Responses and revisions to reviewers1

Comment 1: How to reflect the influence of TLR2 and TLR4 co-recognition?

Response 1: Thank you for your reasonable comment. Gut microbial components regulate gastrointestinal motility via binding to TLR2 and TLR4. TLR2 and TLR4 are located on the membranes of enteric nerve cells, muscles and glial cells to regulate motility. TLR4 recognizes bacterial lipopolysaccharides(a major membrane component of gram-negative bacteria), while TLR2 recognizes lipopeptides and peptidoglycans(the main components of the intestinal gram-positive bacteria,). In addition, both TLR2 and TLR4 maintain nitrergic neurons and neurogenesis in the intestine through their specific signaling and modulate the immune response, playing important roles in the ENS and intestinal transport.

Comment 2: Any relationship with constipation may be due to inverse causality; in other words, CRC may cause constipation before the clinical manifestation of cancer. In other words, CRC may cause constipation before the clinical manifestation of cancer. On what basis?

Response 2: Thanks for your valuable advice. Patients with CRC often seek medical attention for digestive symptoms, such as abdominal discomfort, constipation and blood in the stool from time to time, so the first symptom that may appear in CRC is constipation. The patients do not pay attention to constipation, and do not evaluate whether there was constipation, resulting in memory bias. When describing the clinical symptoms of colorectal cancer, patients do not state that they have constipation symptoms at the first time. Besides, uncertainty about whether constipation is caused by colorectal cancer contributes to inaccurate assessment.

Comment 3: Is there a clear difference between the production of "two well-defined genotoxins by bacteria: cytolethal distending toxin (CDT) and colistin" mentioned in

the article?

Response 3: Thank you for your suggestions. Colistin and CDT have certain differences but also have similarities. The main toxins that intestinal bacteria produce are colistin and CDT, which are made by Escherichia coli (E. coli) and other gram-negative bacteria in the large intestine and directly damage DNA. CDT can trigger a DNA damage response, which leads to the arrest of the cell cycle and causes cellular senescence or death. CDT has a critical effect on the carcinogenic effect that campylobacter jejuni induces. CDT-derived campylobacter jejuni cause injury to host cell DNA, and promote colorectal tumorigenesis by triggering cell multiplication and the enhancing of nuclear translocation of β-catenin protein. Not much research has been done on colistin.Certain strains which produce colistin often have an association with CRC. Colibactin can cause the breakage of double-strand DNA, chromosome instability, and cell senescence in eukaryotic cells. Bacteria which produce E. coli can modify the tumor microenvironment, which leads to cellular aging and can also influence tumor progression through the secretion of growth factors.

Comment 4: Which group of bacterial pathogens produce CDT and which produce colistin?

Response 4: Thank you for your helpful comments. CDT is produced by selective enteric pathogen strains such as Salmonella, Escherichia and Campylobacter spp. Colistin is produced by Escherichia coli (E. coli) and other gram-negative bacteria in the large intestine.

Comment 5: The writing is not standard, for example, the specific content of core tip in the manuscript is blank; The TLR in line 161 of the "Microbiome Induced Constipation Regulation" section is written instead of LTR. Please check the writing carefully again.

Response 5: Thank you for your helpful comments. I have corrected the error about the Toll-like receptors (TLRs).

Comment 6: The overall year of references is earlier, so more references in recent five years.

Response 6: I have added references which study recent years to properly justify the work.

For example:

Jia W, Xie G, Jia W. Bile acid-microbiota crosstalk in gastrointestinal inflammation and carcinogenesis. Nature reviews Gastroenterology & hepatology. 2018;15(2):111-28.

Sun L, Cai J, Gonzalez FJ. The role of farnesoid X receptor in metabolic diseases, and gastrointestinal and liver cancer. Nature reviews Gastroenterology & hepatology. 2021;18(5):335-47.

Tremblay W, Mompart F, Lopez E, Quaranta M, Bergoglio V, Hashim S, et al. Cytolethal Distending Toxin Promotes Replicative Stress Leading to Genetic Instability Transmitted to Daughter Cells. Front Cell Dev Biol. 2021;9:656795.

Fang Y, Yan C, Zhao Q, Xu J, Liu Z, Gao J, et al. The roles of microbial products in the development of colorectal cancer: a review. Bioengineered. 2021;12(1):720-35.

Responses and revisions to reviewers2

Comment 1: To further understand the relationship between the gut microbiota, constipation and CRC, ongoing preclinical and clinical research is required. Which future clinical studies would the authors wish to suggest? Any study designs?

Response 1: In this revision, we revised the discussion section and described the study designs in the last paragraph.

Comment 2: In the introduction they state that constipation is a common problem for 16% of individuals overall and 33.5% of seniors (60-101 years). How is old age classified? Why is the cut off age 60 years?

Response 2: Thank you for underlining this deficiency. There is no universal agreement on what constitutes old age, however, some studies indicate that constipation appears to increase in frequency around age 60.

Comment 3: Please explain the mechanism of how the double-strand DNA breaks through deoxyribonuclease activity and leads to mutations which lead to CRC.

Response 3: Thank you for your suggestions, and we added the mechanism of how the double-strand DNA breaks through deoxyribonuclease activity and leads to mutations which lead to CRC.

Comment 4: Which mutations are caused by B. fragilis toxins?

Response 4: Thank you for your reasonable comment. We added how B. fragilis toxins cause mutations.

Comment 5: Please also explain the mechanism of how Clostridium perfringens increases reactive oxygen species, DNA damage and which mutations are caused leading to CRC?

Response 5: Thanks for your valuable advice. Clostridium perfringens belongs to the genus Clostridium, which produces bile acid hydrolases that catalyze the production of secondary bile acids (such as deoxycholic acid and lithocholic acid). Increased deoxycholic acid and lithocholic acid levels in the colon activate the Wnt/β-linked protein and NF-B signaling pathways, resulting in oxidative DNA damage, increased mitotic activity, and activation of intrinsic apoptotic pathways such as mitochondrial oxidative stress, cytochrome C release, and cytoplasmic cysteine aspartase. Secondary bile acids also influence CRC by activating the bile acid receptors TGR5 and FXR.

Comment 6: Would you consider all patients that are constipated irrespective of age to have a colonoscopy to prevent CRC?

Response 6: Thank you for your helpful comments. Although constipation itself is not an indication for colonoscopy, colonoscopy should be considered to screen for CRC in patients with severe chronic constipation or alarm symptoms. In addition, at

age 50, both men and women need to have a full colonoscopy if there are no factors that increase their risk of developing cancer.

(see NCCN Clinical Practice Guidelines in Oncology: Colorectal Cancer Screening Version 3.2022)

- **Footnote modified: The panel has reviewed existing data for beginning screening of average-risk individuals at age <50 years. Based on their assessment, the panel agrees that the data are stronger to support beginning screening at 50 years, but acknowledges that lower-level evidence supports a benefit for screening earlier. When initiating screening for all eligible**

Answering reviewers for re-review

Responses and revisions to reviewers 1

Comment 1: The crosstalk between the gut microbiota, constipation, and CRC, and their specific mechanisms of action were discussed by authors. It provides a theoretical basis for reducing the risk of colorectal polyps and CRC and preventing disease progression.The authors suggest that gut flora may soon become a powerful tool in the fight against colorectal cancer, making meaningful assumptions for future research.

Response 1: Thank you for your comment.

Responses and revisions to reviewers 2

Comment 1: It would be helpful to explain the mechanism of how clostridium perfringens causes DNA damage and hence which mutations leading to CRC in a flow diagram.

Response 1: Thanks for your valuable advice. The specific mechanism is explained in P4 L152.

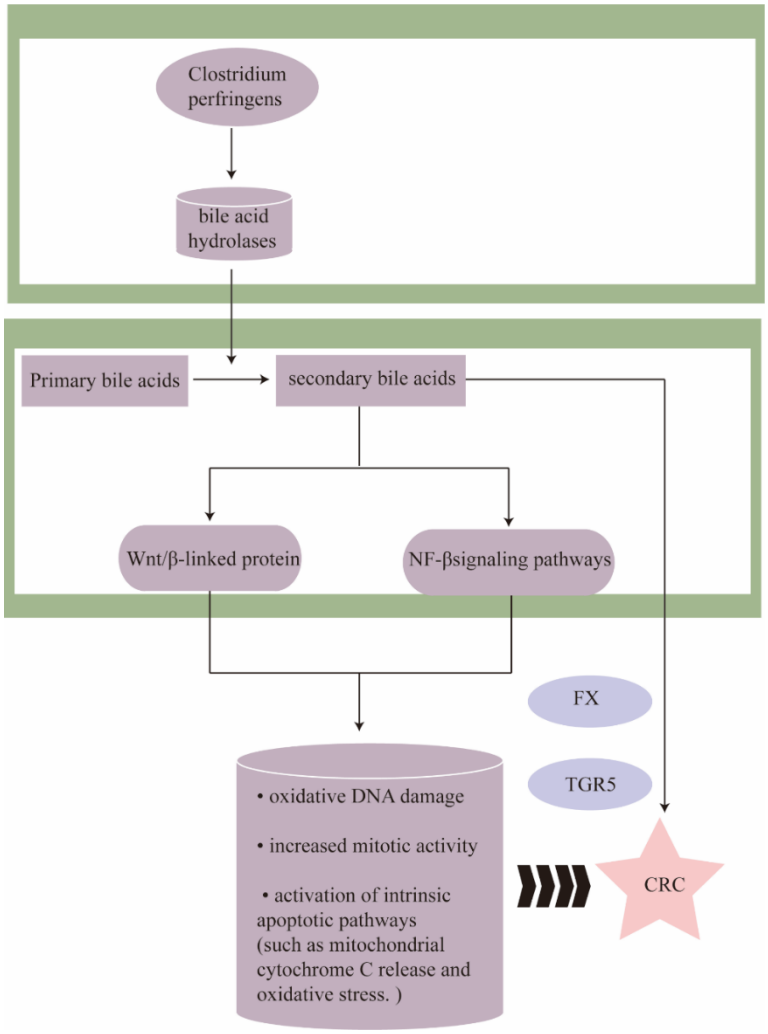

Figure 2. A flow diagram of the mechanism of how clostridium perfringens causes DNA damage and hence which mutations leading to CRC.

Clostridium perfringens can produce bile acid hydrolases that catalyze the production of secondary bile acids. Increased secondary bile acids levels activate the Wnt/β-linked protein and NF-βsignaling pathways, resulting in oxidative DNA damage, increased mitotic activity, and activation of intrinsic apoptotic pathways. Secondary bile acids also influence CRC by activating the bile acid receptors TGR5 and FXR.

Comment 2: Is there any reason why the guidelines propose age 50, both in men and women to have a full colonoscopy if there are no factors that increase their risk of developing cancer.

Response 2: Thank you for your reasonable comment. The recommendation to start CRC screening at age 50 was established based on the observed increased incidence of

CRC above this age in the general population(1). Given the varying prevalence of known CRC risk factors, CRC screening is not a “one size fits all paradigm”. Currently it is accepted that some patients identified as higher risk for CRC such as patients having a family history of CRC or having CRC-associated genetic diseases are screened at younger ages and preferentially with colonoscopy. The guidelines recommend that colonoscopy be started before age 50 or even at age 45 for patients with associated risk factors, or after age 50 if there are no associated risk factors. We added the sentence in P9 L336.

(see NCCN Clinical Practice Guidelines in Oncology: Colorectal Cancer Screening Version 3.2022)

1.Force USPST. Screening for colorectal cancer: recommendation and rationale. Ann Intern Med. 2002;137(2):129-31.

- **Footnote modified: The panel has reviewed existing data for beginning screening of average-risk individuals at age <50 years. Based on their assessment, the panel agrees that the data are stronger to support beginning screening at 50 years, but acknowledges that lower-level evidence supports a benefit for screening earlier. When initiating screening for all eligible individuals, the panel recommends a discussion of potential harms/risks and benefits, and the consideration of all recommended CRC screening options. Ladabaum U, et al. Gastroenterology 2019;157:137-148; *Knudsen, AB, et. al. JAMA 2021;325:1998-2011.***