revealed that when the gut was colonized with *Bacteroides theitaiotaomomer* and *Methanobrevibacter smithii*, there was a significant increase in suppression of *Fiaf*, which leads to obesity.\textsuperscript{26}

More than just the bacteria living in the gut microbiota may influence energy homeostasis. Zhang et al. reported an association between methanogenic *Archaea* (microorganisms which produce methane as a byproduct during anoxic conditions) and obesity.\textsuperscript{27} Increased levels of *Archaea*-derived gene fragments were detected in obese mice compared to their lean relatives suggesting that methanogens in the gut may play a pivotal role in fermentation, and ultimately lead to production of SCFAs with the net result being energy harvest and weight gain.\textsuperscript{28,29} A proposed explanation is that methanogens remove fermentation intermediates, such as H2 (hydrogen gas) or formate, relieving thermodynamic limitations and allowing greater production of SCFAs that are available to be absorbed across the intestinal epithelium, while at the same time extracting more energy from indigestible polysaccharides.\textsuperscript{27} The study concluded that interspecies H2 transfer between bacterial and archaeal species affects energy uptake in humans and puts patients at risk for obesity.\textsuperscript{27} SCFAs also regulate gut hormones via Free Fatty Acid Receptors 2 (FFAR2) and 3 (FFAR3), which promote energy storage by stimulating adipogenesis and inhibiting lipolysis. This decrease in energy expenditure ultimately leads to obesity and other metabolic diseases.\textsuperscript{28-30}

**Bottom line**

Obesity is clearly a strong risk factor in the pathogenesis of NAFLD with a prevalence twice that of lean comparators. High fat diets increase the accumulation of FFAs within the liver, ultimately leading to NAFLD. The gut microbiota has been shown to be intimately involved in this pathway, as a characteristic increase in *Firmicutes* and reduction in *Bacteroidetes* has been widely documented. This alteration in the normal ratio affects the regulation of gut hormones such as PYY and also number of regulatory factors for lipolysis and lipogenesis, including *Fiaf*, LPL, FFAR2, and FFAR3. Continued investigation
into the alterations in the gut microbiota in obesity may help to further our understanding NAFLD and explain key differences in environmental versus genetic factors.

Insulin Resistance

Environmental factors and host genetics play major roles in establishing and maintaining gut microbiota, while in turn interacting to sustain the homeostasis of gut, weight control, and insulin sensitivity, which may have a role in the development of obesity and insulin resistance. TLR2-deficient mice, under germ-free conditions, are protected from diet-induced insulin resistance. It is possible that the presence of gut microbiota could reverse the phenotype of an animal, inducing insulin resistance in an animal genetically determined to have increased insulin sensitivity, such as the TLR2 KO mice. In the present study, we investigated the influence of gut microbiota on metabolic parameters, glucose tolerance, insulin sensitivity, and signaling of TLR2-deficient mice. We investigated the gut microbiota (by metagenomics Previously discussed inflammatory mediators such as TNF-α, IL-6, inducible nitric oxide, and Nuclear Factor (NF-κB) have already been shown to be increased when the gut microbiota is altered or disrupted. Here we will discuss the mechanisms behind which changes in gut microbiota may promote insulin resistance.

Certain inflammatory mediators involved in the development of insulin resistance are controlled by Toll-like receptor 4 (TLR4) activated by Lipopolysaccharide (LPS) from gram negative bacteria, highlighting a link between insulin resistance and liver inflammation through several pathways responsible for the regulation of hepatocyte apoptosis and insulin signaling. Important functions of TLR4 in relation to insulin resistance are the upregulation of both c-Jun NH2-terminal Kinase (JNK) and IkB kinase complex (IKKβ) and also decreased phosphorylation of Insulin Receptor Substrate (IRS)-1. The IRS-1 is needed for insulin signaling cascades that affect glucose transport in muscle and adipose tissue, glycogen synthesis, and lipogenesis. In this respect, alterations in the gut microbiota that affect activation of immune response can potentially modify insulin resistance.

Other bacterial factors that play a role in the development of insulin resistance could be Nucleotide Oligomerization Domain (NOD)-1 and -2 proteins. These NOD proteins are intracellular pattern recognition receptors that can sense bacterial cell wall Peptidoglycan (PGN) moieties, which then induce stress and inflammation pathways. NOD-1 detects PGN found in gram-negative bacteria whereas NOD-2 detects gram-positive bacteria. Activation of NOD-1 in adipocytes leads to impaired insulin signaling and decreased insulin-stimulated glucose uptake, while activated NOD-2 leads to muscle cell-autonomous insulin resistance.

Adenosine Monophosphate-activated Protein Kinase (AMPK) is an enzyme which plays an active role in energy homeostasis. It is activated to offset the energy deprived state by stimulating fatty acid oxidation, ketogenesis, glucose uptake, and insulin secretion while inhibiting cholesterol synthesis, lipogenesis, and triglyceride synthesis. Bäckhead et al. demonstrated that the expression of AMPK is suppressed by microbiota thereby predisposing the host to obesity and insulin resistance.

A few animal studies have also investigated the link between insulin resistance and the gut microbiota, in particular, how the translocation of gut microorganisms and their byproducts into portal and systemic circulation may cause hepatic inflammation and insulin resistance. It has been shown that mice on a HFD have greater accumulation of bacteria close to the mucosa of the intestinal lumen, which facilitates their translocation through the epithelium. This high level of bacteria at the Menteric Adipose Tissue (MAT) triggers inflammatory markers through LPS released by bacteria, eventually leading to systemic inflammation and insulin resistance. Interestingly, mice given one month of probiotics showed complete normalization of insulin sensitivity, inflammation, and fasting hyperinsulinemia, further supporting the gut microbiota as a potential target in insulin resistant diabetic patients. Another study done by Caricilli et al. looked at gut microbiota on a molecular level in association with insulin resistance. Their results showed, that in TLR2 knockout mice, conventionalization (as opposed to “germ-free” condition) results in a phenotype reminiscent of metabolic syndrome, characterized by different gut flora, with a 3-fold increase in *Firmicutes* and a slight increase in *Bacteroidetes* compared with control; further, antibiotics were able to reverse these adverse outcomes. Once again, LPS absorption, subclinical inflammation, insulin resistance, and glucose intolerance are all sequelae of these changes in microbiota.

**Bottom-line**

As compared to obesity, which primarily predisposes to NAFLD on the basis of increased FFA within the liver, insulin resistance appears to affect a wider variety of biochemical pathways involved in the pathogenesis of NAFLD. Insulin resistance is closely linked to inflammatory mediators and regulation of signaling cascades that affect glucose transport in muscle and adipose tissue, glycogen synthesis, and lipogenesis. In this respect, alterations in the gut microbiota that affect activation of immune response can potentially modify insulin resistance.

Cellular Immunity and Inflammation

While obesity and metabolic syndrome are undoubtedly the most important risk factors for the development of NA-
FDL, the relationship between the immune system and the gut microbiota appears to have a more essential role in the inflammatory processes that drive the change from NAFLD to NASH. The pathogenesis of NASH was originally described as a “two-hit” hypothesis in which the “first hit,” hepatic steatosis, acts to sensitize the hepatocytes for the “second hit,” either genetic factors, oxidative stress, gut-derived endotoxins, or inflammatory cytokines. More recently, new evidence has emerged suggesting that inflammation may be able to proceed steatosis in some cases, suggesting that multiple parallel hits may occur to initiate the progression to NASH. 10%-20% of patients who have fatty liver develop inflammation and fibrosis Non-alcoholic steatohepatitis (NASH) While there a number of factors involved in this complex pathway leading to NASH, this review will focus on the role of the innate immune system and its relationship to endotoxin and gut derived signals.

During the progression from NAFLD to NASH, injured cells and necrotic tissues release molecules such as Damage-associated molecular patterns (DAMPs), which trigger inflammation through the binding of several receptors. These receptors can be specific or shared with Pathogen-associated molecular patterns (PAMPs) that recognize molecular patterns associated with microbial pathogens or cellular stress. The essential foundation for the relationship between the immune system and the gut microbiota is the recognition of these PAMPs and DAMPs via Toll-like receptors (TLRs) or Nod-like receptors (NLRs). Both TLRs (located on the cell surface or within endosomes) and NLRs (located within the host cytosol) function to recognize microbial products and activate signaling pathways of both innate and adaptive immune responses. In order to understand the impact that gut microbiota alterations can have on the immune system, it is important to more closely analyze the major receptors in each of the families.

**Toll-like Receptors**

The TLRs often represent a first line of defense based on their cell surface location and recognition of a variety of microbial signals. In the liver, TLRs are an essential piece of immunity as the portal system has the potential to be a significant source of microbial products and any disruption in the balance can lead to excess inflammation within the liver. The four main TLRs involved in NAFLD and NASH are: TLR2, recognizing peptidoglycan and lipoteichoic acid, both components of gram-positive bacterial cell walls; TLR4, recognizing Lipopolysaccharide (LPS) from gram-negative bacteria; TLR5, a receptor for bacterial flagellin; and TLR9, recognizing unmethylated CpG motifs in bacterial DNA.

To date, a number of studies performed in animal models have helped to explain the significance of these receptors in the development of NAFLD. Evidence for the relationship between the gut microbiota and TLRs is multifocal, although key factors are alterations in the gut microbiota along with a related increased intestinal permeability. These factors have been demonstrated in rodent models through a variety of diets including High-fat diet (HFD), Methionine-choline deficient diet (MCD), and Choline-deficient amino acid defined diet (CDAA). For example, it has been shown that rodents placed on a High-fat diet (HFD) have increased inflammation through the induction of TLR4, which leads to increased intestinal permeability and increased endotoxin levels, further accelerating obesity; importantly, this effect was not reproducible with the HFD in TLR4 deficient mice. Additionally, a number of other studies have shown that TLR4 mutant mice are resistant to the development of NAFLD. Similar models using a Methionine choline-deficient (MCD) diet were able to induce NASH, evidenced by increased liver triglyceride accumulation, lipid peroxidation, serum ALT, TNF-α, NADPH, and markers of liver fibrosis.48 When knockout mice deficient for TLR4 and its co-receptor MD-2 (Myeloid Differentiation factor) were also placed on the MCD diet, however, these increases were attenuated. The authors of this study suggest that these results demonstrate a role for LPS recognition via TLR4 and MD-2 for inducing liver steatosis and fibrosis in a NASH model in mice.49 This conclusion is supported by several mouse models in which LPS injections in NAFLD mice were able to further promote liver injury through increased levels of proinflammatory cytokines.50,51 This represents an important finding, as levels of LPS in humans are also elevated in those with metabolic syndrome and NAFLD.

Among patients with biopsy-proved NAFLD, increased small intestine bacterial overgrowth has been associated with disrupted intercellular tight junctions, leading to increased intestinal permeability and delivery of LPS to the portal system, including genetic differences, insulin resistance and intestinal microbiota, account for the progression of Non-alcoholic steatohepatitis (NASH) In patients with type 2 diabetes mellitus, circulating levels of LPS were shown to be 76% higher than in matched controls and further associated with significant increases in TNF-α and IL-6. Another mechanism by which TLR increases inflammation is through the potent activation of Kupffer cells within the liver. This activation of Kupffer cells can induce a pathological effect by inducing Reactive Oxygen Species (ROS) - dependent activation of X-box binding protein-1 (XBP-1), which is a key transcription factor mediating unfolded protein responses in ER (Endoplasmic Reticulum) stress. Additionally, in this rodent model of NASH, Kupffer cell depletion led to an abrogation of the high-fat, high-cholesterol diet induced TLR4 expression; this suggests that Kupffer cells are a major source of proinflammatory mediators through an increased expression of TLR4.

Toll Like Receptor 9, which recognizes unmethylated CpG motifs in bacterial DNA, has also been shown to play an important role in the progression to NASH. Using a CDDA diet induced NASH model, researchers were able to show that TLR9 signaling induced IL-1β production leading to steatosis, inflammation, and fibrosis, which was also associated with insulin resistance and weight gain; in this same model, TLR9 deficient mice showed less steatosis, inflammation, liver fibrosis, insulin...
resistance, and weight gain compared to controls.\textsuperscript{55}

One of the major changes in the gut microbiota associated with obesity and high fat diets is a significant decrease in the gram-negative \textit{Bacteroidetes} and a proportional increase in the gram-positive \textit{Firmicutes}.\textsuperscript{18} This change in the gut microbiota represents a major shift in the balance of the gram-negative to gram-positive bacteria that has the potential for alteration of the inflammatory activity secondary to TLR activation. In this environment TLR2, which recognizes components of gram-positive cell walls, likely acts in concert with TLR4 to mediate changes in the proinflammatory cytokines and alterations in intestinal permeability. Interestingly, while TLR2 deficient mice on a high-fat diet (HFD) when placed on an MCD diet these mice have significantly enhanced histological and molecular evidence of steatohepatitis compared to controls.\textsuperscript{58,59} The proposed mechanism for this phenomenon is increased sensitivity of TLR4 to LPS in the absence of TLR2.\textsuperscript{47} These results would also suggest a protective role of TLR2 against the development of liver injury, a potential mechanism of which would be maintenance of the mucosal integrity, as evidenced by disruption of tight junctions in TLR2 deficient mice that was preserved in wild type mice with a TLR2 agonist.\textsuperscript{60}

Toll Like Receptor 5, which recognizes bacterial flagellin, may also play a protective role, as a study with TLR5 deficient mice showed the development of obesity and steatosis, which was further exacerbated by a high-fat diet.\textsuperscript{61} Subsequent decimation of the gut microbiota in these TLR5 deficient mice corrected the metabolic syndrome relative to the wild type mice. Looking more closely at the gut microbiota in these TLR5 deficient mice, both \textit{Firmicutes} and \textit{Bacteroidetes} were similar with wild type mice; however, more specific analysis showed that species concentrations within these two phyla were significantly different.\textsuperscript{61} When the gut microbiota from TLR5 deficient mice was transplanted into wild type germ-free mice, phenotypic aspects of the TLR5 deficient mice were transferred to wild type mice including hyperphagia, obesity, hyperglycemia, insulin resistance, colomegaly, and elevated proinflammatory cytokines.\textsuperscript{51}

In summary, a wide range of rodent models have shown the significance of interactions between immune regulation through TLRs and the gut microbiota in the development of NAFLD. The primary mechanisms behind these changes are increased proinflammatory cytokines and altered intestinal permeability that create a predisposition to the major risk factors for NAFLD, namely obesity, insulin resistance, and metabolic syndrome.

\textbf{Nod-like Receptors}

In contrast to the TLRs, which function primarily to recognize extracellular ligands, the NLRs are located intra-cellularly and have a more complex mechanism of action including activation of inflammasomes. NLRs are complicated receptor proteins that have a variable N-terminal domain and a centrally located Nucleotide-binding Oligomerization Domain (NOD) and a C-terminal leucine rich repeat region that recognizes PAMPs.\textsuperscript{45} Within the host cytosol these NODs recognize specific microbial molecules; NOD1 recognizes iE-DAP (γ-D-glutamyl-meso-diaminopimelic acid) which contains fragments from most gram-negative and some gram-positive bacteria, while NOD2 recognizes Muramyl dipeptide (MDP) found in the majority of both gram-positive and gram-negative bacteria.\textsuperscript{45} Within the N-terminal domain there are further protein modules involved in downstream signaling pathways, including a Caspase recruitment domain (CARD). These CARDs are particularly important, as multiple NLRs can join together through an adaptor protein such as ASC (Apoptosis-associated speck-like protein) to form an inflammasome, which controls caspase activation and subsequent production of pro-inflammatory cytokines.\textsuperscript{45,62}

These inflammasomes and caspases play critical roles in the immune response through regulation of inflammation and also cell death. Caspase-1 activation by inflammasomes leads to the cleavage of pro-IL-1β and pro-IL-18 into their biologically active forms, causing recruitment of inflammatory cells, production of INF-γ, and enhancement of natural killer cell activity.\textsuperscript{45} One inflammasome in particular, NLRP6, appears to have a critical role in controlling intestinal homeostasis; NLRP6 deficiency has been associated with: decreased levels of IL-18, increased concentrations of \textit{Bacteroidetes} and the bacterial phylum \textit{TM7}, enhanced activation of MAP kinase and NF-Kβ upon TLR ligation, defective autophagy of goblet cells, impaired mucin secretion into the gut lumen, and improved resistance to infection with \textit{Listeria, Salmonella}, and \textit{E. coli}.\textsuperscript{62-66} We show that deficiency of NLRP6 in mouse colonic epithelial cells results in reduced IL-18 levels and altered fecal microbiota characterized by expanded representation of the bacterial phyla \textit{Bacteroidetes} (Prevotellaceae As such NLRP6 may serve to dampen certain inflammatory signals by promoting bacterial dissemination and colonization of systemic organs, while at the same time clearing enteric pathogens from the mucosal surface to maintain intestinal homeostasis (Table 1).

In this manner inflammasome function is intrinsically related to the gut microbiota and regulation of TLR activation, which also has an important role in controlling the progression of liver injury. This has been evidenced in animal studies showing that NLRP6 and NLRP3 along with IL-18 negatively regulate progression of injury in NAFLD and NASH.\textsuperscript{64} Further, inflammasome deficiency may lead to increased TLR4 and TLR9 agonists into the portal circulation, thereby triggering increased inflammation and driving progression of the injury, mainly through hepatic TNF-α production. A key regulator of this increased TLR4 and TLR9 agonist production may be microbiota-induced subclinical colonic inflammation through chemokine.
CCL5 secretion. Additionally, some of the metabolic alterations in these inflammasome-deficient mice can be horizontally spread with the resulting altered gut microbiota negatively impacting NAFLD progression.

**Proinflammatory Cytokines**

Both the TLRs and the NLRs ultimately affect downstream pathways that lead to alterations in the levels of proinflammatory cytokines. Among these cytokines, TNF-α and IL-1β are the major cytokines driving liver injury and progression of NAFLD. The primary role of TNF-α is in the regulation of immune cells. Dysregulation of TNF production has been implicated in a variety of human diseases including a spectrum of rheumatologic diseases and inflammatory bowel disease. Animal models have also shown that TNF-α and IL-1β deficiencies confer resistance to NAFLD and NASH, respectively, while on a HFD.

The cytokine TNF-α is involved in a number of pathways that can ultimately affect the predisposing factors for NAFLD. Most importantly, TNF-α causes increased insulin resistance through alteration of insulin receptor function and also increasing cholesterol accumulation in hepatocytes through the inhibition of LDL receptors and efflux transporters. Increased lipid levels in these hepatocytes alters normal signaling and leads to an increase in reactive oxygen species, which drives cell death signaling. In addition, increased cholesterol accumulation with in hepatocytes can result in increased TLR4 through suppression of the endosomal-lysosomal degradation pathway of TLR4, including genetic differences, insulin resistance and intestinal microbiota, account for the progression of Non-alcoholic steatohepatitis (NASH). In recent years, researchers have been able to identify that Kupffer cells, resident macrophages in the liver, seem to play a crucial role in detecting DAMPs and activating inflammasome responses. Studies with human biopsies have shown an increase in CD68, a pan-macrophage marker in patients with NASH, as compared with simple steatosis, and that Kupffer cell depleted animals develop less features of NASH. Non-alcoholic fatty liver disease (NAFLD) Additionally, Kupffer cell phagocytosis of excess cholesterol also leads to increased expression of proinflammatory cytokines and TLR4 activation. Including genetic differences, insulin resistance and intestinal microbiota, account for the progression of Non-alcoholic steatohepatitis (NASH) Similarly, IL-1β is involved in lipid accumulation within hepatocytes, however, IL-1β suppresses PPARs causing accumulation of triglycerides within hepatocytes, thereby leading to increased expression of pro-apoptotic pathways.

**Bottom-line**

The immune response to changes in the gut microbiota is complex and multifocal; however, it remains clear that increased knowledge of these pathways leads to a more comprehensive understanding of the potential interactions between the risk factors for NAFLD and cellular immunity. This immune response is an important factor in driving the progression of
NAFLD to NASH through induction of inflammation. Additionally, these immune pathways may serve as potential therapeutic targets, as restoring the normal microbiota has been shown to attenuate NAFLD in a number of animal studies.

**TREATMENT OPTIONS FOR ALTERING THE GUT MICROBIOTA**

There are a number of treatment options available for NAFLD aimed at a variety of pathways involved in the development of NAFLD. Among these, the use of diabetes medications clearly functions to combat the increased insulin resistance, while pentoxifylline aims to decrease levels of inflammation. In this manner, available treatment options focus on different aspects of disease pathogenesis, including risk factors and progression. In our review we will focus on those treatment options that alter the gut microbiota as a predominant mechanism of action. These include antibiotics, prebiotics, and probiotics.

**Antibiotics**

At present there is no concise evidence supporting the use of antibiotics in the treatment of NAFLD. There are, however, a number of potential mechanisms by which antibiotics can alter the gut microbiota in favor of attenuating the severity of NAFLD. As discussed earlier in the paper, levels of endotoxemia and inflammation secondary to activation of TLRs by microbial products represents major factors in the progression of NAFLD liver injury. Antibiotic administration leading to a reduction in these bacterial products, in particular LPS, would then theoretically attenuate the inflammation. This in turn would allow for decreased intestinal permeability through increased expression of tight junction proteins. Both of these mechanisms are supported by evidence from animal models where antibiotics decreased circulating LPS and TLR4 activation in addition to increasing expression and function of tight junction proteins.

Rifaximin, a non-absorbable antibiotic, is one potential candidate for the treatment of NAFLD. Rifaximin has been shown in a number of studies to improve liver injury in patients with cirrhosis, most notably for its effects in treating hepatic encephalopathy. Currently, there is an ongoing randomized trial assessing efficacy of rifaximin in NAFLD/NASH through measurements of proinflammatory cytokine and endotoxin levels, including TNF-α and TLR4 activation. Given the high cost and adverse effects associated with chronic antibiotic use, however, results of this study and others will be needed before rifaximin or other antibiotics can be recommended as a therapeutic option for NAFLD.

**Prebiotics**

Prebiotics were originally defined as “nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon”; however, they are now more loosely defined as “selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the gastrointestinal microflora, that confer benefits.” In order for a food to be classified as a prebiotic it must resist gastric acidity, hydrolysis by mammalian enzymes, and absorption in the upper gastrointestinal tract, such that it is able to be fermented by the gut microbiota into Short-chain fatty acids (SCFAs), including acetate, propionate, and butyrate, that can be used for energy. The primary prebiotics used the two inulin-type fructans, Oligofructose (OFS) and Fructo-oligosaccharides (FOS), and the galactan, Galacto-oligosaccharides (GOS). The fructans are the most extensively studied prebiotics for use in metabolic syndrome, with the differences between the fructans being only the number of repeating units of D-fructose in the polymer chain.

The role of prebiotics in the treatment of NAFLD centers largely around the functional roles of improved glucoregulation and modified lipid metabolism. Specific alterations of the gut microbiota by these prebiotics include favored growth of indigenous bifidobacteria and/or lactobacilli and decreased luminal pH, which impedes the growth of pathogens. Modification of lipid metabolism by prebiotics is centered on regulation of de novo fatty acid synthesis. While healthy individuals usually have minimal hepatic de novo lipogenesis, NAFLD patients with hyperinsulinemia can have up to 26% of the hepatic triglyceride content as a result of de novo lipogenesis. Importantly, this increased de novo lipogenesis is also an important phenotypic factor in genetically obese mice, another clinical feature that has strong implications in the development of NAFLD in humans. Prebiotics have been shown to attenuate de novo lipogenesis, likely through a mechanism of action that includes alterations in gene expression of regulatory enzymes for lipogenesis. Additionally, prebiotics may decrease lipogenesis by altering the by-products of microbiota fermentation. Of the SCFA by-products, acetate and propionate are the major constituents delivered to the liver, whereas most butyrate is metabolized in the colon; in the liver, acetate promotes lipogenesis, while propionate inhibits lipogenesis. One suggested mechanism of prebiotics in NAFLD is an increased ratio of propionate to acetate, which may promote a decrease in hepatic lipogenesis.

Alteration of the gut microbiota by prebiotics may also affect the levels of proinflammatory cytokines secondary to changes in intestinal permeability and levels of LPS. Using the prebiotic (oligofructose) in mice fed a HFD, gut microbiota showed an increase in the levels of *Bifidobacterium*, which was positively associated with decreased endotoxemia and proinflammatory cytokines as a result of decreased levels of LPS. The complexity of this relationship between the gut microbiota and intestinal permeability is further highlighted as researchers have also shown decreased intestinal permeability and LPS absorption in prebiotic treated mice who have increased production of glucagon-like-peptide 2.

There is currently little data from human studies con-
cerring the use of prebiotics as it pertains to alterations in inflammation, with only one randomized placebo controlled pilot study of 7 patients with NASH showing decreased levels of aminotransferases after 8 weeks; however, there is some evidence for prebiotics in lowering lipid levels, improving both weight loss and insulin resistance. In eight studies using prebiotics in human subjects with diabetes or hyperlipidemia, levels of cholesterol and triglycerides were shown to decrease between 6-20% and 14-27%, respectively.88 One randomized control trial assigned patients to receive either the prebiotic oligofructose or placebo for 12 weeks and found a significant reduction in weight of 1.03±0.43 kg in the prebiotic group versus a weight gain of 0.45±0.31 kg in the placebo group (P=0.01).87 Additionally, patients in the prebiotic group reported a decreased caloric intake that was associated with decreased ghrelin and increased peptide YY levels.

In summary prebiotics may serve a role in the modification of lipid metabolism by attenuating de novo lipogenesis and alerting byproducts of microbial fermentation. Other potential benefits of prebiotics include decreased intestinal permeability and alteration of gut hormones that may lead to decreased caloric intake. While there is insufficient clinical evidence to support routine use of prebiotics in NAFLD patients, the evidence from animal studies supports consideration for the use of prebiotics in select patients who may not have responded to other therapeutic options.

Probiotics

Probiotics are live microorganisms that, when administered in adequate quantities, confer a health benefit to the host.88 Probiotics have been used in a number of disease processes, including NAFLD, in an attempt to produce a health benefit through the correction of gut dysbiosis. The use of probiotics in NAFLD is focused on the basis that many patients with NAFLD have increased intestinal permeability secondary to Small Intestinal Bacterial Overgrowth (SIBO).11 As discussed earlier, the increased intestinal permeability results from disruption of intercellular tight junctions and leads to increased translocation of bacterial products into the bloodstream, causing increased endotoxemia and delivery of these products to the liver activating inflammatory cytokines. There are several different mechanisms to justify a potential role for the use of probiotics in the treatment of NAFLD. First, probiotics have been shown to produce a number of antimicrobial factors which lead to a decreased pH and inhibition in the growth of pathogenic gram negative bacteria.89 In addition, some probiotic strains can compete with and displace pathogenic bacteria from epithelial surface receptors in the gut.89 Intestinal permeability is also improved as lactobacillus and bifidobacteria mixtures have been shown to increase mucin secretion through upregulation of the mucin producing genes MUC2 and MUC3.89 Overall, the activity of probiotics should lead to improvements in NAFLD by partially correcting the dysbiosis of the gut microbiota and by limiting SIBO and its resultant increased intestinal permeability and endotoxemia.

The efficacy of probiotics in NAFLD animals models has been well established in a variety of Lactobacillus species, with a number of studies showing reductions in LDL, cholesterol, and triglycerides along with histological improvement and amelioration of the inflammation and steatosis.89 Despite this, there have been a limited number of human trials investigating the efficacy of probiotics in NAFLD, largely related to the complex pathology of the disease and the ethical considerations required with invasive diagnostic procedures and histological sampling. To date, the best clinical evidence in humans comes from a recent meta-analysis covering 134 patients from four randomized control trials receiving probiotics (including Lactobacillus, Bifidobacterium, and Streptococcus species) for the treatment of NAFLD or NASH. Results showed that, compared to placebo, probiotics significantly decreased ALT, AST, total cholesterol, HDL, and TNF-α10; however, no significant changes in BMI, glucose, or LDL2 were associated with probiotic use.10 Some limitations exist when interpreting this data, namely the difficulties in ascertaining changes in liver fatty infiltration as it requires a histologic specimen. Of the three studies using histologic analysis, only one had post-treatment histology results. The remaining study used ultrasonography, which cannot identify fatty infiltration of the liver below a threshold of 30%.86 Lastly, there remains a potential for confounding, as dietary restrictions, exercise and physical activity were not reported.

In summary, probiotics appear to be a potential treatment option for NAFLD. Numerous studies have shown improvements in the intestinal dysbiosis, leading to decreasing intestinal permeability, endotoxemia and subsequent inflammation. While the majority of evidence supporting the use of probiotics is from animal studies with only a few clinical trials, given the technical difficulties of performing this research in humans, the positive findings from the clinical trials should be encouraging for efficacy of probiotics in NAFLD (Table 2).

Bottom-line

There are number of potential therapeutics roles for antibiotics, prebiotics, and probiotics in the treatment of NAFLD based on alterations of the gut microbiota. While currently there is limited evidence to support the use of antibiotics, both prebiotics and probiotics have encouraging results in animal studies for improving the gut dysbiosis and potentially inducing a clinical benefit in NAFLD patients. As such clinicians should be aware of these options and consider them for patients either not responding to other treatment approaches or who desire an adjunctive treatment option.

1Mean differences: ALT [-23.71, 95% CI (-33.46, -13.93), p=0.00001]; AST [-19.77, 95% CI (-32.55, 7.00), p=0.002]; Total cholesterol [-0.28, 95% CI (-0.55,-0.01), p=0.04]; TNF-α [-0.32, 95% CI (-0.48, -0.17), p=0.0001]
2Mean differences: BMI [0.05, 95% CI (0.18-0.29), p=0.64]; Glucose [0.05, 95% CI (-0.25, -0.35), p=0.76]; LDL [-0.38, 95% CI (-0.78,0.02), p=0.06]
with NAFLD. Additionally, the gut microbiota may be a potential effective therapeutic target for improving outcomes associated with NAFLD. Moreover, the gut microbiota may also play a role in the development and progression of type 2 diabetes.

Accordingly, it is essential that clinicians understand the modifiable risk factors for NAFLD. The gut microbiota has long been understood to play a role in the pathogenesis of various diseases; however, recent advances in technology have greatly increased our ability to analyze the composition of the gut microbiota and its alterations relative to specific diseases. Current evidence strongly supports the existence of certain characteristic changes in the gut microbiota, affecting signaling pathways and immune responses, which play a role in the development and progression of NAFLD. Additionally, the gut microbiota may be a potential effective therapeutic target for improving outcomes associated with NAFLD.

CONCLUSIONS

The global epidemic of obesity and the increasing prevalence of type 2 diabetes has propelled NAFLD as the most common chronic non-viral liver disease. The complication of NASH in this population is formidable given the numbers of patients affected. Additionally, the burden of NAFLD on the healthcare system is expected to increase, as by 2020, this is projected to be the number one indication for liver transplantation in the US.2 Accordingly, it is essential that clinicians understand the modifiable risk factors for NAFLD. The gut microbiota has long been understood to play a role in the pathogenesis of various diseases; however, recent advances in technology have greatly increased our ability to analyze the composition of the gut microbiota and its alterations relative to specific diseases. Current evidence strongly supports the existence of certain characteristic changes in the gut microbiota, affecting signaling pathways and immune responses, which play a role in the development and progression of NAFLD. Additionally, the gut microbiota may be a potential effective therapeutic target for improving outcomes associated with NAFLD.

CONFLICTS OF INTEREST: None.

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The Role of Energy Metabolism in Driving Disease Progression in Inflammatory, Hypoxic and Angiogenic Microenvironments

James J. Phelan, C. O’Hanlon, John V. Reynolds and Jacintha O’Sullivan

Department of Surgery, Institute of Molecular Medicine, Trinity College Dublin, St. James’s Hospital, Dublin 8, Ireland

ABSTRACT

Cellular metabolism plays a crucial role in primed inflammatory, hypoxic and angiogenic microenvironments by supporting disease progression in a range of disease entities. To adapt to fluctuating stress-induced microenvironments, pre-neoplastic and neoplastic tissue must utilise a diverse range of molecular mediators to alter their metabolism. Despite being widely documented to play independent roles in disease prevalence, these complex processes exploit a range of key cellular components that act in tandem to restore metabolic equilibrium. Therefore, this review examines the primary molecular mechanisms linking energy metabolism with inflammation, hypoxia and angiogenesis. Furthermore, the review considers a diverse range of conventional and novel mediators that link energy metabolism and hypoxia. Moreover, to investigate their reciprocal relationship and the mechanisms employed to execute their functional effect in greater detail, the roles of glycolysis and oxidative phosphorylation in rheumatoid arthritis and circadian rhythms respectively are reviewed. Lastly, this review explores some current metabolic-based treatments and multi-targeted therapies that could potentially target these fundamental cellular processes.

KEYWORDS: Energy metabolism; Inflammation; Hypoxia; Angiogenesis.

INTRODUCTION

Otto Warburg’s initial observation in 1956 demonstrated that tumours exhibit increased levels of aerobic glycolysis. This observation has since resulted in numerous studies investigating the role of mitochondrial energy metabolism in disease progression across many disease entities. As a reflection of its importance in the development of various cancers, the reprogramming of cellular energetics is now beginning to establish itself as one of the new hallmarks of cancer. In addition to significant quantities of adenosine triphosphate (ATP), metabolically demanding tumours require glucose for lipid and protein synthesis and de novo synthesis of nucleotides for rapid proliferation. More importantly, this altered metabolic phenotype allows tumours to maintain higher proliferative rates and resist apoptosis orchestrated by increased oxidative damage. Moreover, these metabolic phenotypes persist and are sometimes altered in distinct metabolically demanding microenvironments. Therefore, elucidating how diverse metabolic processes converse with distinct functional inflammatory, hypoxic and angiogenic pathways may infer significant insights into how several heterogeneous malignancies arise and subsequently advance beyond therapeutic intervention.

It has been widely documented that inflammation, hypoxia and angiogenesis all play independent roles in disease prevalence and in its subsequent stepwise progression. Some studies, however, have uncovered close associations between energy metabolism and these extensive processes. Therefore, this review focuses on the primary molecular mechanisms that link energy metabolism with inflammation, hypoxia and angiogenesis. In addition to investi-
gating conventional mediators that link energy metabolism with inflammation and hypoxia, novel mediators that link energy metabolism to hypoxia, hypoxia and angiogenesis will also be discussed. This review also explores the mechanisms linking energy metabolism with hypoxia by exploring the roles of glycolysis in rheumatoid arthritis and oxidative phosphorylation (OXPHOS) in circadian rhythms. This review subsequently focuses on the connection between energy metabolism and inflammation in greater detail by examining some of the reciprocal mechanisms linking both processes throughout the gastrointestinal tract. To conclude, this review explores contemporary metabolic-based treatments and multi-targeted therapies that target these key processes.

**MOLECULAR MEDIATORS LINKING ENERGY METABOLISM WITH INFLAMMATION, HYPOXIA AND ANGIOGENESIS**

**Conventional Mediators Linking Energy Metabolism with Inflammation and Hypoxia**

**HIF1α**

Hypoxia Inducible Factor-1α (HIF1α) is an oxygen sensitive transcription factor subunit involved in various cellular processes including hypoxia, angiogenesis, cell survival, inflammation and energy metabolism.6 Interestingly, cells in hypoxic regions tend to be more resistant to the effects of radiotherapy and other conventional chemotherapeutic agents.7 As a result, these more resistant cells have been implicated in disease resistance and recurrence, and can lead to more aggressive phenotypes and contribute to subsequent metastasis.7,8 It is important to note, however, that hypoxia-induced alterations in energy metabolism are physiologically normal, for example, cardiomyocytes upregulate glycolytic ATP production under hypoxic stress.9 As Figure 1 shows, hypoxia affects metabolism by inducing the overexpression of various glycolytic enzymes, lactate dehydrogenase (LDH) and carbonic anhydrase in addition to inhibiting pyruvate dehydrogenase, a key enzyme that converts pyruvate into acetyl-CoA.10 However, it has been shown that hypoxia, specifically HIF1α, plays a key role on T-cell function by modulating T-cell metabolism.

Upon activation, the metabolic demands of T-cells increase dramatically since activated T-cells are highly anabolic and exhibit marked increases in glycolytic metabolism.11,12 Interestingly, one study hypothesises that one possible mechanism responsible for T-cell anergy is failure to upregulate key meta-

![Figure 1: Conventional mediators linking energy metabolism with inflammation and hypoxia.](image-url)
bolic machinery, since blocking energy metabolism mitigates T-cell activation and inhibition of these metabolic pathways during activation leads to anergy in Th1 cells. Hypoxia has differential effects on T-cell function, however, as lack of glucose in human CD4+ T lymphocytes results in increased dead cell numbers and increased reactive oxygen species under normoxia but not under hypoxic conditions. Hypoxia also stimulates increased levels of interleukin-1β (IL-1β), IL-10 and IL-8 in these cells, but the lack of glucose reduces secretions of these cytokines, implying that CD4+ T cells are highly metabolically adaptable allowing for proper immune function under highly fluctuating bioenergetic microenvironments. HIF1α also regulates the balance between regulatory T cell and helper T cell differentiation. This differentiation has been shown to be regulated in both regulatory T cells and helper T cells via the glycolytic pathway in a HIF1α-dependent manner. In stimulated T\textsubscript{h}17 cells, glycolysis and various glycolytic enzymes were actively upregulated, although blocking glycolysis inhibited T\textsubscript{h}17 development while promoting T\textsubscript{reg} differentiation. HIF1α activity is key for mediating glycolytic activity and subsequently contributes to lineage choices between T\textsubscript{h}17 and T\textsubscript{reg} cells, whereas lack of HIF1α results in reduced T\textsubscript{h}17 development but enhances T\textsubscript{reg} differentiation. Some evidence suggests that HIF1α mediates this effect through mammalian target of Rapamycin (mTOR). These studies support the view that hypoxia mediates T-cell function and drives chronic inflammation through HIF1α by regulating T-cell metabolism.

**AMPK**

AMP-activated protein kinase, or AMPK, is a sensor of cellular energy metabolism and exhibits anti-Warburg effects by promoting fatty acid oxidation, mitochondrial biogenesis and the expression of genes necessary for oxidative metabolism (Figure 1). As aerobic glycolysis is a common entity in many cancer types, it is exciting to speculate that drugs that activate AMPK might therefore have therapeutic and clinical utility. Any metabolic imbalance that either inhibits the generation of ATP or accelerates ATP consumption results in increases in the ADP/ATP ratio resulting in AMPK activation due to the accumulation of ADP. As a result, activated AMPK acts to switch off ATP-consuming anabolic processes and restores energy imbalances by switching on alternative catabolic pathways that increase cellular ATP. One of these mechanisms involves down-regulating protein synthesis. For example, AMPK down-regulates protein synthesis of target of rapamycin complex 1 (TORC1), which is known to promote HIF1α translation, thereby reducing HIF1α expression and decreasing the expression of key glycolytic and glucose transporters required for aerobic glycolysis. Using a mouse model of Peutz-Jeghers syndrome, deficiency of either AMPK or Liver Kinase B1 (LKB1), the protein kinase responsible for induction of AMPK activation, led to the upregulation of HIF1α, hexokinase 2 and glucose transporter member 1 (GLUT1).

Activated immune cells tend to favour aerobic glycolysis whereas quiescent cells preferentially utilise oxidative metabolism. Therefore, agents that activate AMPK may have anti-inflammatory effects. LPS-induced activation of dendritic cells results in reduced activation of AMPK, whereas knockdown of AMPK leads to the maturation of dendritic cells that exhibit increased glucose uptake. Interestingly, AMPK downregulation in macrophages results in increased expression of various pro-inflammatory cytokines whereas expression of AMPK had the reverse effect. Therefore, AMPK promotes macrophage polarisation towards an anti-inflammatory M2 phenotype rather than the pro-inflammatory M1 phenotype. In addition, AMPK has been shown to monitor metabolic stress in cytotoxic T lymphocytes and control the differentiation switch from metabolically active cytotoxic T lymphocytes to metabolically quiescent CD8+ T cells, highlighting the important role of AMPK in various metabolic, immune and inflammatory processes.

**p53**

The transcription factor p53 regulates metabolism by lowering aerobic glycolysis and promoting oxidative phosphorylation through a variety of molecular mechanisms. p53 primarily supports oxidative phosphorylation by functioning as a mitochondrial checkpoint protein, regulating mitochondrial DNA copy number and mediating mitochondrial biogenesis. p53 promotes mitochondrial health via the p53-inducible protein Mieap that controls mitochondrial quality by repairing or eliminating unhealthy mitochondria. In intestinal metastasizes patients with progressive disease, oxidative-induced damage results in telomere shortening and mutations in the p53 gene abrogate p53’s role as the checkpoint of proliferation and apoptosis. Other studies have also shown that p53 plays a vital role in the synthesis of key components of the electron transfer chain.

p53 mediates its central metabolic role through TP53-induced glycolysis and apoptosis regulator, or TIGAR, act as a phosphatase and degrades fructose-2,6-Bisphosphate (F26B) thereby decreasing the activity of phosphofructo kinase 1 (PFK1), a key enzyme of the glycolytic pathway. p53, via TIGAR, decreases glycolysis by diverting glycolytic intermediates into the pentose phosphate pathway (PPP). p53 also negatively regulates the expression of Pyruvate dehydrogenase kinase 2 (PDK-2) thereby inactivating the pyruvate dehydrogenase complex responsible for converting pyruvate to acetyl-CoA. Thus p53, activating the pyruvate dehydrogenase complex, favours oxidative phosphorylation through the production of acetyl-CoA. Furthermore, p53 directly downregulates the expression of GLUT1 and GLUT4.

The role of p53 is highlighted in hypoxic microenvironments. Through a hypoxia-induced HIF1α dependent mechanism, TIGAR has been shown to form a complex with hexokinase 2 at the mitochondria resulting in an increase in hexokinase 2 activity. This complex reduces glycolytic flux supporting pentose phosphate pathway activity, generates NADPH in the process and promotes antioxidant function thereby limiting reac-
tive oxygen species-associated apoptosis and autophagy.\textsuperscript{36} p53 also represses the expression of monocarboxylate transporter 1 (MCT1) preventing the efflux of lactate under hypoxic conditions.\textsuperscript{40} It has been speculated that aberrant p53 expression may even promote tumour progression as some evidence suggests that p53 may enhance aerobic glycolysis rather than inhibit it.\textsuperscript{27,28,41} In addition, the mechanism by which p53 regulates the glycolytic pathway may be tissue and context specific which is thought to reflect different types of cellular stress, that is, metabolic, oxidative and hypoxic stress.\textsuperscript{27,42}

**NOVEL MEDIATORS LINKING ENERGY METABOLISM WITH INFLAMMATION, HYPOXIA AND ANGIOGENESIS**

**NFκB**

Despite some early studies linking nuclear factor kappa B (NFκB) with energy metabolism, recent studies have increasingly shown NFκB to possess an equally important central role in various metabolic and pathological diseases.\textsuperscript{43} Inflammation is a key factor in the development of metabolic diseases such as atherosclerosis, insulin resistance, type 2 diabetes and obesity.\textsuperscript{43-45} The central role of NFκB in immunity, inflammation and carcinogenesis has been well documented.\textsuperscript{46-48}

NFκB regulates cellular respiration in a p53-dependent manner (Figure 2).\textsuperscript{49} Translocation of the NFκB family member RelA to mitochondria is inhibited by p53, however, in the absence of p53, RelA is transported into mitochondria and recruited to the mitochondrial genome where it represses mitochondrial gene expression, oxygen consumption and cellular ATP levels, thereby contributing to the switch to glycolysis.\textsuperscript{49} Indeed, it was reported that the RelA subunit also upregulates transcription of GLUT3 resulting in increases in glucose uptake and glycolytic flux.\textsuperscript{50} The elevated glycolytic flux stimulates further IKK/NFκB pathway activity in a positive feedback loop that subsequently promotes H-Ras-induced oncogenic transformation in mouse embryonic fibroblasts.\textsuperscript{50} This was the first functional study to show that NFκB promotes cell growth and carcinogenesis by metabolic manipulation, but crucially, p53 was central to this pathway, as introduction of p53 disrupted the link between NFκB and glycolysis.\textsuperscript{50}

Intriguingly, the role of NFκB is reversed in normal mouse embryonic fibroblasts upon glucose starvation, whereby NFκB inhibition causes cellular reprogramming to aerobic metabolism.\textsuperscript{50}

**Figure 2. Novel mediators linking energy metabolism with inflammation, hypoxia and angiogenesis.** NFκB regulates cellular respiration in a p53-dependent manner. In the absence of p53, the NFκB family member, RelA, represses mitochondrial gene expression, oxygen consumption and cellular ATP levels thereby promoting glycolysis. NFκB promotes OXPHOS through an AMPK-p53-mediated mechanism by upregulating SCO2, a key electron transfer chain component. Furthermore, endothelial tip cells increase their glycolytic rate and promote angiogenesis by upregulating numerous glycolytic constituents such as LDH and GLUT1, primarily through HIF1α, VEGF, VEGFR2 and PFKFB3 signalling. However, the activation of VEGFR2 in tip cells induces the expression of Notch ligand DLL4 in neighbouring stalk cells activating Notch signalling. As a result, this reduces VEGF2 and PFKFB3 expression thereby lowering glycolytic flux and promoting OXPHOS in stalk cells. VEGF can also control angiogenesis through the glycolytic metabolite lactate, as lactate inhibits PHD resulting in HIF1α activation subsequently promoting OXPHOS and glycolysis. In addition, lactate has been shown to induce the production of the pro-inflammatory cytokine IL8. mTOR, succinate and STAT3 can also mediate metabolism. mTOR plays an important role in the modulation of both adaptive and innate immune function. TORC1, one of mTOR’s signalling forms, upregulates glycolysis, glutaminolysis and the expression of SNAT-2. Moreover, mTOR inhibition results in a metabolic bias towards OXPHOS. Succinate can mediate metabolism through the inhibition of PHD and HIF1α. Similarly, STAT3 can regulate tumour cell metabolism through HIF1.
glycolysis. The role of NFκB in upregulating mitochondrial respiration in this circumstance involves the p53-mediated upregulation of mitochondrial synthesis of cytochrome c oxidase 2 (SCO2), a key component of complex IV of the electron transport chain. Hence, NFκB can act as a focal checkpoint of metabolic homeostasis in conjunction with AMPK and p53 to regulate the response to low cellular ATP levels. Therefore, despite its prominent role in the Warburg effect, the metabolic plasticity of NFκB confers adaptivity in cells to adapt to fluctuating oxidative and hypoxic microenvironments.

**VEGF and PFKFB3**

In response to hypoxia-induced pro-angiogenic stimuli, endothelial cells rapidly switch from a metabolically inactive state of quiescence to an active migratory and proliferative state. Effective vascular sprouting relies on coordinated navigating tips cells and on proliferating stalk cells that elongate the sprout. Until recently, only genetic signals were known to play a role in this angiogenic switch. However, the angiogenic switch also requires a change in endothelial cell metabolism. Interestingly, endothelial cells are thought to be addicted to glycolysis as they rely minimally on oxidative phosphorylation for ATP generation. For instance, the glycolytic inhibitor 2-deoxy-D-glucose induces significant endothelial cell death.

Endothelial cells increase their glycolytic rate by upregulating a range of glycolytic constituents including GLUT1, LDH and 6-phosphofructo-2-kine/fructose-2,6-bisphosphatase-3 (PFKFB3). PFKFB3 has been shown to be critical for angiogenic sprouting, and its inactivation reduces endothelial cell proliferation and migration, and impairs motility and formation of endothelial cell lamellipodia and filopodia. Conversely, PFKFB3 overexpression stimulates the sprouting of mitotically-inactivated endothelial cells and promotes tip cell formation. This entire process of tip and stalk cell differentiation, however, is under tight control of vascular epithelial growth factor (VEGF) and Notch signalling (Figure 2).

VEGF promotes tip cell induction and filopodia formation inducing the expression of the Notch ligand Delta-like 4 (DLL4). One of the main genetic signals of vessel sprouting is orchestrated through Notch. DLL4 subsequently activates Notch signalling in neighbouring cells and suppresses VEGF receptor 2 expression and tip cell behaviour. Therefore, the activation of VEGF receptor 2 in tips cells upregulates PFKFB3 levels and glycolysis but induces the expression of the Notch ligand DLL4 in neighbouring stalk cells activating Notch signalling, lowering VEGF receptor 2 expression resulting in lower PFKFB3 expression and glycolytic flux. Interestingly, overexpression of PFKFB3 overcomes the pro-stalk activity of Notch signalling thereby promoting tip cell behaviour, indicating that highly glycolytic endothelial cells can overcome inhibitory genetic signals.

VEGF also controls angiogenesis through the glycolytic metabolite lactate. Once taken up by endothelial cells through MCT1, lactate competitively inhibits the oxygen-sensing propyl hydroxylase domain protein 2 (PHD2), resulting in activation of HIF1α and an increase in VEGF receptor 2 expression. Lactate signalling also induces VEGF expression. In addition to its angiogenic role, lactate also indirectly releases NFκB inducing IL8 expression, another promoter of angiogenesis. VEGF has also been shown to induce the production of IL8 in endothelial cells. In addition to promoting aerobic glycolysis, VEGF stimulates mitochondrial biogenesis through Akt-dependent signalling, and plays a significant role in fatty acid metabolism. These studies demonstrate a close relationship between VEGF-induced metabolism, hypoxia, angiogenesis and inflammation in endothelial cells and highlight how stressed endothelial cells adapt to an altering milieu that could potentially favour tumour progression.

**mTOR, Succinate and STAT3**

mTOR is a serine/threonine kinase that controls cell proliferation and metabolism in response to a range of extracellular stimuli such as the availability of nutrients, growth factors and stress. mTOR plays an important role in the modulation of both innate and adaptive immune function (Figure 2). As discussed, activated T cells switch to an anabolic metabolism using aerobic glycolysis as a major supply of ATP to fuel the rapid synthesis of proteins, nucleotides and other biosynthetic products. TORC1, one of two currently recognised signalling forms of mTOR, has been shown to be heavily involved in the upregulation of enzymes involved in glycolysis, glutaminolysis, the pentose phosphate pathway, surface expression of GLUT1 and expression of the glutamine transporter, SNAT-2. Similarly, inhibition of mTOR results in a metabolic bias towards oxidative phosphorylation and has been shown to produce a larger CD8 memory T cell pool. Ongoing clinical trials investigating the efficacy of mTOR inhibitors suggest that mTOR-mediated metabolism does play a central role in regulating biological outcomes within immune cells, however, a key question remaining is how mTOR-mediated metabolism is coupled to immune function.

Increasing evidence also proposes that succinate, a citric acid cycle metabolite that accumulates due to succinate dehydrogenase mutations, transmits an oncogenic signal from the mitochondria to the cytosol, directly inhibiting PHD and resulting in HIF1α stabilization under normoxic conditions, with resultant increased expression of genes that facilitate angiogenesis, metastasis and glycolysis (Figure 2). By adding succinate to glioblastoma multiforme-derived cells cultured under hypoxic conditions, HIF1α stabilization is induced which increases stem cell fractions and preserves the tumour stem cell niche thereby promoting tumour survival. Recently, it has been reported that succinate as a metabolite in innate immune function enhances IL-1β production during inflammation through HIF1α thereby promoting disease progression.
The signal transducer and activator of transcription factors (STATs) are a family of transcription factors that regulate cell growth, survival, differentiation and motility. One of the STAT members, STAT3, has long been recognised as a critical regulator of tumour cells. STAT3 has been recently found to act as one of the central mediators of aerobic glycolysis through both HIF1α and independent mechanisms (Figure 2). Upon translocation to the mitochondria, serine phosphorylation of STAT3 contributes to tumour cell transformation and tumourigenesis.

EXPLORING THE MOLECULAR MECHANISMS THAT LINK ENERGY METABOLISM AND HYPOXIA

Glycolysis, Hypoxia and Rheumatoid Arthritis

It has been 35 years since the link between increased glycolytic activity and rheumatoid arthritis (RA) was first established. In normal synovial tissues, glycolysis is the primary pathway for mitochondrial substrate oxidation of pyruvate. Levels of two major glycolytic enzymes glyceraldehyde 3-phosphate dehydrogenase and LDH were found to be significantly increased in the synovial cells from fresh non-rheumatoid and rheumatoid synovial tissue. More recently, one study detected elevated lactate and reduced glucose levels in the synovial fluid in RA. Moreover, it is plausible that metabolic alterations that favour aerobic glycolysis are a result of hypoxia-induced mitochondrial mutagenesis and dysfunction. Despite studies lacking strong evidence of a direct relationship between inflammation and glycolysis in RA, it is interesting that some glycolytic components are characterised as being autoantigens, for example, glucose-6-phosphate isomerase, aldolase and enolase. However, studies need to be undertaken to examine the role of metabolic autoantigens in cancer initiation and progression.

On the other hand, the link between hypoxia and inflammation has been well documented in-vivo. Significantly higher levels of synovial fluid tumour necrosis factor-α (TNFα), IL-1β, interferon-γ and macrophage inflammatory protein-3α were found in patients with inflammatory arthritis. Interestingly, TNFα blocking therapy reverses joint inflammation and hypoxia. Another study also demonstrated that hypoxia-induced IL-17A expression is localised to neutrophils, mast cells and T cells within inflamed synovial tissue supporting the concept that IL-17A is a key mediator in inflammatory arthritis.

Numerous mechanistic processes within the inflammatory joint may alter energy metabolism profiles. RA is associated with increased levels of HIF1α and HIF2α. HIF1 also induces the expression of GLUT1 and GLUT3. Furthermore, HIF has been shown to regulate the levels of hexokinase II, glyceraldehyde 3-phosphate dehydrogenase, LDH and cytochrome oxidase in the inflammatory synovium. RA is also commonly associated with mutations in p53. As discussed, p53 can regulate glucose metabolism through NRF2B, however, loss of p53 promotes the positive feedback cycle between the I KK-

AMPK, Hypoxia and Circadian Rhythms

Significant time-of-day oscillations in glucose metabolism are observed in both humans and rodent models, at both the whole body and cellular level. It has been speculated that various mitochondrial functions may be regulated by the circadian clock thereby serving as a central coordinator between the clock and cellular energy metabolism. For example, cytochrome c oxidase activity is increased in the brains of 2 month old wistar rats during wakefulness compared to sleep to meet increased energy demands. AMPK is one of the main metabolic sensors responsible for transmitting energy dependent signals to the mammalian clock.

A molecular oscillator exists whereby the transcription factors CLOCK and BMAL1 work together to drive the expression of many genes responsible for the mammalian molecular clock, including those encoding their own inhibitors, the period (PER1, PER2 and PER3) and cryptochrome (CRY1 and CRY2) proteins. These PER and CRY proteins are transcriptional repressors that are necessary for circadian clock function. The E3 ligase component F-box/LRR-repeat protein 3 (FBXL3) catalyzes the polyubiquitination of CYR1 and CRY2 and thus stimulates their proteosomal degradation. AMPK-mediated selective phosphorylation of CRY1 and CRY2 initiates the interaction between CRY1, CRY2 and FBXL3 and stimulates the degradation of both cryptochromes. Casein kinases, CK1ε and CK1δ, are also important modulators of circadian rhythm in mammals. Genetic disruption or pharmacological inhibition of these casein kinases alters behavioural and cellular circadian rhythms in mice. Casein kinases phosphorylate serine in PER2, however, AMPK was reported to phosphorylate CK1ε at serine 389 thereby increasing its enzymatic activity and indirectly leading to destabilisation of PER2 and alterations in circadian rhythm.

AMPK has also been implicated in circadian rhythm entrainment in mice as pharmacological activation of AMPK by intraperitoneal injection of both 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) or metformin causes a phase shift of the liver clock. In addition, AICAR stimulation altered clock gene expression in wild type mice but not in mice lacking the AMPKγ3 subunit implying that AMPK activation may play a role in circadian entrainment. Furthermore, AMPK possesses a close relationship with silent mating type information regulation 2 homolog 1 (SIRT1), another fuel-sensing molecule key to nutritional status and circadian regulation. AMPK
not only enhances SIRT1 activity by increasing NAD⁺ levels but activation of SIRT1 causes AMPK phosphorylation via LKB1 activation. AMPK is also associated with regulating other metabolic sensors known to have key roles in circadian regulation such as poly (ADP-ribose) polymerase 1 and nicotinamide phosphoribosyltransferase. These studies suggest that the effectiveness of widely prescribed drugs that regulate glucose homeostasis, such as metformin, may be ameliorated by altering the timing of treatment through AMPK-mediated control of circadian function by pharmacological intervention.

**RECIProCAL MECHANISMS LINKING ENERGY METABOLISM TO INFammATION IN GASTROENTEROLOGICAL DISEASES**

It is apparent thus far, that the combined effect of various molecular processes, can act in tandem to significantly alter the local microenvironment and attenuate disease progression. Little is known about how energy metabolism profiles cooperate with inflammatory processes to facilitate metaplastic progression in gastroenterological disease entities. However, some recent research has provided some insight on metabolic signatures in Barrett’s oesophagus, oesophageal adenocarcinoma, Inflammatory Bowel Disease (IBD), gastritis and gastric cancer.

Recent research has demonstrated that both oxidative phosphorylation and glycolysis are reprogrammed early in the inflamed Barrett’s disease sequence and may act mutually to promote disease progression in Barrett’s oesophagus. Subsequent to screening 84 genes using a PCR microarray, validations utilising *in-vitro* and *in-vivo* models found that 3 genes associated with mitochondrial energy metabolism, ATP12A, COX412 and COX8C, were differentially expressed across the Barrett’s sequence. In addition, tissue microarrays demonstrated significant epithelial and stromal alterations using surrogate protein markers of oxidative phosphorylation, ATP synthase subunit 5 beta and heat shock protein 60, or ATP5B and HSP60 respectively. Moreover, significant alterations across the Barrett’s sequence were also demonstrated using surrogate protein markers of glycolysis, pyruvate kinase isozyme M2 and glyceraldehydes 3-phosphate dehydrogenase, or PKM2 and GAPDH respectively. Interestingly, ATP5B in sequential follow up surveillance biopsy material segregated Barrett’s non progressors and progressors to high grade dysplasia and adenocarcinoma thereby highlighting the prognostic advantage of metabolic profiles in these pre-neoplastic patients. Finally, utilising the *in-vitro* model, the authors present evidence that Barrett’s and adenocarcinoma cells exhibit significantly altered levels of various oxidative parameters, whereby the adenocarcinoma cell line maintains an equilibrium between both metabolic pathways while the Barrett’s cell line favours a more detrimental oxidative phenotype that may be selected for during early stages of disease progression.

Other studies, although mostly indirectly, link inflammation to energy metabolism. IL-6, documented as being increased in myofibroblasts of Crohn’s disease patients, has also been shown to increase the expression of hexokinase 2 and PFKFB3 in murine embryonic fibroblasts. Moreover, increased secreted and immunological levels of IL-6 have been found in Barrett’s tissue compared to matched normal adjacent squamous epithelium. Aberrant expression of p53 is also associated with an increased risk of neoplastic progression in patients with Barrett’s oesophagus. Therefore, since mutated p53 enhances IL-6 promoter activity in renal cell carcinoma, it may be plausible that p53, known to modulate oxidative phosphorylation and glycolysis, simultaneously alters inflammatory and metabolic profiles in pre-neoplastic and neoplastic microenvironments of the oesophagus. Similarly, HIF1α, known to mediate hypoxia-induced alterations in glycolytic metabolism and to possess an intrinsic relationship with p53, has been shown to be differentially expressed across the Barrett’s sequence.

Interestingly, despite increased oxidative phosphorylation in Barrett’s oesophagus, ulcerative colitis is associated with low levels of this metabolic pathway. Loss of oxidative phosphorylation precedes the development of dysplasia in ulcerative colitis and thus could potentially be utilised to predict cancer. Furthermore, following peneoplastic progression, cancer cells restore mitochondria indicative of an increase in energy demands for growth and proliferation. In addition, one study showed that increasing mucosal levels of ATP can protect mice from colitis and thus increasing ATP synthesis could be a plausible therapeutic approach for ulcerative colitis. Such reductions in oxidative phosphorylation, thought to be caused by defects in complex I of the electron transport chain, have also been reported in atrophic and active chronic gastritis. Gastric cancer is additionally associated with a complex I-induced defective electron transport chain. As well as decreased mitochondrial respiration, gastric cancer exhibits shifts to glycolysis, Decreased fructose-1,6-bisphosphatase (FBP), the enzyme which functions to antagonise glycolysis though NfKb, has been shown to be decreased in both gastric cell lines and gastric carcinomas thereby promoting glycolysis. Moreover, PKM2 are also overexpressed in gastric and colorectal tumour tissue and their expression is associated with poor survival. Moreover, knockout of PKM2 has been shown to repress the proliferative and migratory capabilities of colorectal cancer cells.

IBD patients are known to have high levels of HIF1α and HIF2α. IBD patients also exhibit increased colonic expression of various glycolytic enzymes and these alterations in metabolism are thought to be triggered by hypoxic stress. Such extensive regulation of various glycolytic intermediates could be mediated by central regulators of metabolism known to associate with HIF, for example, PFKFB. One study in gastric cancer cell lines and tissue found that both PFKFB3 and PFKFB4 significantly responded to hypoxia through HIF1α and this subsequently promoted the Warburg effect. Interestingly, alterations in the gut microbiome, as demonstrated by the fusoclytansferase 2 polymorphism in Crohn’s disease patients for example, could also affect the host mucosal state and thus increase disease susceptibility. Therefore, further studies directly linking inflam-
mation with energy metabolism profiles through these distinct processes would enhance our understanding on the mechanisms involved in inflammatory-induced neoplastic progression in gast-erological diseases.

INNOVATIVE METABOLIC-BASED TREATMENTS AND MULTI-TARGETED THERAPIES

In order to replicate and divide, tumour cells need to possess the ability to acquire large quantities of proteins, lipids and nucleotides. As these processes are highly metabolically demanding, cells additionally require vast quantities of ATP. Consequently, targeting glucose metabolism and nucleotide biosynthesis could have significant advantages on combating metabolic transformation. In addition, altering the metabolism of susceptible or predisposed pre-neoplastic or neoplastic tissue may prevent subsequent disease progression.

Table 1 highlights some of the diverse therapeutic strategies currently being employed to target various aspects of energy metabolism. Significant research has begun to focus on targeting upstream regulators of metabolic pathways such as HIF, phosphoinositide 3-kinase (PI3K), Akt, mTOR and AMPK. For example, PI3K inhibitors such as BEZ235 have been shown to target metabolism leading to cancer regression in Kras-mutant murine lung adenocarcinomas. The AMPK activator metformin, primarily used to treat patients with type 2 diabetes, has been shown to be protective as those treated with metformin were found to be cancer free over 8 years versus those on alternative treatment regimes. Additional AMPK activators are also being investigated for their potential therapeutic use. Interestingly, methotrexate, a chemotherapeutic known to target nucleotide biosynthesis, enhances the antianabolic and antiproliferative effects of AICAR, an alternative AMPK agonist. Moreover, targeting nucleotide biosynthesis may be more favourable as nucleotide building blocks necessary for proliferating tumour cells can be synthesised by endogenous glucose and glutamine due to poor vascularisation. Therefore, blocking ribose-5-phosphate synthesis, with 5-fluorouracil (5-FU) for example, could provide a better therapeutic window. Dichloroacetate, an inhibitor of PDK-1, has also been shown to re-sensitise gastric cancer cells with hypoxia-induced resistance to 5-FU through the alteration of glycolysis.

Therapeutic agents that target the glycolytic pathway such as 2-deoxyglucose, lonidamine, 3-bromopyruvate and TLN-232 have also shown significant promise. Despite not showing substantial effects on tumour growth as monotherapeutic drugs, their use in conjunction with other chemotherapeutic reagents seems to sensitive tumours by reducing ATP levels and perhaps by indirectly limiting the availability of macromolecules synthesised through anapleurotic interactions. In addition to being combined with radiotherapy, some glycolytic inhibitors are currently being used in phase I, II and III clinical trial. Inhibitors that target glucose transport across the plasma membrane, such as phloridzin and phloretin, have also shown efficacy in inhibiting in-vitro, xenograft and in-vivo tumour growth. Inhibition of PFKFB3 with 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one, or 3-PO, known to be a selective agent against neoplastic cells, has been shown to reduce cellular lactate, ATP, NAD and other cellular metabolites within several human malignant hematopoietic and adenocarcinoma cell lines.

Additional therapeutic modalities that can be exploited include targeting HIF1α, lactate transporters, amino acid metabolism and lipid metabolism. Altering diet is also a unique

<table>
<thead>
<tr>
<th>Drug</th>
<th>Target</th>
<th>Mechanism</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-deoxyglucose</td>
<td>Glycolytic Pathway</td>
<td>Inhibits the production of glucose-6-phosphate</td>
<td>(5,142)</td>
</tr>
<tr>
<td>3-bromopyruvate</td>
<td>Glycolytic Pathway</td>
<td>Inhibits GAPDH</td>
<td>(5)</td>
</tr>
<tr>
<td>3-PO</td>
<td>Glycolytic Pathway</td>
<td>Inhibits PFKFB3</td>
<td>(145)</td>
</tr>
<tr>
<td>5-FU</td>
<td>Nucleotide Biosynthetic Pathway</td>
<td>Inhibits cell proliferation</td>
<td>(5)</td>
</tr>
<tr>
<td>BEZ235</td>
<td>PI3K/mTOR Pathways</td>
<td>Inhibits PI3K signalling &amp; mTORC1/2</td>
<td>(138)</td>
</tr>
<tr>
<td>Dichloroacetate</td>
<td>Glycolytic Pathway</td>
<td>Inhibits PDK-1</td>
<td>(146)</td>
</tr>
<tr>
<td>Lonidamine</td>
<td>Glycolytic Pathway</td>
<td>Inhibits hexokinase and mitochondrial respiration</td>
<td>(5)</td>
</tr>
<tr>
<td>Metformin</td>
<td>AMPK agonist</td>
<td>Activates AMPK</td>
<td>(137)</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>AMPK / Nucleotide Biosynthetic Pathway</td>
<td>Activates AMPK</td>
<td>(5,139)</td>
</tr>
<tr>
<td>Phloretin</td>
<td>Glucose Transport</td>
<td>Inhibits sodium-glucose transporters 1 &amp; 2</td>
<td>(142,144)</td>
</tr>
<tr>
<td>Phloridzin</td>
<td>Glucose Transport</td>
<td>Inhibits sodium-glucose transporters 1 &amp; 2</td>
<td>(142,144)</td>
</tr>
<tr>
<td>PX-478</td>
<td>HIF1α</td>
<td>Inhibits HIF signalling</td>
<td>(5)</td>
</tr>
<tr>
<td>Salicylate</td>
<td>AMPK agonist</td>
<td>Activates AMPK</td>
<td>(138)</td>
</tr>
<tr>
<td>TLN-232</td>
<td>Glycolytic Pathway</td>
<td>Inhibits PKM2</td>
<td>(5)</td>
</tr>
</tbody>
</table>

Table 1: Metabolic-based compounds.
and beneficial therapeutic approach. For example, a ketogenic diet relies on food that does not increase plasma glucose but produces ketone bodies that can be used as a carbon source thereby bypassing glycolysis. Even though the ketogenic diet has been shown to have mixed results, further studies may reveal that it is a cancer specific therapy. More recently, micro RNAs have shown promise at targeting cancer metabolic pathways. A recent study demonstrated that mir-122 targets PKM2 and affects metabolism in hepatocellular carcinoma. Despite the encouraging evolution of metabolic-based treatments and multi-targeted therapies, more work is required to understand which pathways are activated in distinct tumour types thereby allowing the identification of pharmacological targets that can avert disease progression and alleviate tumour burden.

CONCLUSION

Cellular energy metabolism plays a crucial role in inflammatory, hypoxic and angiogenic microenvironments by supporting malignant progression in a range of disease entities. Pre-neoplastic and neoplastic tissue must use a diverse range of molecular components to alter their metabolism to adapt to fluctuating oxidative, hypoxic and metabolic stresses. This involves exploiting various molecular elements such as HIF1α, AMPK or p53 that have the potential to function rapidly to acute onsets of stress. It is evident from ongoing research, however, that tumour cells can survive these stresses by adjusting their metabolism through a range of alternative pathways and novel mediators such as NFκB, VEGF and mTOR. Substantiating the reciprocal relationship between energy metabolism in inflammatory and hypoxic diseases is evident in RA and circadian rhythms. In addition, it is clear that the inflammatory microenvironment of the gastrointestinal tract presents clear indication of this mutual association. Therefore, understanding the underlying mechanisms that permit premalignant cells to transform, survive, thrive and subsequently adapt in response to a range of metabolic-based therapies will aid considerably in the development of effective and specific multi-targeted therapies.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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Short Communication

Mucinous Tumour in Ileal Pouch Post Restorative Proctocolectomy and Ileal Pouch Anal Anastomosis for Familial Adenomatous Polyposis

James Wei Tatt Toh¹,²*, Kasim Rahman² and Daniel Kozman²

¹Conjoint lecturer, University of Western Sydney, University of New South Wales, James Ruse Dr, Parramatta NSW 2150, Sydney NSW 2052, Australia
²Bankstown Hospital, 68 Eldridge Road, Bankstown NSW 2200, Australia

E-mail: james.toh@unsw.edu.au

CLINICAL CONTEXT

We present a fifty-six years old female having surveillance gastroscopy and colonoscopy ten years after a restorative proctocolectomy and ileal-pouch anal anastomosis (IPAA). This was performed for Familial Adenomatous Polyposis (FAP). The patient was found to have unusual patchy nodular areas of abnormal mucosa in the ileal pouch and at the level of the anastomosis.

Magnetic Resonance Imaging (MRI) was performed which showed a large fluid collection (12x10x13 cm) within the pelvis. Histopathology from biopsy was reported as a low grade mucinous tumour with abundant mucoid material containing scattered degenerate cellular material and histiocytes. Computed Tomography (CT) of chest and pelvis did not reveal any evidence of metastatic disease.

A pelvic exenteration was performed and patient underwent adjuvant radiotherapy. Histopathology showed cystic mass 50 mm x 50 mm x 30 mm in size containing mucin of intermediate nuclear grade with 0/18 nodes involved (Figure 1).

DISCUSSION

There have been only a few case reports of adenocarcinoma within ileal pouches after restorative proctocolectomy and IPAA in FAP.¹ In a recent literature review, only 21 cases were identified.² While malignancy is rare, risk of pouch adenomas is common and ranged between 6 to 75%.²-⁵ Risk factors for development of pouch adenomas include >1000 colonic polyps and...
age >50 years old 5, age of pouch, with pouches >10 years old at high risk of adenoma² and those who have previously developed pouch adenomas.³

It is unclear why the risk of malignant transformation in these patients is low, but most cohort studies have shown adenomas to be tubular or tubulovillous with only a small percentage demonstrating dysplasia. To the best of our knowledge, there have been no reports of mucinous tumours within an ileal pouch in the literature.

While the incidence of malignancy within an ileal pouch is low, this case illustrates the importance of surveillance for patients who have had restorative proctocolectomy and IPAA for FAP. Apart from ileal pouch malignancy, patients may also develop anastomotic site tumours and anal transitional zone tumours, although this is also a rare entity.⁶ Older pouches are more at risk of malignancy. In this case, the mucinous tumour was found thirteen years after restorative proctocolectomy and IPAA.

The Australian Clinical Practice Guidelines and European guidelines recommend annual pouch surveillance post restorative proctocolectomy and IPAA for FAP.⁷⁸ As the risk of pouch adenoma and adenocarcinoma, anal transitional zone and anastomotic tumours increases with time, FAP patients post restorative proctocolectomy and IPAA should have lifelong surveillance.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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