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REVIEW

Taurocholic acid metabolism by gut microbes and colon cancer

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ABSTRACT

Colorectal cancer (CRC) is one of the most frequent causes of cancer death worldwide and is associated with adoption of a diet high in animal protein and saturated fat. Saturated fat induces increased bile secretion into the intestine. Increased bile secretion selects for populations of gut microbes capable of altering the bile acid pool, generating tumor-promoting secondary bile acids such as deoxycholic acid and lithocholic acid. Epidemiological evidence suggests CRC is associated with increased levels of DCA in serum, bile, and stool. Mechanisms by which secondary bile acids promote CRC are explored. Furthermore, in humans bile acid conjugation can vary by diet. Vegetarian diets favor glycine conjugation while diets high in animal protein favor taurine conjugation. Metabolism of taurine conjugated bile acids by gut microbes generates hydrogen sulfide, a genotoxic compound. Thus, taurocholic acid has the potential to stimulate intestinal bacteria capable of converting taurine and cholic acid to hydrogen sulfide and deoxycholic acid, a genotoxin and tumor-promoter, respectively.

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Introduction

With estimated overall costs of 201.5 billion dollars in 2008, cancer is among the most significant contributors of health care spending in the United States (US).[1] Colorectal cancer (CRC) is the third most frequent cancer worldwide resulting in 142,882 new CRC cases and 50,830 CRC related deaths in 2013.[1] CRC is a multifactorial disease in which both nature and nurture interact in varying degrees between individuals. Only about 15% of CRC incidence can be explained by heredity alone,[2] implicating environmental risk factors, particularly the sedentary, overindulgent 'Western lifestyle'. Physical activity, smoking status, alcohol, types and quantity of dietary fiber, micronutrient intake, and diets high in animal protein and saturated fat all appear to play a role in the disease.[3] The goal of much observational and mechanistic work spanning many decades is to establish how we can alter our *environment* so as to lower the risk for CRC. Intriguingly, each of these factors also alters the gut microbiome.[4,5] Several studies have attempted to apply high-throughput sequencing followed by network correlation analysis to identify gut bacteria associated with colorectal tumors.[6-11] While each study concluded that CRC is associated with gut dysbiosis, a significant shift in the gut microbiome community structure relative to healthy control populations, there has been a lack of consistently observed dysbiotic microbiota patterns.

Identification of organisms such as *Fusobacterium nucleatum* DNA in CRC tumors correlate with reduced survival; however, it is unclear whether *F. nucleatum* causes tumor formation or simply thrives in the tumor environment.[12-14] In addition, toxin formation by some strains of *Bacteroides fragilis* and *Escherichia coli* provide potential mechanisms of carcinogenesis worth further exploration.[15-17]

Bile acids have become recognized as a particularly important class of steroid molecule regulating host and microbial physiology.[18-20] The gut microbiota have the capacity to significantly alter the physicochemical properties of bile acids, generating high affinity ligands to host nuclear receptors (farnesoid X receptor, Vitamin D receptor (VDR), pregnane X

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receptor) and G-coupled protein receptors (TGR-5), in addition to sphingosine-1-phosphate receptor 2, and activate a range of signal transduction pathways (JNK, ERK, Akt).[19,20] The levels and composition of the human bile acid pool affects gut microbiome structure, and gut microbiome structure and gene content affects bile acid pool composition. The antimicrobial nature of bile acids prevents bacterial overgrowth in the small intestine; however, microbial metabolism of bile salts in the large intestine represents long-term exposure to metabolic end-products that are implicated in CRC. The dietary context appears to be a major environmental factor determining the extent to which bile acid metabolism becomes a risk factor for the disease, as we shall demonstrate.

Here we focus on the natural history of the biliary metabolite, taurocholic acid (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid N-(2-sulfoethyl)amide) (TCA), whose components, taurine and cholic acid support the growth of microbial groups of low abundance that due to their metabolic by-products, are implicated mechanistically in DNA-damage and tumor-promotion. The proportion of TCA and its toxic metabolites increases directly with a Western diet high in animal protein and fat, and low in complex carbohydrates. These observations highlight the critical link between Western diet, bile salt metabolizing microorganisms, and CRC.

Taurine

Taurine (2-aminoethanesulfonic acid) is the most abundant free amino acid in humans and plays a variety of important physiological functions. Taurine is synthesized in hepatocytes from methionine and cysteine in adults, but is considered semi-essential due to the limited synthetic capacity of neonates, and declining biosynthetic capability with age and certain diseases.[21] The taurine content of various foods and food products are summarized in Table 1. Diets rich in taurine include those high in animal protein and seafood; while vegetarian diets, particularly vegan result in significantly lower levels of taurine measured in blood and urine.[21] Energy drinks are a particularly modern and Western phenomenon, supplemented with roughly 8x (8 mmoles) the daily intake of taurine (~1–1.5 mmoles, 125–188 mg) on a typical Western diet.[21,22] The body maintains homeostatic levels, but is unable to metabolize taurine. Consequently, excess taurine is excreted either in urine, or through bile, conjugated to bile acids.

Table 1. Taurine content of common foods.

Product	Taurine content range per 100 mi in nmol	Reference
Energy drinks	1600	Reissig et al. 2009
Mollusks	2850 ± 759 – 6614 ± 123	Reissig et al. 2009
Dark Meat Poultry	1355 ± 299 – 2445 ± 551	Laidlaw et al. 1990
Fish	332 ± 102 – 1375 ± 428	Laidlaw et al. 1990
Beef and Pork	307 ±78–489 ±85	Laidlaw et al. 1990
Processed Meats	251 ± 32–981 ±42	Laidlaw et al. 1990
Light Meat Poultry	89 ± 9–236 ±55	Laidlaw et al. 1990
Shrimp	84 ± 11–315 ± 102	Laidlaw et al. 1990
Breast milk	40	Sturman 1993
Infant feeding formulas	27.2 ±0.5–60.8	Laidlaw et al. 1990
Dairy	15 ±2–62.4 ±7.2	Laidlaw et al. 1990
Fruit	<1	Laidlaw et al. 1990
Vegetables	<1	Laidlaw et al. 1990
Grains	<1	Laidlaw et al. 1990
Nuts and Seeds	<1	Laidlaw et al. 1990
Legumes and soy milk	<1	Laidlaw et al. 1990

Synthesis and enterohepatic circulation of taurocholic acid

Bile acids are water-soluble, amphipathic molecules functioning primarily to solubilize lipids in an aqueous environment. The liver is the only organ in the body expressing all 14 enzymes required for synthesis of primary bile acids from cholesterol.[23] Indeed, bile acid synthesis is the principal means whereby cholesterol is removed from the body. Bile acids stimulate bile flow due to osmotic effects and further aid in removal of cholesterol by formation of mixed micelles in bile that are secreted into the small intestine. While the diversity of primary bile acids produced between vertebrates is immense,[24,25] humans make primarily 2 major bile acids, cholic acid (CA; 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid) and chenodeoxycholic acid (CDCA; 3α,7α-dihydroxy-5β-cholan-24-oic acid). Rodents form both CA and CDCA; however, CDCA is converted to muricholic acids by enzymatic 6α- or 6β-hydroxylation in the liver.

Free bile acids are conjugated (amidated) to the amino acids taurine and/or glycine in hepatocytes. Conjugation (amidation) of bile acids has several important biological functions. The pK_a of glycine (~4.0) and taurine (<2.0) is lower than the "free" C_{24} bile acid precursor (~5.0). Consequently, conjugated bile acids are fully ionized at physiological pH and impermeable to cell membranes (hepatocytes, cholangiocytes, enterocytes). Conjugated bile acids are also soluble in aqueous solution at acidic

Table 2. Sulfidogenic and 7a-dehydroxylating organisms, substrates, and end products.

Group	Genotoxic/tumor promoting end product	Substrate	Example organism
Sulfate reducing bacteria	H_2S	**Inorganic sulfate** Diet **Organic sulfate** Organosulfates Sulfomucins Sulfated bile acids Estrogen-3-sulfates	*Desulfovibrio* spp
Taurine respiring bacteria	H_2s	Phenylsulfates Dietary Taurine Taurine conjugated bile acids	*Bilophila wadsworthia*
Cysteine fermenting bacteria	H_2s	Dietary Cysteine Organosul fates Assimilatory sulfite reduction	*Fusobacterium nucleatum*
7a-dehydroxylating bacteria	Deoxycholic acid	Taurocholic acid Cholic acid	*Clostridium scindens*

pH and resist Ca^{2+} precipitation.[26] The amide bond of bile acids conjugated to taurine or glycine is highly stable, and resistant to hydrolysis by pancreatic carboxypeptidases.

The N-acyl amidation of bile acids is catalyzed by 2 sequential enzymatic steps in the liver. The first reaction is catalyzed by a microsomal enzyme, cholyl-CoA synthetase ($_{EC}$ 6.2.1.7), resulting in an acyl-CoA thioester,[27,28] followed by transfer of the bile acid moiety from the CoA-thioester to either glycine or taurine, forming *N*-acyl bile acid conjugate by a single cytosolic enzyme bile acid-CoA:amino acid *N*-acyltransferase (BAT) ($_{EC}$ 2.3.1.65).[28,29] BAT shows high substrate specificity for both glycine (K_m 5 mM) and taurine (K_m 1 mM).[30] A major difference between rodents and humans is that taurine conjugation in rodents is genetically determined nearly all murine bile acids are taurine-conjugated. However, in humans, bile acid taurine:glycine conjugation ratio is diet-dependent. Measurements from Native Africans consuming their traditional low fat and protein diet have taurine:glycine ratios of 1:9, while the ratio has been measured to be 10:1 in those consuming a diet high in meat and seafood.[31,32] Feeding taurine, but not glycine can substantially alter the taurine:glycine ratio.[32] The biochemical basis for this is likely owing to the higher affinity of taurine to BAT, and the fact that bile is a major route of excretion of excess taurine.[33]

The bile acid pool in healthy humans is about 2–3 g, cycling several times with each meal (4–6 g), or roughly 12 to 18 g/day circulates from liver to gallbladder through the small intestine and from ileum back to the liver.[34] A recent review by Dawson describes the transport of bile acids in the intestines and the influence of the gut microbiome on this process.[35]

Production of deoxycholic acid

A major theme in the liver-gut microbiome axis is that the liver is conjugative and oxidative; while the gut microbiota is hydrolytic and reductive. Bile acids synthesized by the host liver are termed *primary bile acids* whereas bacterial metabolites of host primary bile acids are termed *secondary bile acids*. In general, bacterial metabolism of bile acids in the anaerobic environment of the gut is limited to oxidation-reduction reactions of the 3, 7, 12-hydroxy groups catalyzed by stereo-specific and position-specific hydroxysteroid dehydrogenase (HSDH) enzymes forming oxo-bile acids and bile acid epimers (iso or epi bile acids). In humans, oxo and iso bile acids are, as Hofmann puts it "repaired" in the liver;[36] however, a small group of bacteria can remove the 7α-hydroxy group converting CA or CDCA to deoxycholic acid (3α,12α-dihydroxy-5β-cholan-24-oic acid) and lithocholic acid (LCA; 3α-hydroxy-5β-cholan-24-oic acid), respectively, which are not "repaired" in humans, but are in rodents. Some gut bacteria capable of converting CA to DCA are also capable of converting 7β-hydroxy bile acids such as ursodeoxycholic acid (UDCA; 3α, 7β, 12α-dihydroxy-5β-cholan-24-oic acid) to LCA. UDCA is a primary bile acid in some vertebrates, such as bears, but is considered a secondary bile acid (or tertiary) in humans, found in small amounts in bile, generated by the concerted action of microbial 7α-HSDH and 7β-HSDH or orally ingested as a therapy for chronic liver and biliary disorders.

While glycine and taurine conjugated bile acids are not recognized by host carboxypeptidases, bacterial bile salt hydrolases (BSH) cleave bile acid conjugates liberating taurine or glycine (reviewed recently[37]). To

date, organisms that express BSH are not capable of converting CA to deoxycholic acid (DCA).[37,38] Likewise, organisms capable of producing DCA and LCA from host primary bile acids lack BSH and are incapable of converting conjugated primary bile acids such as TCA to DCA without the presence of BSH expressing taxa capable of converting TCA to free CA.[39,40]

To survive in the gut environment, microorganisms have to contend with numerous host selection pressures, particularly anti-microbial agents such as lectins, defensins, and bile acids. The detergent nature of bile acids lead to membrane damage, collapse of proton motive force, and oxidative stress and DNA damage, resulting in top-down selection pressure on gut microbiome structure.[37] Microbes have evolved resistance mechanisms such as expression of HSDH capable of producing iso-bile acids (3β-hydroxy) or ursobile acids (7β-hydroxy) that are less toxic.[41,42] Indeed, the therapeutic use of ursodeoxycholic acid (UDCA) (3α,7β-dihydroxy-5β-cholan-24-oic acid) is due to its hydrophilicity, it is able to dilute out toxic secondary bile acids and render the bile acid pool less damaging to the host. UDCA has been used for over a millennium in traditional Chinese medicine, and is a principle therapy for a number of GI diseases including cholestasis, and potentially a preventative treatment for patients with adenomatous polyposis.

Phillip B. Hylemon's laboratory discovered the genes involved in secondary bile acid production and worked out much of the complex biochemistry and molecular biology of bile acid 7α-dehydroxylation of CA in *Clostridium scindens*.[38] It is interesting to note that only a small number of intestinal *Clostridium* spp. have evolved a multi-gene bile acid inducible operon capable of bile acid 7α-dehydroxylation of CA and CDCA and 7β-dehydroxylation of UDCA.[37,38,43] While the proximal explanation of this metabolic capacity is that the bile acid 7α/β-dehydroxylation pathway yields a net 2 electron reduction, the ultimate explanation might relate to production of a toxic, antimicrobial compound.[44,45] In addition, the fact that secondary bile acids (DCA, LCA and derivatives) are high affinity ligands to several host nuclear and G-coupled protein receptors suggests perhaps these metabolites are involved in interkingdom signaling.[19,37,38]

During enterohepatic circulation, secondary bile acids are produced in the cecum and colon and returned to the liver by passive diffusion through the gut epithelium into the portal circulation.[39] Evidence is emerging that as animal-based diets stimulate more bile input into the gut the levels of DCA-producing bacteria increase.[47,48] LCA is also produced from the 7α-dehydroxylation of CDCA or the 7β-dehydroxylation of UDCA and is highly toxic (potentially genotoxic and tumor-promoting). However, LCA does not tend to accumulate in the bile acid pool because it is efficiently excreted in feces by 2 mechanisms: 1) Being monohydroxylated, it is insoluble in fecal water and efficiently precipitates out in the acidic pH of the colon particularly in the presence of Ca^{2+} ions; and 2) binding of LCA to VDR results in enzymatic sulfation of LCA[49] whose hydrophilicity prevents passive absorption in colonocytes and allows removal from the body. Indeed, LCA makes up a small proportion (<2%); whereas DCA can reach upwards of 70% of the human biliary pool.[39] DCA is more hydrophobic than CA,[50] but more water-soluble than LCA allowing passive diffusion through the colon into the portal circulation where it can return to the liver. Unlike LCA, DCA does not activate the signaling cascade resulting in VDR-dependent sulfation.[49] The human liver cannot 7α-hydroxylate DCA and so secondary bile acids can accumulate in some individuals, and hence the reason for our focus primarily on TCA rather than taurochenodeoxycholic acid (TCDCA) or other taurine-conjugated bile acids. Importantly, diets high in animal protein and fat, and low in fiber result in greater secretion of bile, decreased transit time, and higher intestinal pH resulting in increased fecal DCA and greater accumulation of DCA in the biliary pool.

Diet and gut microbial respiration of taurine

Given that taurine is an organosulfonate common in diets high in animal protein, it is not surprising that a high meat diet significantly increases both bile acid tauro-conjugation,[31,32] as well as production of fecal sulfide.[51] We want to emphasize that any bile acid, not just CA, can be conjugated to taurine and thus, from the perspective of taurine metabolism, our argument extends beyond TCA. Milk fat (MF) fed mice and not lowfat (LF) or polyunsaturated fat (PUFA) fed mice increased the abundance of the taurine respiring sulfidogenic organism *B. wadsworthia*. Dextran sodium sulfate (DSS) treatment of MF-fed SPF C57BL/6 mice induced more severe colitis than in their LF and PUFA fed counterparts, as well as consistent observations of *B. wadsworthia*.[52] In mono-associated germ-

free *Il10*$^{-/-}$mice, colonization of *B. wadsworthia* could only be obtained in MF fed mice, and abundance was greater mainly in the mucosa. *B. wadsworthia* grown in culture medium was selectively stimulated by gallbladder bile from MF-fed mice. When low fat (LF)-fed SPF *Il10*$^{-/-}$ mice were gavaged with either TCA or glycocholic acid, only TCA fed mice exhibited increased growth of *B. wadsworthia*.[52] Thus, at least in a colitis-susceptible mouse model, an organosulfonate conjugated to the bile acid CA was upregulated by MF and enhanced growth of the taurine respiring bacterium *B. wadsworthia*, particularly in the mucosa, and this promoted colitis, a known risk factor for CRC.[52] It is not known if *B. wadsworthia* can deconjugate TCA, and the genome sequence of *B. wadsworthia* ATCC 49260 appears to lack a BSH gene, suggesting the requirement of BSH expressing members of the microbiome capable of liberating free taurine.

B. wadsworthia was first identified in 1988 during a study characterizing the microbiome of gangrenous appendicitis. The organism is a common anaerobic isolate in infections,[53] including ear, biliary tract and liver abscess,[54] and is the third most common isolate detected in appendicitis, with abundance correlating with severity of disease. As a β-lactamase producer, *B. wadsworthia* is resistant to most antibiotics, with metronidazole being the only effective agent against all tested strains. The organism has endotoxic activity, and has been observed to produce intraabdominal abscess in mice when injected directly into the peritoneal cavity. Evolutionary analysis of 16s rRNA genes place *B. wadsworthia* within the *Desulfovibrionaceae* family, and interestingly *Desulfovibrio spp* has also been identified in pathogenic roles.[55,56] *B. wadsworthia* is asaccharolytic, and while it is able to ferment pyruvate and reduce nitrate, the organism grows most efficiently in the presence of taurine, making it uniquely suited to thrive in the taurine and hydrogen rich environment of the colon.

Bilophila wadsworthia respires taurine in 3 enzymatic steps, resulting in the release of ammonia, acetate, carbon dioxide, and H_2S (Fig. 1). In the last enzymatic step, sulfite (SO_3^{2-}) reduction can be achieved using formate, lactate, pyruvate, or the taurine carbon as electron donors, but is most efficient when utilizing hydrogen. SO_3^{2-}reduction is carried out by the multi-subunit enzyme known as dissimilatory-sulfite reductase (*DSR*), which catalyzes the 6-electron reduction of SO_3^{2-} to H_2S.[57] Desulfoviridin type *DSRs*, as the type held by *B. wadsworthia*, were originally thought to be comprised of α, β, and γ subunits designated as *dsrA*, *dsrB*, and *dsrC* respectively. Subsequent evidence revealed that *dsrC* was not only expressed at a separate locus, but is a separate protein which interacts with the *dsrAB* complex with a cysteine containing C-terminal residue. *dsrAB* type dissimilatory (bi)sulfite reductases are highly conserved and abundant in many bacteria and archaea due to lateral gene transfer.[58] The enzyme has been characterized in *Salmonella enterica* serovar Typhimurium[59] and *Clostridium pasteurianum*;[60] however, the majority of bacteria with this activity in humans are found in the *Desulfovibrionaceae*, a subdivision of the δ-Proteobacteria.[61]

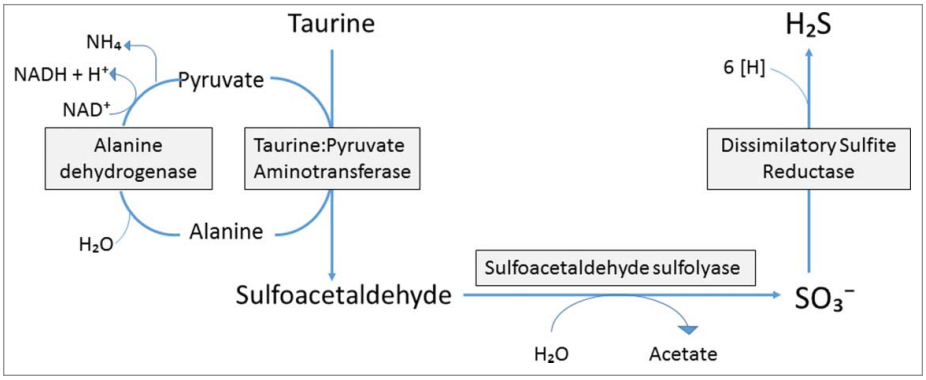

Figure 1. Taurine respiration by *Bilophila wadsworthia*. *B. wadsworthia* respires taurine in 3 enzymatic steps. First, taurine is transaminated from pyruvate using the enzyme taurine:pyruvate amino transferase to produce alanine and sulfoacetaldehyde. Sulfoacetaldehyde is sulfonated with water by sulfoacetaldehyde sulfolyase to produce acetate and sulfite. Sulfite is then reduced by dissimilatory sulfite reductase to hydrogen sulfide using electron donors like hydrogen and formate. Alanine is then converted back to pyruvate using alanine dehydrogenase.

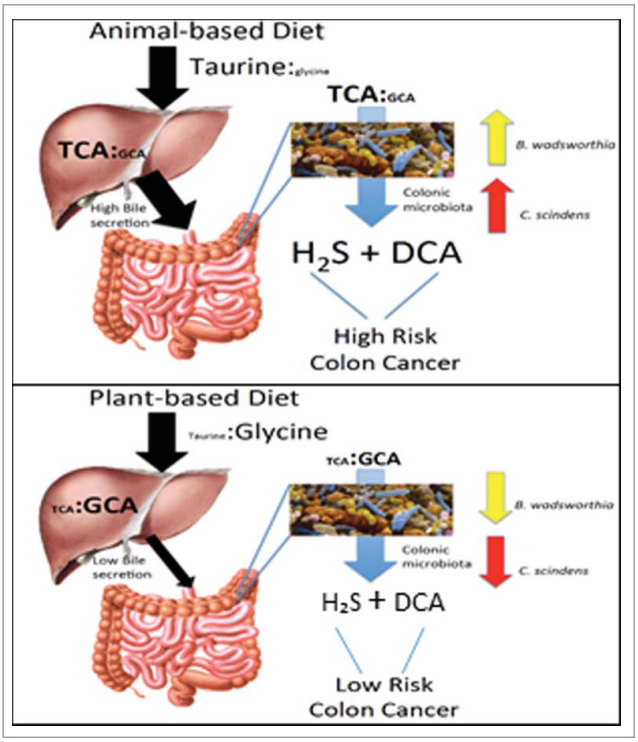

Figure 2. Hypothesis linking diet, TCA metabolism, and colon cancer risk. Animal-based diet high in taurine increases TCA levels in liver and gut, and results in higher levels of DCA in the gut than the plant-based diet owing to higher bile secretion due to higher fat consumption. TCA is metabolized to genotoxin H_2S by *B. wadsworthia* and tumor-promoter, deoxycholic acid (DCA), by *C. scindens*. Plant-based diet, low in taurine, results in low levels of genotoxic H_2S taurine metabolism, and lower fecal DCA levels.

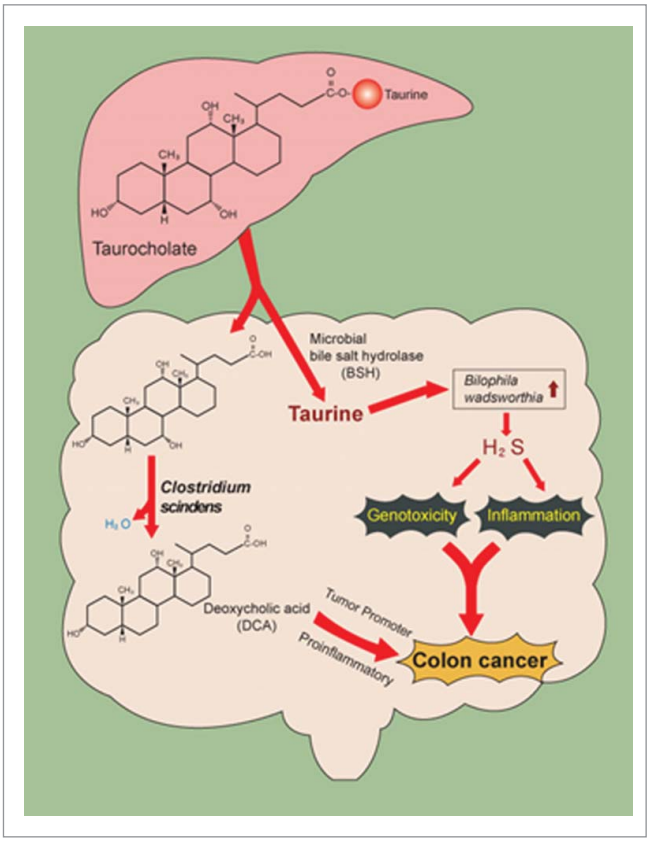

Figure 3. Metabolism of bile salt, taurocholic acid, by the gut microbiome. Gut microbes in the ileum and colon deconjugate bile salts to free bile acids by bile salt hydrolase. Taurine, because of sulfite moiety, can provide pathobionts in the gut with a terminal electron acceptor, allowing for their growth and expansion in the gut. High-fat diet is associated with increased taurine-conjugation in humans. Free primary bile acids are further metabolized to toxic secondary ones that can accumulate in the bile pool in humans and alter host physiology.

Sequencing analysis groups *B. wadsworthia's dsrAB* in the Deltaproteobacteria supercluster, and similar to other Desulfoviridin type DSR harboring organisms, *B. wadsworthia's* DSR contains a single *dsrAB* transcriptional unit that is coordinately expressed. Usually, Desulfoviridin type *DSR*s contain an additional dsrD gene with unknown purpose at the terminal end of the *dsrAB* operon, but *B. wadsworthia* is unique in that it does not have a separate *dsrD* gene, but rather has a merged *dsrBD* unit. The d*srA* gene has been a useful marker for quantification of SRB, and *B. wadsworthia* specific *dsrA* primers have proven to be most suitable for quantification of the organism in intestinal and mucosal samples.[62]

Deoxycholic acid and colon cancer

During the 1970s epidemiological data correlated populations consuming diets high in fat with significantly higher rates of colon cancer.[63] A sharp contrast was drawn between colon cancer rates in Japanese consuming traditional diets, and Americans consuming a "Western diet."[64] However, as Japanese immigrated to Hawaii and began consuming a 'Western diet', colon cancer rates rose midway between Japanese and American populations within two generations.[64,65] Another population that received particular attention were the Seventh Day Adventists, who consume a vegetarian diet and have significantly lower incidence of CRC relative to age-matched and socioeconomically similar cohorts in the American population who consume a Western diet.[66]

Wynder et al. (1967) first proposed an association between dietary fat and CRC,[67] and further proposed that dietary fat influences the fecal microbiota in a way that promotes CRC.[64] Indeed, observations of high risk American populations on Western diets relative to low-risk groups (Japanese, Chinese, Seventh Day Adventists),[68,69] reveal that patients with CRC

and adenomas have higher total fecal bile acid levels,[70] resulting from animal protein and fat.[71] In developing countries, CRC rates were shown to be significantly higher in cities relative to rural areas, consistent with different dietary patterns.[63] Reddy et al. (1974) conducted a small diet exchange study based on their hypothesis linking geographical observations of CRC risk and Western diet, and while they did not observe intra-individual changes in total bile acid levels, they did observe significant increases in DCA and its metabolites in subjects who consumed the animal protein and fat rich Western diet.[72]

The Native black African population (NA) has received considerable study due to their low CRC risk (<1 :100,000) compared to high-risk South African whites (SW) (17:100,000) and African Americans (AA) (65:100,000).[73,74] Interestingly, SW consume diets higher in nutrients and vitamins, higher in insoluble fiber; however, NA consume a diet higher in resistant starch and importantly significantly lower in animal protein and fat.[73] However, as NA adopt a Western diet, their incidence of CRC approaches that of SW and AA. Indeed, migrant studies have shown that one generation is sufficient for rates of CRC to elevate to that of the host western population[75,76] and that dietary change is likely to be the cause. Berg (1973) observed a similar incidence of CRC in 1st and 2nd generation descendants of NA immigrants to Western countries who eschewed the traditional diet.[75]

The work of Wynder and Reddy during the 1970s pointed to higher fecal concentrations of bile acids in high risk populations of Americans on Western diets relative to low-risk groups (Japanese, Chinese, Seventh Day Adventists),[68,75,77] that CRC and patients with adenomas have higher fecal bile acids,[13] and that this was a result of animal protein and saturated fat.[14] Similarly, the NA population was found to have significantly lower fecal total bile acids and significantly lower fecal DCA than AA consistent with lower meat intake.[48,78]

The role of DCA as a tumor promoter was established in animal models during this period. Single-dose intrarectal infusions of the carcinogen N-methyl-N′nitro- N-nitrosoguanidine (MNNG) followed by repeated rectal infusion doses of DCA significantly induced colorectal neoplasms, while DCA infusion alone was insufficient to induce tumor formation in both germ-free and conventional rats.[79,80] Subsequent studies with rectal infusion of CA or CDCA in MNNG-treated rats showed significantly higher rates of tumor formation in conventional versus germ-free rats, suggesting the conversion of primary to secondary bile acids by intestinal bacteria was key to tumor promotion.[81] Rats fed 20% corn oil or lard secreted significantly more fecal bile acids, and were more susceptible to colon cancer than rats fed normal levels of dietary fat (5%) in a 1,2-dimethylhydrazine-induced model.[82] In the 1990s it was shown that: i) DCA was significantly elevated in serum of patients with colorectal adenomas;[83-85] ii) serum DCA correlates with DCA in fecal water;[83] and iii) colonic mucosal proliferation is correlated with DCA in serum.[86]

The activation of cellular signaling cascades and membrane perturbing effects generated by secondary bile acids, but not primary bile acids, provide mechanisms by which DCA and LCA promote CRC. The detergent properties of DCA (but not CA) cause membrane perturbations leading to the release of arachidonic acid, which is converted by the enzymes cyclooxygenase 2 (COX-2) and lipooxygenase, to pro-inflammatory and pro-angiogenic prostaglandins, and reactive oxygen species which damage DNA and inhibit DNA repair enzymes.[87,88] DCA also induces *COX-2* expression through transactivation of the epidermal growth factor receptor,[87,89] and activates the β-catenin cell-signaling pathway resulting in colon cancer cell proliferation and invasiveness.[90] Notably, a large body of published work demonstrates the effectiveness of non-steroidal anti-inflammatory drugs in significantly reducing polyp formation and reducing CRC risk by inhibiting the activity of COX-2.[88] Mutations at the adenomatous polyposis coli (APC) locus are a common and early somatic event in polyp formation and CRC.[25] Conspicuously, *COX-2* can be downregulated by wild type but not mutant APC.[91,92] APC regulates β-catenin levels in the cell, failure of which results in accumulation of transcriptionally active β-catenin, which both binds to the *COX-2* promoter region activating transcription, and *COX-2* mRNA increasing stabilization.[93] In addition, DCA can activate proteosomal degradation of the tumor suppressor p53 selecting for cells resistant to apoptosis in spite of DNA damage.[94]

Taken together, diets high in animal protein and fat appear to promote colon carcinogenesis by selecting for bacteria capable of converting primary host bile acids to toxic, tumor-promoting secondary bile acids. Epidemiological, animal models, in vitro cell culture

studies converge on DCA in particular as a metabolite whose serum levels correlate with disease and whose membrane-perturbing and activation of cellular signaling pathways associated with cell proliferation and apoptosis provide mechanisms for long term environmental risk of neoplasia. It is now important to identify additional bacterial metabolites than could serve *genotoxic* roles than might synergistically work with DCA to transform normal colonocytes into adenomas and adenocarcinomas. We will here argue that hydrogen sulfide (H_2S) fits this bill.

Hydrogen sulfide is genotoxic

Sulfidogenic bacteria, including the cysteine fermenting *Fusobacterium nucleatum*, the taurine respiring *Bilophila wadworthia*, and SRB like *Desulfovibrio* spp., metabolize organic and inorganic sources of sulfur to produce H_2S (Table 2). Several lines of evidence implicate H_2S and SRB in the pathogenesis of the inflammatory bowel disease Ulcerative Colitis (UC), a risk factor for CRC. Fecal H_2S is significantly elevated in UC patients experiencing active disease.[95-98] However, other reports have reported no significant increase.[99] It has been argued that UC is an "energy deficiency" disease due to the observation that high levels of H_2S inhibits butyrate oxidation in vitro[100-102] and in vivo,[100] the primary energy source for colonocytes. Measurement of breath CO_2 and luminal bicarbonate are significantly reduced in UC patients relative to controls after rectal infusion of butyrate.[103,104] A difficulty inherent in fecal sulfide measurement may explain discrepancies between studies. It is estimated that 95% of H_2S is absorbed through the colon[105] owing to a natural diffusion gradient between colonic lumen (mM) and blood (μM). Furthermore, due to multiple dissociation states (HS^- and H_2S), which are affected by pH, roughly 2 thirds dissociates into the anionic form.

One study showed that H_2S increased proliferation in cells in the upper colonic crypts by over 54%.[106] This proliferation was reversed by infusion of butyrate. Butyrate enemas are effective in treating the clinical symptoms of UC.[107,108]

Sulfide is thought to inhibit β-oxidation of butyrate through persulfide formation of the coenzyme A prosthetic group of the enzyme short-chain acyl CoA dehydrogenase.[109] Inability to oxidize butyrate results in significant reduction in ion absorption, the formation of protective mucus, and cellular detoxification.[99] Colonocytes express protective enzymes on the mucosal surface that oxidize H_2S.[110] At low concentration (μM), H_2S is oxidized by the mitochondrial electron transport chain by H_2S:quinone oxidoreductase, a gene in mitochondria, which originated in eubacteria exposed to sulfide-rich aquatic environments.[111-112] Sulfide can thus increase ATP synthesis, resulting in a mix of oxidized metabolites including $S_2O_3^{2-}$, HSO_3^-, SO_4^{2-}. At mM concentrations H_2S inhibits cytochrome oxidase decreasing the electrochemical potential inhibiting basic cellular physiology. Loss of sulfide-detoxification has been reported to occur in active UC and CRC.[113] Such a situation is conducive to genotoxicity leading to genetic changes and CRC.

Two studies in rats have also demonstrated that increased protein intake and increased protein fermentation were associated with genotoxicity.[114,115] Fecal hydrogen sulfide levels were significantly higher in patients with neoplasm of the colon and those who have undergone surgery for sigmoid cancer.[116] Animals fed DSS, but not DSS + metronidazole, had significantly higher development of dysplasia as well as adenoma and carcinoma.[117] Two European studies demonstrate associations between sulfidogenic bacteria (*Fusobacterium* spp.) and the tumor surface in a subset of CRC[118,119] Indeed, the Gaskins' lab demonstrated that sulfide is genotoxic at doses found in the colon providing a reasonable explanation for these observations.[120-123] The rationale for this hypothesis is also supplemented by our long-term study of mammalian cell responses to the effects of exogenous sulfide. In a series of prior publications, we demonstrated that sulfide induces proliferative and inflammatory pathways in non-transformed intestinal crypt epithelial cells[120,121] and provided unequivocal evidence that sulfide is genotoxic at concentrations less than those measured previously in human colon leading to cell-cycle arrest; and DNA damage, which is produced by oxidative stress.[120-123] More recently, the genotoxic properties of sulfide have been demonstrated in non-transformed human intestinal epithelial cells with further evidence that sulfide modulates the expression of genes involved in cell-cycle progression, triggering both inflammatory and DNA repair responses.[121] Together with these data, our own published work showing that sulfide is directly genotoxic at high concentrations suggests that H_2S could be a significant bacterial metabolite that initiates colon cancer.

Taurocholic acid: metabolic potential for carcinogen and *tumor-promoter* in one

The abovementioned animal study by Devkota et al. (2012) is intriguing because it suggests a link between an animal-fat diet and a substrate that has the potential to become both a genotoxin (H_2S) and a tumor-promotor (DCA) in the context of a taurine respiring immunogenic bacteria and bile acid 7α-dehydroxylating bacterial species, respectively (Fig. 2).[52] Animal-based diets that favor taurine-conjugation also lead to enhanced bile acid secretion. Enhanced bile secretion provides more bile acid substrate to support a larger population of DCA-producing bacteria.[19] The relationship between sulfidogenesis in the Devkota et al. (2013) model[52] and bile acid 7α-dehydroxylation have not been explored. However, in vitro studies of human fecal isolates have shown that deconjugation of TCA by a sulfide-producing *Bacteroides* sp. strain R1 stimulated bile acid 7α-dehydroxylation of *Clostridium* sp strain 9/1.[124] Narushima et al. (2006) established germ-free mice with a minimal microbiota and observed a significant stimulation of DCA-formation by addition of an isolate identified as closely related to *B. wadsworthia*.[125] Higher levels of fecal secondary bile acids have been shown to worsen the severity of colitis in DSS-treated mice.[126] These data suggest stimulation of secondary bile acid formation by sulfide, and exacerbation of inflammation by the combination of DCA and H_2S.

Recent diet-exchange studies in humans have demonstrated rapid changes in the microbiota over short periods, confirming the role of diet on levels and activities of sulfidogenic, methanogenic, butyrogenic, and bile acid modifying bacteria.[47,48] David et al. (2013) exchanged a plant-based and animal-based diet in healthy American volunteers and measured gut microbiome and key metabolites, including SCFA and bile acids.[47] They showed that *Prevotella*, the major bacterial genus in NA, was reduced in subjects consuming an animal-based diet. Animal-based diet significantly increased the abundance of bile-resistant members such as *B. wadsworthia, Alistipes putredinis*, and *Bacteroides sp*., cluster 29.[47] Total fecal bile acids and DCA in particular were significantly upregulated with the animal-based relative to plant-based diet. Genes involved in bile acid metabolism, including bile *bsh* and *dsrA* were significantly upregulated on animal based diet.[47] SCFA associated with carbohydrate metabolism (acetate, butyrate) were significantly reduced on the animal-based diet, while SCFA (isovalerate, isobutyrate) associated with fermentation of amino acids were significantly upregulated on the animal-based diet.[47]

A recent 2-week food exchange study between NA and AA (NA consumed animal-based diet, AA consumed vegetarian-based diet) demonstrate rapid reciprocal changes in levels of bacteria responsible for sulfide production (*B. wadsworthia, Desulfovibrio spp*) and protective butyrate production, as well as levels of DCA producing bacteria (*C. scindens*) and DCA in stool.[48] These results confirm a recent short-term diet exchange focusing on animal- and plant-based diets which found enhanced DCA formation and increased levels of taurine-utilizing, sulfide-producing *B. wadsworthia*.[47] AA were dominated by *Bacteroides* while NA by *Prevotella* consistent with the study by David et al. (2013).[47] NA had higher abundance of starch degrading gut bacteria (diet high in resistant starch), carbohydrate fermenters and butyrate producers and metabolites, while Americans had higher levels of potentially pathogenic proteobacteria (*Escherichia* and *Acinetobacter*) and bile acid deconjugators and their metabolic end products.[48,78] O'Keefe et al. (2015) also measured colonic inflammation and found that it went below NA baseline when AA were placed on NA diet, while NA colonic inflammation went above AA baseline when NA were placed on AA diet.[48] Butyrate production was associated with lower mucosal proliferation both in NA consuming high-fiber diet and AA switched to NA diet. NA switched to AA diet had significantly lower butyrogenesis. NA diet stimulated methanogenesis, sulfidogenesis, and acetogenesis, improving butyrate production by removing hydrogen. This study also reiterated an important connection between the gut microbiota, Western diet, and disease was the significant upregulation of choline.

Previous studies have shown that gut microbes convert choline to trimethylamine, which is absorbed and converted by the liver to the pro-atherogenic trimethylamine *N*-oxide (TMNO).[127,128] AA had significant choline at baseline, and an observed increase in urinary TMNO in NA given the western diet. Prior studies by the same group showed that significantly higher levels of total fecal bile acids in AA than NA, and DCA in particular was significantly correlated (r^2=0.65; P=0.01) with *baiCD* gene abundance- a gene in the bile acid-inducible operon of bile acid

7α-dehydroxylating bacteria such as *Clostridium scindens* and related organisms responsible for DCA production.[78]

In summary, we propose a novel mechanism to explain why consumption of a high red meat and saturated fat diet imparts risk for CRC development involving primary microbial risk factors (bile acid metabolizing and sulfidogenic bacteria) that are modifiable by diet (Fig. 3). The focus is on taurine, an overlooked sulfur amino acid that is abundant in red meat or provided by bacterial deconjugation of the bile salt TCA, which is increased in subjects consuming a diet high in saturated fat. The taurine provided by bacterial deconjugation of TCA is used as a substrate by *B. wadsworthia* for anaerobic respiration generating genotoxic H_2S. And once deconjugated, free primary bile acids are further metabolized by colonic bacteria to genotoxic and proinflammatory secondary bile acids. Specifically, the production of the secondary bile acid DCA acts as a tumor promoter by causing membrane perturbations leading to the release of arachidonic acid, which is converted by the enzymes COX-2 and lipooxygenase, to pro-inflammatory and pro-angiogenic prostaglandins, and reactive oxygen species which damage DNA and inhibit DNA repair enzymes. Together these mechanisms support a compelling link among diet, levels of TCA, the metabolic end products of TCA metabolism by intestinal bacteria, and development of CRC.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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