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14. ABSTRACT Colorectal cancer (CRC) stages II/III are optimally treated with chemotherapy (e.g. FOLFOX) and surgery. However, up to 50% of CRC patients experience chemotherapy-induced diarrhea (CID), which can lead dose reduction and be life-threatening. There are no biomarkers for predicting risk or severity of CID. The purpose of this study is to understand the dietary and microbial factors that are associated with incidence and severity of CID and develop a predictive biomarker. I aim to study patient-derived biospecimens and dietary recall to identify microbial and dietary changes that are predictive of incidence and severity of CID to inform future studies of CRC treatment response.						
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1. INTRODUCTION:

Colorectal cancer (CRC) stages II/III are optimally treated with chemotherapy (e.g. FOLFOX) and surgery. However, up to 50% of CRC patients experience chemotherapy-induced diarrhea (CID), which can lead dose reduction and be life-threatening. There are no biomarkers for predicting risk or severity of CID. The purpose of this study is to understand the dietary and microbial factors that are associated with incidence and severity of CID and develop a predictive biomarker. I aim to study patient-derived biospecimens and dietary recall to identify microbial and dietary changes that are predictive of incidence and severity of CID to inform future studies of CRC treatment response.

2. KEYWORDS:

Colorectal cancer, chemotherapy-induced diarrhea, diet, microbiome, toxicity, biomarkers

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1

Major Task 1: Quantify the gut microbiota prior to surgery and treatment, and at 3 time points during FOLFOX, and correlate with CID incidence and severity

Extract DNA from stool and tissue and perform 16S rRNA sequencing

To be completed by month 30

Percentage of completion: 0%

Statistical analysis of 16S rRNA sequencing

To be completed by month 30

Percentage of completion: 0%

Microbiome-CID analysis

To be completed by month 36

Percentage of completion: 0%

Major Task 2: Quantify microbial pathway abundance and fecal metabolite levels prior to, and during treatment, and determine their associations with CID incidence and severity

Extract, quantify, and analyze microbial metabolites prior to surgery and during treatment

To be completed by month 30

Percentage of completion: 0%

Metabolite-CID analysis

To be completed by month ***clerical error, no month assigned – should be month 30**

Percentage of completion: 1%

Major Task 3: Quantify circulating and tumor tissue cytokine levels prior to, and during treatment, and determine their associations with the gut microbiota, and their associations with CID incidence and severity

Process blood and tissue samples, perform cytokine analysis

To be completed by month 30

Percentage of completion: 0%

Cytokine-CID analysis

To be completed by month 30

Percentage of completion: 0%

Analyze the interaction between the microbiome and microbial metabolites prior to surgery and during treatment

To be completed by month 30

Percentage of completion: 0%

Milestone: Local IRB/HRPO already approved #017-342

To be completed by month 4

Percentage of completion: 100%

Milestone: All subject consented/enrolled

To be completed by month 24

Percentage of completion: 0%

Milestone: Microbiome and metabolite data analysis complete

To be completed by month 30

Percentage of completion: 0%

Milestone: All data on CID incidence and severity completed

To be completed by month 30

Percentage of completion: 0%

Specific Aim 2:

Deploy dietary questionnaires to patients; collect responses

To be completed by month 30

Percentage of completion: 0%

Perform microbiome/metabolite-diet analysis

To be completed by month 30

Percentage of completion: 1%

Analyze associations between dietary factors and risk of developing CID

To be completed by month 36

Percentage of completion: 0%

Milestone: Dietary analysis complete

To be completed by month 26

Percentage of completion: 0%

Milestone: Diet-CID risk analysis complete

To be completed by month 36

Percentage of completion: 0%

What was accomplished under these goals?

Major Activities: The Greathouse Lab has made progress towards Aims 1 and 2 in the areas of methods development, creation of shared resources and knowledge dissemination as detailed below:

- Major task 2 – In an effort to better understand and prepare to analyze tryptophan and indole metabolites to address the outstanding gap in knowledge regarding the bacterial indole effects on CRC we published the following manuscript → Madhur Wyatt and *K. Leigh Greathouse. Targeting dietary and microbial tryptophan-indole metabolism as therapeutic approaches to colon cancer. *Nutrients*. 2021; Apr 3;13(4):1189. doi: 10.3390/nu13041189. (**Appendix**)
- Major task 2 - *Extract, quantify, and analyze microbial metabolites prior to surgery and during treatment* → Co-I Dr. Ramon Lavado has established and optimized the methodology to extract and quantify metabolites from stool samples.

1. SCFA Analysis

1.1. Derivatization

The derivatization of SCFA was done according to (Jin et al. 2018) with minor modifications. 20 µL of sample was transferred to a reacti-vial tube for further derivatization. The derivative reagent (2 mL) was constituted of 1 mL of 200 mmol/L 3-NPH HCl (3-nitrophenylhydrazine hydrochloride) in 50% acetonitrile, 200 µL of 600 mmol/L EDC HCl 1-ethyl(3-aminopropyl)-3-ethylcarbodiimide hydrochloride) in 50% acetonitrile, and 800 µL 7.5% pyridine in 50% acetonitrile. The samples were mixed with derivative reagent at the ratio of 1:1 (v/v; 40 µL in total). The whole process was performed in ice. Afterwards, the mixture was permitted to react in at 40 °C for 30 min. A mixed standard solution containing 4 mM of acetic acid (D₄), 2 mM

of propionic acid (D₅), and 1 mM of each of the other six SCFAs (butyric-D₇, valeric-D₉, caproic-D₁₁, and octanoic-D₁₅) were derivatized as previously described. After the reaction, the mixture was cooled down and finally diluted to 1 mL with 10% acetonitrile. Before injection, the mixed system was centrifuged at 10,000g for 10 min, and 720 µL of the supernatant was added with 30 µL (internal standard, IS).

1.2. Preparation of standard solutions, calibration and quality control (QC) samples

The mixture of stock standard solution containing acetic (4 mmol/L), propionic (2 mmol/L), butyric (1 mmol/L), valeric (1 mmol/L), isovaleric (1 mmol/L), caproic (1 mmol/L), heptanoic (1 mmol/L) and caprylic (1 mmol/L) was derivatized as previously described and diluted with 50% acetonitrile to perform standard calibrations. Quality control (QC) samples were prepared from each biological sample to ensure broad metabolite coverage. All solutions were stored at 4 °C before use.

1.3. UHPLC-MS/MS analysis

The samples were analyzed on a Thermo Scientific UltiMate 3000 Rapid Separation Quaternary HPLC System, connected to a QExactive™ Focus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (Thermo Scientific, Madison, WI). A Waters Acquity BEH C₁₈ column (2.1 × 100 mm, 1.7 µm) was used to separate the SCFAs. The mobile phases were composed of 0.01% formic acid in water (v/v) (A) and 100% acetonitrile (B). The mobile phases were eluted at 0.5 mL/min with the gradient as follows:

1. 0-10 minutes – 80% A / 20% B.
2. 10-12.5 minutes – 40% A / 60% B.
3. 12.5-12.6 minutes – 80% A / 20% B.
4. Re-equilibrate at 20% B for 5 min.

The column temperature was maintained at 40°C, and the injection volume was 2 µL. The MS/MS detection was performed via electrospray ionization source in the negative mode. The parameters of mass spectrometer included a transfer tube temperature of 350°C; a sheath gas of 35 arb; an auxiliary gas of 10 arb; a sweep gas of 0 arb; an ion spray voltage of 3.0 kV; a collision energy of 30 eV and a capillary temperature of 275 °C. Selective Reaction Monitor (SRM) conditions for the quantification of each compound were summarized in Table 1.

The quantitation of SCFAs was carried out using Xcalibur Quan Browser (Thermo Finnigan, U.S.A). Calibration curves for the targeted SCFAs were constructed by plotting each SCFA/IS peak area ratios obtained from calibration standards versus amounts of SCFA and fitting these data using linear regression with the correlation coefficient (R²).

Table 1. Chromatographic and mass spectrometric characteristics of the analytes.

Compound	RT (min)	Parent ion (m/z)	Product ion (m/z)	Collision energy (eV)
Acetic acid	3.00	194.06	151.06	30
Propionic acid	4.89	208.07	165.07	30

Compound	RT (min)	Parent ion (m/z)	Product ion (m/z)	Collision energy (eV)
Butyric acid	6.68	222.10	179.09	30
Isovaleric acid	8.08	236.10	193.10	30
Valeric acid	8.37	236.10	193.10	30
Caproic acid	9.84	250.10	207.12	30
Enanthic acid	10.01	262.12	208.07	30
Caprylic acid	10.89	278.09	231.12	30

2. Fecal extraction of SCFAs

Fecal extraction for short-chain fatty acid determination was optimized by diluting fresh fecal samples (0.5 g) with 95% ethanol and then homogenized by sonication for 10 min. After that, mixing and extracting by vortex for 5 min to extract all SFCA. The extracts were centrifuged at 10,000g for 10 min at 10°C and the supernatant filtered through 0.22 µm PTFE syringe filter. Samples were stored at −80 °C until analyzed. The optimized fecal extractions of SCFA are shown in Table 2.

Table 2. Fecal extraction efficiency of the different SCFA analyzed. Data are presented as average ± SD

Compound	Systematic name	Carbons	Extraction efficiency (%)
Acetic acid	Ethanoic acid	2	94.76 ± 10.89
Propionic acid	Propanoic acid	3	88.36 ± 9.01
Butyric acid	Butanoic acid	4	92.63 ± 2.34
Isovaleric acid	Isopentanoic acid	5	90.23 ± 12.11
Valeric acid	Pentanoic acid	5	87.12 ± 13.53

Compound	Systematic name	Carbons	Extraction efficiency (%)
Caproic acid	Hexanoic acid	6	83.02 ± 6.98
Enanthic acid	Heptanoic acid	7	79.69 ± 15.32
Caprylic acid	Octanoic acid	8	65.23 ± 8.41

3. References

- Jin Y-Y, Shi Z-Q, Chang W-Q, Guo L-X, Zhou J-L, Liu J-Q, et al. 2018. A chemical derivatization based uhplc-ltq-orbitrap mass spectrometry method for accurate quantification of short-chain fatty acids in bronchoalveolar lavage fluid of asthma mice. *Journal of Pharmaceutical and Biomedical Analysis* 161:336-343.
- Major task 3 - Milestone: Local IRB/HRPO already approved #017-342 → all IRBs have been approved. **NOTE:** We finalized approval with BSWH and DoD HRPO for addition of a third hospital site at BSWH Round Rock for recruitment within the Baylor Scott and White Hospital System (30 min from the primary site) to increase enrollment opportunities. In addition, we are planning on adding two additional large BSWH medical centers in Dallas and College Station, TX. This will significantly increase the catchment area for patient enrollment.
 - Major task 3 - Deploy dietary questionnaires to patients; collect responses → As part of our efforts to streamline and optimize dietary data collection from participants we have added a 3-day food log to the stool collection kit to improve recall for 24-dietary recalls. Importantly, we are also writing a review for the journal *Neoplasia* (invited review) on this topic of the importance of dietary collection and collection tools needed for cancer patients; especially for microbiome-cancer studies. This manuscript is being written together with two physicians and three dietitians at major cancer treatment centers and will be submitted for publication on 12/01/2021.
 - Major task 3 - Perform microbiome/metabolite-diet analysis → In an effort to establish new methods for longitudinal diet-microbiome analysis in preparation for this study we have obtained a large dataset from the ongoing ColoCare Study (<https://uofuhealth.utah.edu/huntsman/labs/ulrich/research/colocare-study.php>) in which I am part of the dietary and microbiome working groups. I submitted a protocol to obtain these data which include 16S rRNA microbiome sequencing as baseline diagnosis and at 6 months, and food frequency questionnaire data at 6 months from 398 individuals with CRC. I obtained these data in September 2021. Unfortunately, only one case of chemotherapy-induced diarrhea was captured as this was not their primary goal, however, these data have allowed us to begin to test out two new approaches to modeling the diet-microbiome relationship over time with other key cancer outcomes including survival and recurrence. First, we are developing a structural equation model (SEM) utilizing parcels of microbiome data to predict recurrence or survival. Utilizing an SEM approach allows us to account to individual differences and measurement error in microbiome abundance. One of the complexities of using an SEM approach with microbiome data is the high dimensional nature of microbiome data would require an immense amount of data not practical for microbiome research. A parceling approach is implemented to create subsets of microbiome

to use as indicators in the SEM model that predicts recurrence or survival. One of the unique aspects of this approach to modeling is that we are able to identify which parcel(s), subsets of taxa, predict recurrence most strongly and also account for differences in predictive performance across parcels when making a final inference about the predictiveness of the microbiome as a whole. The modeling approach described above is being developed in collaboration with colleagues from Texas Tech, Dr. Caleb Phillips and Dr. Todd Little, expertise in SEM, we are conducting initial modeling attempts with these data. The second approach, is using a clustering method previously used for microbiome data and diet data separately but not together. This approach using Partitioning Around the Mediod (PAM), a type of k-means clustering, groups similar objects that share associations and attempts to create novel clusters. These clusters are called community state types (CST). We are currently working to produce CSTs for both the microbiome data and diet data in the following manner. **First Step:** Construct community state types (CST-M) for all microbes from samples with either survival data or recurrence data - using PAM (**Figure 1**); **Second Step:** Construct community state types (CST-D) for all dietary data from samples with microbiome data, and either survival or recurrence data - using PAM or similar; **Third Step:** Identify the change in microbiome CST from baseline to 6 months; **Fourth Step:** Determine correlations between CST-M and CST-D; **Fifth Step:** Determine correlations between change in CST-M and CST-D; **Sixth Step:** Determine if change in CST-M is predictive of survival or recurrence; **Seventh Step:** Determine if CST-D is predictive of survival or recurrence. The intent is to be able to use the CSTs to predict which groups have better/worse survival or recurrence. The goal is to use this method on the data collected from our study to predict chemotherapy-induced diarrhea.

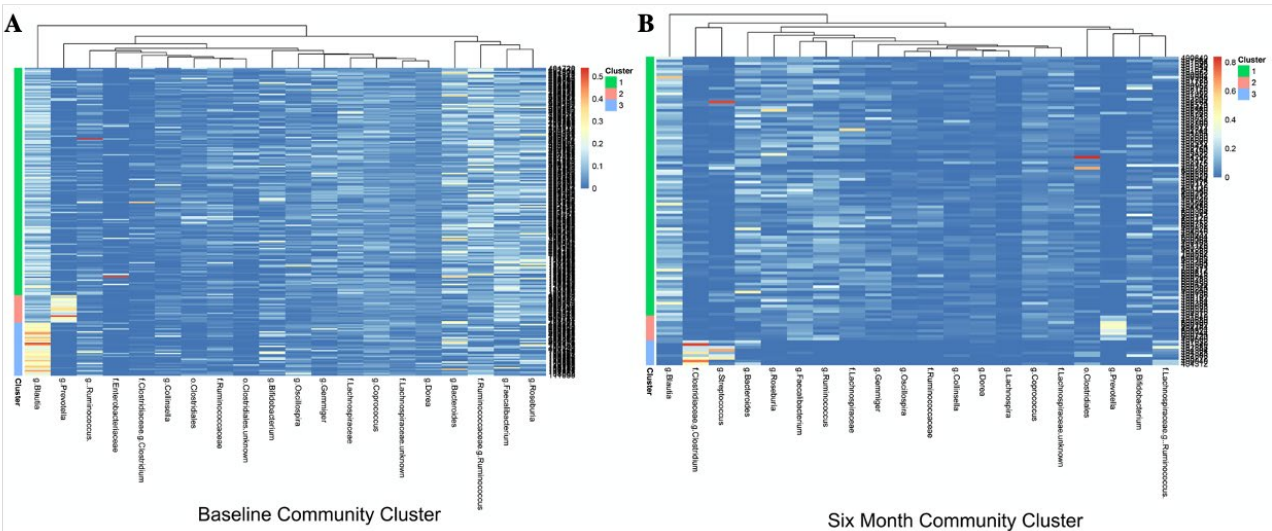


Figure 1. Community state clusters (CST) for microbial taxa present in fecal samples at baseline (A) and at 6 months after CRC diagnosis (B). Clusters were constructed using partitioning around the mediod (PAM) approach with microbial taxa identified from 16S rRNA sequencing from the ColoCare Study.

Other achievements:

In an effort to further elucidate the tryptophan-indole metabolite profiles in stool samples from CRC patients, we worked with our Co-I Dr. Jun Chen from the Mayo Clinic to obtain 7 stool samples from their ongoing CRC microbiome study. At the beginning of this study, we established a

collaboration with the company GALT (<https://www.galt-inc.com/prospector/>) to use their novel Prospector device in order to produce large quantities of clinical isolates that were both associated with CRC and had the potential to produce indoles. We accomplished this by designing 6 qPCR probes that were targeted to select for genus-level *Fusobacterium*, *Peptrostreptococcus*, and *Bacteroides*, and genus-specific genes for *tnaA* and *fldAIBC* (indole producers). They recently provided us with over 1,360 clinical isolates (**at no cost to us or this funding source**) for strain-typing. Our next steps are to utilize internal funds to identify strain and gene presences for all isolates using Baylor University's high-throughput DNA isolation and qPCR system, originally acquired for COVID testing of our students and staff. This enormous isolate library will be made available to the public at no cost, and will provide critical information for our research to better understand the mechanisms by which bacterial indoles improve or worsen CRC treatment response.

Excitingly, in an effort to overcome the perceived drawback to collecting stool samples by patients enrolling into the study, I worked with a Film and Digital Media professor here at Baylor, David Garcia, to create a video that makes collecting stool seem much easier and important for CRC research. Professor Garcia utilized his senior film students to write a skit, which they pitched to me first for approval. Then, the students acted and directed the short video, and Professor Garcia produced and edited the video called "Back to the Toilet" (**no project funds were used to produce this video**). A secure copy is available through YouTube for enrolled patients to view at this location: https://www.youtube.com/watch?v=HyK_Kx82FPw&t=2s

Discussion of goals not met: In our SOW we enumerated an estimated enrollment timeline whereby we would recruit and enroll 18 participants each quarter, starting in Q2. Unfortunately, we have not met this goal; 0 CRC cases and 1 control have been recruited or enrolled. A detailed discussion in this regard is provided in the section "**Changes/Problems**" as indicated by the DoD Scientific Officer.

What opportunities for training and professional development has the project provided?

As part of this study, I have been able to provide training to multiple individuals. First, I have been working with my doctoral student Madhur Wyatt one-on-one to increase her knowledge and skills related to scientific writing as evidenced by her recent publication (see previous manuscript publication noted earlier in *Nutrients*), human subjects training, project and database management, and computer language skills development. Second, I have been training another one of my doctoral

students, Julia Xu, in microbiome analysis skills and scientific writing. Specifically, one of her current projects is focused on identification of bacteria with indole producing capabilities and relationship to CRC. She is working with my collaborator, Mary Lauren Benton (Bioinformatician) here at Baylor, and she has identified 52 CRC-microbiome databases with sufficient information for this analysis. Dr. Benton will be assisting Julia in computationally identifying all of the bacteria with the gene *tnaA* or similar orthologues capable of producing indoles. Julia will be using the 16S rRNA data to look for association between putative indole producing bacteria and risk of CRC and survival in these databases through meta-analysis. Furthermore, she attended a Genomics Workshop through Cold Spring Harbor, where she obtained microbiome-specific analysis skills that she is utilizing in her project. Third, as part of this project I am training two undergraduate students on how to isolate DNA and RNA from stool samples and prepare them for 16S rRNA sequencing. One of these undergraduates with exceptional bioinformatic skills I have trained in microbiome analysis, and she has already successfully produced inferred metabolomic data using the tool PICRUSt from our in-house CRC datasets. Finally, I am currently training my new postdoc, Ankan Choudhury, by one-on-one training in scientific writing and other aspects of microbiome study design and analysis. Specifically, we recently co-authored an invited spotlight manuscript together – “Spiking immunotherapy with a bacterial cocktail brings T cells back to the fight”. Greathouse, K. Leigh and Ankan Choudhury, *Cell Reports Medicine*, Volume 2, Issue 10, 100430. Also, he is working with me on the PAM analysis mentioned earlier as part of our methods development for these study data.

How were the results disseminated to communities of interest?

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?

The major objective for this next study period is to recruit sufficient numbers of CRC patients for analysis of chemotherapy-induced diarrhea. Our current plan for increasing enrollment is multi-faceted. First, we added a third hospital site in regional area, Baylor Scott and White Health Round Rock, TX. They are ~30 min from the primary site in Temple, TX and 1 hour from the secondary site in Waco, TX. This has already been approved through the BSWH Research Institute as well. This should increase the opportunity to enroll CRC patients. In addition, we are planning on adding

additional sites in Dallas, TX and College Station, TX that are part of the BSWH Research Institute. In this regard, I have also established collaboration with Dr. Rajesh Shah at BSWH Round Rock as the physician champion at this site, and likewise have developed a collaboration with Dr. Christopher Johnson at the McClinton Cancer Center (2nd enrollment site) in Waco, TX. We have also produced study flyers and have made them available at all locations and specifically to the nurses at the endoscopy/colonoscopy clinic to enhance awareness and enrollment. In an effort to promote and keep all study staff aware of this study and open enrollment, I will be giving regular talks and study updates on a quartile basis in rotation through each of the medical teams associated with this study at each study site, GI, surgery, pathology and oncology. Also, study staff at Site 1 (Temple) will be sending regular study promotion and update emails to all sites. Lastly, I will be giving a “Focus on Research” talk about this study and the cancer microbiome, which includes all BSWH medical centers across Texas, and is usually attended by ~500 people each week.

Apart from enrollment goals, we plan to continue to develop our methodology for longitudinal microbiome-diet analysis and predictor development using ColoCare data sets. If either of these methods are successful, we plan to publish these methodologies as manuscripts.

All major tasks will be continued to be addressed as biospecimens are obtained from enrolled participants; specifically, data entry and data checking on a rolling bases to ensure data integrity.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Although we do not yet have our own data collected, we have been very resourceful in utilizing other similar datasets that have allowed us to develop novel methods for longitudinal microbiome and dietary analysis. Currently, there is a lack of robust and validated methods for identifying the impact of dietary changes on the changes in the gut microbiome over time, especial in the context of cancer. Our goal is to develop these methods by testing SEM and PAM on similar diet-microbiome data sets from other CRC studies to predict treatment response. The long-term goal from this study would be to design precision nutrition therapy for patients to improve response to treatment and reduce toxic side effects, as well as prolong survival and reduce recurrence. This would be a substantial step forward for this field; as such currently there is *only* dietary management of symptoms. Thus, these results would be highly impactful.

Additionally, the new bacterial CRC isolates collection we have now obtained, and are currently annotating, will be a substantial contribution to the scientific community, specifically as a resource for the study of bacteria-associated mechanisms of CRC. Specifically, we plan to utilize these isolates

for multiple future studies to further interrogate indole effects on CRC using *in vivo* and *in vitro* models (tumor spheroids) and other related mechanisms. This collection will make a large impact on the public knowledge base and resources for microbiome studies of CRC.

What was the impact on other disciplines?

If our novel SEM or PAM modeling of the diet-microbiome relationship in predicting cancer outcomes for CRC is shown to be successful, this could potentially be useful in multiple other cancer types and other disease states where the diet-microbiome relationship is known to mediate disease and treatment response, such as the kidneys, liver, pancreas, and GI tract.

The CRC bacterial isolate collection will most certainly be impactful far beyond CRC, and likely be a resource for other microbiologists and other researchers studying diseases that are known to be associated with *Fusobacterium*, *Bacteroides*, and *Peptostreptococcus*.

What was the impact on technology transfer?

It is possible the new CRC isolate collection may represent a new commercial or public use technology for other companies and scientists.

What was the impact on society beyond science and technology?

It is possible that the development of this unique video “Back to the Toilet” demonstrating the ease of collecting stool and the importance of studying the gut microbiome in CRC would enhance awareness of CRC screening and improve screening attitudes. But this would need to be analyzed in a separate study to confirm its impact.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Actual or anticipated problems or delays and actions or plans to resolve them

Issues related to COVID-19 pandemic are the major reasons for the hindrance of enrollment. First, we were hit hard especially here in Texas and even more so in our county, McClennan. Our county had and continues to have some of the highest rates of COVID-19 rates and hospitalizations in Texas, in the initial wave and in the second wave after the mask mandate was lifted. Thankfully, this rate is now starting to drop precipitously. This forced a significant part of the hospital staff into other temporary positions to care for COVID-19 patients. This issue was combined with the significant reduction in the numbers of individuals coming in for colonoscopy screening and potential CRC diagnosis for fear of catching COVID while at the hospital surrounded by high-risk patients (see attached letter from study Co-PI, Dr. Wong, in the **Appendix**) as shown in **Table 1**. Importantly, in March-April and July-September of 2021, BSWH closed down all elective surgeries, which further decreased the number of colonoscopies and surgeries for CRC screening and treatment. Second, after receiving official notification of IRB approval through DoD HRPO in November 2020, we received initial IRB approval at BSWH; however, several issues with protocols, consents, and other study documents delayed enrollment until all documents were approved through IRB in April 2021. Thus, we did not begin to screen for enrollment until April 2021. Since this time, study staff at Temple and Waco sites, have screened 5 individuals that match enrollment criteria. One was a screen fail, one could not be contacted, and three did not wish to participate.

Table 1. Site 1 Hospital Colorectal Cases

	2019	2020	2021
Jan	11	8	5
Feb	9	10	2
Mar	10	14	7

Apr	11	4	11
May	12	8	5
Jun	10	4	4
Jul	9	6	13
Aug	8	14	6
Sep	11	11	9
Oct	10	9	NA
Nov	9	12	NA
Dec	14	6	NA
Total	124	106	62

Thankfully, BSWH has now on-boarded the patient and physician tracking system EPIC and coordinated system REDCap at the beginning of 2021, and our study coordinators are becoming more familiar with how to use this system to screen for potential patients to enroll. Of note, we continue to have weekly meetings with study coordinators and PIs from each site, even though the pandemic, which are usually virtual.

Due to this low enrollment, and feedback from study coordinators, we also amended our protocol to remove some of the enrollment criteria in order to capture more individuals with CRC (removed: must be taking FOLFOX therapy → now only required to have chemotherapy as treatment; no previous FAP or Lynch syndrome → now we will accept patients with this history; stage II/III CRC → now we will accept any stage CRC). **Prior to making these changes we obtained email approval from DoD HPRO that these changes did not represent a “significant change to the protocol”.**

In order to improve enrollment, we added a third study site, BSWH Round Rock Medical Center, which is 30 minutes from the primary enrollment site in Temple, TX. This approval is pending DoD HRPO/IRB currently and has not taken effect.

Additionally, I have been in regular contact with Dr. Nick Chia, Director of the Microbiome Center at Mayo Clinic, who runs their microbiome cancer CRC study, which is very similar to ours. I have had numerous conversations about study protocols and methods to improve enrollment since the study began, and have implemented all of his suggestions where possible.

We anticipate with the significant reduction in COVID hospitalizations in our area, booster vaccine approvals, addition of 3 more enrollment sites, collaboration with two new GI physicians’ champions for the study at Round Rock and Waco sites, and familiarization with the EPIC system for patient screening our enrollment number should come up dramatically.

Changes that had a significant impact on expenditures

Since COVID-19 pandemic cancelled some of the travel I had planned as part of my career development training, I utilized this funding instead to pay for the build out of the surveys in REDCap (not previously available when the grant was submitted) to facilitate programming of these surveys for patient use as part of this study, which turned out to be roughly the same cost of my lost travel opportunity \$1273.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Nothing to report

Significant changes in use or care of vertebrate animals

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications. Madhur Wyatt and *K. Leigh Greathouse. Targeting dietary and microbial tryptophan-indole metabolism as therapeutic approaches to colon cancer. *Nutrients*. 2021; Apr 3;13(4):1189.

doi: 10.3390/nu13041189. (federal support not acknowledged as federal dollars were not used to produce this manuscript)

Books or other non-periodical, one-time publications.

Nothing to report

Other publications, conference papers and presentations.

Nothing to report

- **Website(s) or other Internet site(s)**

Nothing to report

- **Technologies or techniques**

Nothing to report

- **Inventions, patent applications, and/or licenses**

Nothing to report

- **Other Products**

Product 1 – YouTube video “Back to the Toilet” – an informative video detailing the process of stool collection and the importance of CRC screening and research for this study
(https://www.youtube.com/watch?v=HyK_Kx82FPw&t=2s)

Product 2 – CRC stool bacterial isolate collection – a publicly available resource of bacterial isolates from individuals with CRC (annotation and cataloguing in progress)

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Madhur Wyatt
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	3
Contribution to Project:	Ms. Wyatt performed work in the area of study and database management, and manuscript writing/publication
Funding Support:	Baylor University, Health and Human Sciences
Name:	Julia Xu
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	2

Contribution to Project: Ms. Xu performed database searching/curation of publicly available CRC data for using in validating results from this study

Funding Support: Baylor University, Department of Biology Doctoral Program

Name: Ramon Lavado
Project Role: Co-I
Researcher Identifier (e.g. ORCID ID): 0000-0002-2774-5104
Nearest person month worked: 1

Contribution to Project: Dr. Lavado optimized methods for extraction and analysis of stool metabolites

Funding Support: This award

Name: Leigh Greathouse
Project Role: PI
Researcher Identifier (e.g. ORCID ID): 0000-0002-6855-8516
Nearest person month worked: 5

Contribution to Project: Dr. Greathouse coordinated IRB approval, managed study coordinators/enrollment, developed study collection methods/protocols, managed study team members, developed new methods for data analysis

Funding Support: This award

Name: Lucas Wong
Project Role: Co-PI (PI Mentor)
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 2

Contribution to Project: Dr. Wong coordinated IRB approval, managed study coordinators/enrollment, and managed study team members

Funding Support: This award

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report

What other organizations were involved as partners?

Organization Name: Mayo Clinic

Location of Organization: Rochester, MN

Partner's contribution to the project: Collaboration and in-kind support – provided CRC stool samples for testing; provided collaboration on study enrollment suggestions for improvement

Organization Name: GALT, General Automation Lab Technologies

Location of Organization: San Carlos, CA

Partner's contribution to the project: In-kind support – provided clinic isolates from CRC stool samples at no cost

Organization Name: Natera Inc.

Location of Organization: San Carlos, CA

Partner's contribution to the project: Collaboration and in-kind support – providing the Signatera test for all CRC patients enrolled in our study to monitor minimal residual disease; they will be providing whole exome sequencing of patient tumors at no cost; the purpose is to improve their predictor by identify circulating bacterial DNA that may enhance their predictive algorithm

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES:

We have included in the appendix the following:

- Letter from study Co-PI Dr. Lucas Wong
- 4 study questionnaires – quality of life, stool collection history, medical history, and bowel function
- Published manuscript

Dear Department of Defense in regard to Dr. Leigh Greathouse and award W81XWH-20-1-0570:

We have collaborated over the past year as a co-investigator in the COLON-MD grant, exploring the gut microbiome and colon cancer. This grant is critically important in the next step in our understanding the interactions between the microbiome, colon cancer, and future therapies for colon cancer.

Our institution has invested many hours and manpower in support of this grant, and we plan to execute the requirements and fulfill the goals of the grant as written. We believe in Dr. Greathouse's research and will continue to provide any support for its completion.

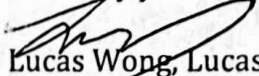
Having been in clear in our support, the execution of this project has been significantly impaired by barriers beyond all of our control. The COVID-19 pandemic has greatly impacted the number of colonoscopies performed due to patients' reluctance to have the procedure, lack of safety equipment for the procedure, and lack of medical resources diverted to the pandemic. We have had two major shutdowns this year and last year for elective procedures, which affected the overall number of colonoscopies during the lifetime of the grant. Meetings were conducted "virtually" which were productive but not as productive as face-to-face meetings.

Going forward, we have made major revisions in screening for patients, processing of samples, and communications with medical staff. Dr. Greathouse has participated regularly in our GI Tumor Boards and Rectal Tumor Boards. I have personally lobbied staff regarding the importance of the study and patient recruitment.

We feel confident we will see an increased number of patients enrolled in the study with our next study amendment. Screening logs show we are making the effort, but the end goal is patient samples which we will see increase significantly in the future.

If you have any questions, please reach out to me.

Sincerely yours,



Lucas Wong, Lucas M.D.
Director of Clinical Research
Division of Hematology/Oncology



EORTC QLQ – CR29

Patients sometimes report that they have the following symptoms or problems. Please indicate the extent to which you have experienced these symptoms or problems during the past week. Please answer by circling the number that best applies to you.

During the past week:	Not at All	A Little	Quite a Bit	Very Much
31. Did you urinate frequently during the day?	1	2	3	4
32. Did you urinate frequently during the night?	1	2	3	4
33. Have you had any unintentional release (leakage) of urine?	1	2	3	4
34. Did you have pain when you urinated?	1	2	3	4
35. Did you have abdominal pain?	1	2	3	4
36. Did you have pain in your buttocks/anal area/rectum?	1	2	3	4
37. Did you have a bloated feeling in your abdomen?	1	2	3	4
38. Have you had blood in your stools?	1	2	3	4
39. Have you had mucus in your stools?	1	2	3	4
40. Did you have a dry mouth?	1	2	3	4
41. Have you lost hair as a result of your treatment?	1	2	3	4
42. Have you had problems with your sense of taste?	1	2	3	4

During the past week:	Not at All	A Little	Quite a Bit	Very Much
43. Were you worried about your health in the future?	1	2	3	4
44. Have you worried about your weight?	1	2	3	4
45. Have you felt physically less attractive as a result of your disease or treatment?	1	2	3	4
46. Have you been feeling less feminine/masculine as a result of your disease or treatment?	1	2	3	4
47. Have you been dissatisfied with your body?	1	2	3	4
48. Do you have a stoma bag (colostomy/ileostomy)? (please circle the correct answer)	Yes		No	

Please go on to the next page

During the past week:

Not at All	A Little	Quite a Bit	Very Much
-----------------------	---------------------	------------------------	----------------------

Answer these questions ONLY IF YOU HAVE A STOMA BAG, if not please continue below:

49. Have you had unintentional release of gas/flatulence from your stoma bag?	1	2	3	4
50. Have you had leakage of stools from your stoma bag?	1	2	3	4
51. Have you had sore skin around your stoma?	1	2	3	4
52. Did frequent bag changes occur during the day?	1	2	3	4
53. Did frequent bag changes occur during the night?	1	2	3	4
54. Did you feel embarrassed because of your stoma?	1	2	3	4
55. Did you have problems caring for your stoma?	1	2	3	4

Answer these questions ONLY IF YOU DO NOT HAVE A STOMA BAG:

49. Have you had unintentional release of gas/flatulence from your back passage?	1	2	3	4
50. Have you had leakage of stools from your back passage?	1	2	3	4
51. Have you had sore skin around your anal area?	1	2	3	4
52. Did frequent bowel movements occur during the day?	1	2	3	4
53. Did frequent bowel movements occur during the night?	1	2	3	4
54. Did you feel embarrassed because of your bowel movement?	1	2	3	4

During the past 4 weeks:

Not at All	A Little	Quite a Bit	Very Much
-----------------------	---------------------	------------------------	----------------------

For men only:

56. To what extent were you interested in sex?	1	2	3	4
57. Did you have difficulty getting or maintaining an erection?	1	2	3	4

For women only:

58. To what extent were you interested in sex?	1	2	3	4
59. Did you have pain or discomfort during intercourse?	1	2	3	4



EORTC QLQ-C30 (version 3)

We are interested in some things about you and your health. Please answer all of the questions yourself by circling the number that best applies to you. There are no "right" or "wrong" answers. The information that you provide will remain strictly confidential.

Please fill in your initials:

--	--	--	--	--

Your birthdate (Day, Month, Year):

--	--	--	--	--	--	--	--	--	--

Today's date (Day, Month, Year):

31

--	--	--	--	--	--	--	--	--	--

	Not at All	A Little	Quite a Bit	Very Much
1. Do you have any trouble doing strenuous activities, like carrying a heavy shopping bag or a suitcase?	1	2	3	4
2. Do you have any trouble taking a <u>long</u> walk?	1	2	3	4
3. Do you have any trouble taking a <u>short</u> walk outside of the house?	1	2	3	4
4. Do you need to stay in bed or a chair during the day?	1	2	3	4
5. Do you need help with eating, dressing, washing yourself or using the toilet?	1	2	3	4

During the past week:

	Not at All	A Little	Quite a Bit	Very Much
6. Were you limited in doing either your work or other daily activities?	1	2	3	4
7. Were you limited in pursuing your hobbies or other leisure time activities?	1	2	3	4
8. Were you short of breath?	1	2	3	4
9. Have you had pain?	1	2	3	4
10. Did you need to rest?	1	2	3	4
11. Have you had trouble sleeping?	1	2	3	4
12. Have you felt weak?	1	2	3	4
13. Have you lacked appetite?	1	2	3	4
14. Have you felt nauseated?	1	2	3	4
15. Have you vomited?	1	2	3	4
16. Have you been constipated?	1	2	3	4

Please go on to the next page

During the past week:

	Not at All	A Little	Quite a Bit	Very Much
17. Have you had diarrhea?	1	2	3	4
18. Were you tired?	1	2	3	4
19. Did pain interfere with your daily activities?	1	2	3	4
20. Have you had difficulty in concentrating on things, like reading a newspaper or watching television?	1	2	3	4
21. Did you feel tense?	1	2	3	4
22. Did you worry?	1	2	3	4
23. Did you feel irritable?	1	2	3	4
24. Did you feel depressed?	1	2	3	4
25. Have you had difficulty remembering things?	1	2	3	4
26. Has your physical condition or medical treatment interfered with your <u>family</u> life?	1	2	3	4
27. Has your physical condition or medical treatment interfered with your <u>social</u> activities?	1	2	3	4
28. Has your physical condition or medical treatment caused you financial difficulties?	1	2	3	4

For the following questions please circle the number between 1 and 7 that best applies to you

29. How would you rate your overall health during the past week?

1 2 3 4 5 6 7

Very poor

Excellent

30. How would you rate your overall quality of life during the past week?

1 2 3 4 5 6 7

Very poor

Excellent

Stool Collection Log

Study Name

MCC#

Principal Investigator:

Co-Principal Investigator:

Co-Investigator:

Research Coordinator:

Study ID: _____

Fecal Collection Information:

Bowel movement date: / /
Month Day Year

Bowel movement time : AM/PM
Hour Minutes

Bristol Stool Chart:

Please select the Type that best approximates the consistency of your bowel movement today.

☐ Type 1	Separate hard lumps, like nuts (hard to pass)	
☐ Type 2	Sausage-shaped but lumpy	
☐ Type 3	Like a sausage but with cracks on its surface	
☐ Type 4	Like a sausage or snake, smooth and soft	
☐ Type 5	Soft blobs with clear-cut edges (passed easily)	
☐ Type 6	Fluffy pieces with ragged edges, a mushy stool	
☐ Type 7	Watery, no solid pieces (entirely liquid)	

Please turn over to answer the questions on the back of this page

Please answer the following questions and provide any additional information:

1. Have you had any antibiotics in the past 2 months?	☐ Yes ☐ No	If yes, specify how long ago and which antibiotic:
2. Do you currently take probiotics or prebiotics?	☐ Yes ☐ No	If yes, specify how long ago and which pro/prebiotics:
3. Do you consider yourself vegan, vegetarian, or pescatarian (fish only, no meat)?	☐ Yes ☐ No	If yes, specify which diet:

I.D. # __ - __ - __ __ __ __ __ __

COLON-MD QUESTIONNAIRE

Baylor University
One Baylor Place 97346
Waco, TX 76798-7346
Phone (254) 710-7619

Baylor Scott & White McClinton Cancer Center
150 Hillcrest Medical Blvd, Waco, TX 76712
Phone (254) 202-4000

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I.D. # ____ - ____ - _____

IDENTIFIER SHEET.....

1. Interviewer's name: _____ 2. Interviewer's ID ____

3. Hospital: _____

4. Date of interview: ____ / ____ / _____

5. Start time: ____:____ am/pm

6. Name _____ / _____ / _____
First Middle Last

7. Date of birth ____ / ____ / _____

8. Gender: () Male () Female

9. Address

Street Apt. No.

City State Zip Code - _____

10. Telephone number Home : (____) _____ - _____
Work: (____) _____ - _____ Ext. _____

11. What is the name; address and telephone number of a person who can help us contact you in the future or your next of kin?

Name Relationship to patient

Street Apt. No.

City State Zip Code - _____

Home telephone number (____) _____ - _____

SOCIO-ECONOMIC INFORMATION

Now I would like to ask you some general information about you.

1. What is your marital status?
☐ ₁ Single, never married
☐ ₂ Married
☐ ₃ Divorced
☐ ₄ Separated
☐ ₅ Has a partner, living as married
☐ ₆ Widowed

2. Do you consider yourself to be:
☐ ₁ White/Caucasian
☐ ₂ Black/African American
☐ ₃ Asian
☐ ₄ Native Hawaiian/Other Pacific Islander
☐ ₅ American Indian/Alaska Native

3. Do you consider your self Hispanic/Latino or Non Hispanic/Latino?
☐ ₁ Hispanic/Latino ☐ ₂ Non Hispanic/Latino

4. What is your age? ____ ____

I.D. # ____ - ____ - ____

TOBACCO HISTORY: GENERAL

Next, I would like to ask you some questions about any smoking history you may have.

1. Have you ever smoked more than 100 cigarettes, which is equivalent to five packs, in your life? ()₀ No **(Skip to next section)** ()₁ Yes

2. *Please tell me about your smoking history.*

Period	1	2
a. In what year did you start smoking cigarettes?	_____	_____
b. What was the average number of cigarettes or packs per day you smoked?	_____ () ₁ cigarettes () ₂ packs	_____ () ₁ cigarettes () ₂ packs

If R stopped smoking more than 6 months ago, Skip to 5

3. Do you currently smoke cigarettes? ()₀ No **(Skip to 5)** ()₁ Yes

4. How many cigarettes have you smoked in the last 48 hours? _____

5. Have you ever smoked at least one cigar a month for more than 6 months?
()₀ No ()₁ Yes

6. Have you ever smoked a pipe on a daily basis for more than 6 months?
()₀ No ()₁ Yes

6. Have you dipped or used chewing tobacco on a daily basis for more than 6 months?
()₀ No ()₁ Yes

TOBACCO HISTORY ()₁ Very good ()₂ Good ()₃ Fair ()₄ Poor

ALCOHOL HISTORY

Now, I would like to ask you some questions about any alcoholic beverages you may drink on a regular basis.

1. In your entire life, have you ever consumed more than 12 alcoholic beverages per year, such as beer, wine, wine coolers or liquor? ()₀ No **(Skip to 3)**
()₁ Yes

2. Tell me about the types of alcohol and when you were drinking them. **Continue to add additional columns as needed on tablet computer.**

Period	1
a. At what age did you first start to drink/when you next began to drink?	____
b. How many cans, bottles or 12 oz of beer did/do you drink?	____ () ₁ Per day () ₂ Per wk. () ₃ Per mo. () ₄ Per yr.
c. How many 4 oz glasses of wine did/do you drink?	____ () ₁ Per day () ₂ Per wk. () ₃ Per mo. () ₄ Per yr.
d. How many 1 ½ oz. shots of liquor, by itself or in a drink did/do you drink?	____ () ₁ Per day () ₂ Per wk. () ₃ Per mo. () ₄ Per yr.
g. What age did you stop drinking?	____

3. Have you had any alcoholic beverages such as beer, wine or liquor in the last 7 days?
()₀ No **(Skip to next section)** ()₁ Yes

4. In the last seven days, how much did you drink of the following?:	Number:
a. Cans, bottles or 12 oz. glass of beer	____
b. 4 oz. glasses of wine	____
c. 1 ½ oz. shots of hard liquor or drinks containing a shot of hard liquor	____

ALCOHOL HISTORY ()₁ Very good ()₂ Good ()₃ Fair ()₄ Poor

MEDICAL HISTORY: GENERAL

Now I would like to ask you some questions about your medical history and your health.

1. Have you ever been diagnosed with cancer (prior to your current diagnosis- if cancer case)?

()₀ No **(Skip to 3)** ()₁ Yes

2. What type of cancer(s)? _____ (cancer organ dictionary, add rows as needed)

3. What is your current weight? _____ lbs

4. What was your weight 10 years ago? _____ lbs

5. What was your weight 2 years ago? _____ lbs

6. How tall are you? _____ feet _____ inches

MEDICAL HISTORY: GENERAL ()₁ Very good ()₂ Good ()₃ Fair ()₄ Poor

I.D. # ____ - ____ - ____

MEDICAL HISTORY (I)

1. Please answer the following questions about pain relievers that you may have taken regularly during the past 3 months, at least 1 pill/week.

Have you taken the following regularly - at least 1/week during the past 3 months?	How many pills per day or week did you take regularly, during the past 3 months?	Did you take regularly one month prior to interview?
a. Antibiotics (Zithromax, Cipro, amoxicillin, doxycycline, metronidazole) ☐ ₀ no ☐ ₁ yes ☐ ₈ Don't know	_____ # pills per: ☐ ₁ day ☐ ₂ week ☐ ₈ Don't know	☐ ₀ no ☐ ₁ yes ☐ ₈ Don't know
b. Diabetic medications (metformin, Amaryl, Glucotrol, insulin) ☐ ₀ no ☐ ₁ yes ☐ ₈ Don't know	_____ # pills per: ☐ ₁ day ☐ ₂ week ☐ ₈ Don't know	☐ ₀ no ☐ ₁ yes ☐ ₈ Don't know

2.	Did a doctor ever tell you that you had?	Yes/No	How old were you when you were first diagnosed? DK = 888, condition at birth =000
a.	Crohn's disease	☐ ₀ No (Skip to 1b) ☐ ₁ Yes	____ _
b.	Ulcerative colitis	☐ ₀ No (Skip to 1c) ☐ ₁ Yes	____ _
c.	Irritable Bowel Disease	☐ ₀ No (Skip to 1d) ☐ ₁ Yes	____ _
d.	Colon polyps	☐ ₀ No (Skip to 1e) ☐ ₁ Yes	____ _
e.	Familial adenomatous polyposis	☐ ₀ No (Skip to 1f) ☐ ₁ Yes	____ _
f.	GI disease, other than cancer (specify)	☐ ₀ No (Skip to 1g) ☐ ₁ Yes	____ _
g.	Diabetes (check all that apply) ☐ Childhood ☐ Adult	☐ ₀ No ☐ ₁ Yes	____ _

MEDICAL HISTORY (I) ☐ ₁ Very good ☐ ₂ Good ☐ ₃ Fair ☐ ₄ Poor

3. Prior to diagnosis, have you lost more than 10% of your body weight without trying?
 ()₀ No ()₁ Yes

4. If Yes, how many pounds would you estimate you have lost?
 ()₀ <10 lbs ()₁ >10 lbs

5. Prior to diagnosis, did you change your diet significantly due to feeling unwell or sick?
 ()₀ No ()₁ Yes

FAMILY HISTORY: GENERAL

Now, I would like to learn about the members of your family.

1. Has anyone in your family that is related to you by blood, ever been told they have cancer, include children, parents, grandparents, brothers, sisters?

()₀ No ()₁ Yes

FAMILY HISTORY: GENERAL ()₁ Very good ()₂ Good ()₃ Fair ()₄ Poor

REPRODUCTIVE HISTORY (I) (If male skip to next section)

This next set of questions may seem personal, but remember that your answers are very important to us.

1. Have you ever been pregnant? ()₀ No **(Skip to 3)** ()₁ Yes
2. How many times have you been pregnant? ____
3. Has a doctor or other health professional ever told you that you had completed menopause or the change in life? ()₀ No ()₁ Yes
4. Have you ever used hormonal medications just before, during or after menopause, such as pills, vaginal creams, shots, suppositories or skin patches?
()₀ No **(Skip to next section)** ()₁ Yes

		At what age did you start to use them?	Total number of years used? 77= still using
a. Estrogen pills (Premarin, Estrace, Estratab, Ogen)	() ₀ No () ₁ Yes	____	____
b. Progesterone pills (Progestins, Provera, Megace)	() ₀ No () ₁ Yes	____	____
c. Estrogen and progesterone pills (Prempo)	() ₀ No () ₁ Yes	____	____
d. Estrogen and testosterone (Estratest)	() ₀ No () ₁ Yes	____	____
e. Estrogen vaginal cream	() ₀ No () ₁ Yes	____	____
f. Estrogen shots	() ₀ No () ₁ Yes	____	____
g. Estrogen skin patches (Estraderm)	() ₀ No () ₁ Yes	____	____
h. Estrogen patch and progesterone pills	() ₀ No () ₁ Yes	____	____
i. Suppository	() ₀ No () ₁ Yes	____	____
j. Other (Write in tablet computers- don't code)_____	() ₀ No () ₁ Yes	____	____

REPRODUCTIVE HISTORY (I) ()₁ Very good ()₂ Good ()₃ Fair ()₄ Poor

GENERAL INFORMATION

1. What was the highest level of education that you completed?
 - ()₁ Elementary School (5th or 6th grade)
 - ()₂ Middle or Junior High School (7th, 8th or 9th grade)
 - ()₃ 10th or 11th grade
 - ()₄ High School or GED (12th grade)
 - ()₅ Some College (includes AA degree)
 - ()₆ Technical School
 - ()₇ College
 - ()₈ Professional School (includes MS, PhD, MD, etc)
2. What is your current level of household income per year?
 - ()₁ Less than \$10,000
 - ()₂ \$10,000-29,999
 - ()₃ \$30,000-59,999
 - ()₄ \$60,000-90,000
 - ()₅ Greater than \$90,000
 - ()₈ Don't Know/Refused
4. Are you having any surgery in the near future?
 - ()₀ No **(Skip to 7)**
 - ()₁ Yes
5. What kind of surgery are you having? _____
6. When are you having this surgery? ____ / ____ / ____ - ____ - ____
7. May we contact you again later if we need to clarify any of the information you have provided.
 - ()₀ No
 - ()₁ Yes
8. Time ended: ____ : ____ ()₁ AM ()₂ PM
9. Interviewer's Signature: _____

First get specimen samples and then provide reimbursement of \$20.

☐☐☐ **Blood Specimen Collected** ☐☐☐☐ **Stool Specimen Collected**

INTERVIEWER REMARKS

1. Interview was conducted: ()₁ Home
 ()₂ Hospital - inpatient
 ()₃ Hospital - outpatient
 ()₄ One of the Study Offices
 ()₅ Other

2. Respondent's cooperation was:
 ()₁ Very good ()₂ Good ()₃ Fair ()₄ Poor

3. The overall quality of the interview was:
 ()₁ Very good ()₂ Good ()₃ Fair ()₄ Poor

4. Did any of the following occur during the interview?

a. R did not know enough information regarding the topics	() ₀ No	() ₁ Yes
b. R did not want to be more specific	() ₀ No	() ₁ Yes
c. R did not understand or speak English well	() ₀ No	() ₁ Yes
d. R was upset or depressed	() ₀ No	() ₁ Yes
e. R had poor hearing or speech	() ₀ No	() ₁ Yes
f. R was confused by frequent interruptions	() ₀ No	() ₁ Yes
g. R was emotionally unstable	() ₀ No	() ₁ Yes
h. Others helped with the answers	() ₀ No	() ₁ Yes
i. R required a lot of probing	() ₀ No	() ₁ Yes
j. Patient was reserved	() ₀ No	() ₁ Yes
k. R was physically ill	() ₀ No	() ₁ Yes
l. Other, specify _____	() ₀ No	() ₁ Yes

5. Comments/Remarks:

Bowel Function Index (BFI): Instructions for use

Instructions for study personnel on the administration of the BFI to study subjects

The Bowel Function Index (BFI) is a 3-item questionnaire to measure constipation from the patients perspective. Study personnel should ask subjects the BFI questions. The BFI is not intended to be given to the subject for completion on their own (self-administration), not even if study personnel explain how the measure should be completed. The BFI should always be administered to the subject by study personnel.

Instructions for administering each item of the BFI are indicated in the grey sections below each item.

Ask subjects each question. If the subject does not understand the question, study personnel may provide clarification as indicated below each question in the grey sections of the measure below. Study personnel should enter each answer provided by the subject in the appropriate section of the case record form (CRF). To avoid any form of response bias, study personnel must not lead the subjects in their answers (e.g. study personnel should not provide examples of answers to a given question).

Bowel Function Index (BFI)	
Please complete all items in this assessment	
1. Ease of defecation (NAS) during the last 7 days according to patient assessment:	
0 = easy / no difficulty 100 = severe difficulty	
Ask the subject: "During the last 7 days, how would you rate your ease of defecation on a scale from 0 to 100, where 0 = easy or no difficulty and 100 = severe difficulty?" If the subject requires clarification, ask: "During the last 7 days, how easy or difficulty was it to have a bowel movement on a scale from 0 to 100, where 0 = easy or no difficulty and 100 = severe difficulty?"	
2. Feeling of incomplete bowel evacuation (NAS) during the last 7 days according to patient assessment:	
0 = not at all 100 = very strong	
Ask the subject: "During the last 7 days, how would you rate your feeling of incomplete bowel evacuation on a scale from 0 to 100, where 0 = no feeling of incomplete evacuation and 100 = a very strong feeling of incomplete evacuation?" If the subject requires clarification, ask: "During the last 7 days, how strongly did you feel that you did not empty your bowels completely? Please indicate how strong this feeling was on a scale from 0 to 100, where 0 = not at all and 100 = very strong"	
3. Personal judgement of patient (NAS) regarding constipation during the last 7 days:	
0 = not at all 100 = very strong	
Ask the subject: "During the last 7 days, how would you rate your constipation on a scale from 0 to 100, where 0 = not at all and 100 = "very strong" If the subject requires clarification, ask: "During the last 7 days, how would you rate how constipated you felt on a scale from 0 to 100, where 0 = not at all and 100 = very strong"	

Review

Targeting Dietary and Microbial Tryptophan-Indole Metabolism as Therapeutic Approaches to Colon Cancer

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Abstract: Tryptophan metabolism, via the kynurenine (Kyn) pathway, and microbial transformation of tryptophan to indolic compounds are fundamental for host health; both of which are altered in colon carcinogenesis. Alterations in tryptophan metabolism begin early in colon carcinogenesis as an adaptive mechanism for the tumor to escape immune surveillance and metastasize. The microbial community is a key part of the tumor microenvironment and influences cancer initiation, promotion and treatment response. A growing awareness of the impact of the microbiome on tryptophan (Trp) metabolism in the context of carcinogenesis has prompted this review. We first compare the different metabolic pathways of Trp under normal cellular physiology to colon carcinogenesis, in both the host cells and the microbiome. Second, we review how the microbiome, specifically indoles, influence host tryptophan pathways under normal and oncogenic metabolism. We conclude by proposing several dietary, microbial and drug therapeutic modalities that can be utilized in combination to abrogate tumorigenesis.



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Keywords: tryptophan metabolism; kynurenine; indoleamine 2,3-dioxygenase; aryl hydrocarbon receptor; microbiome; indole; colon cancer

1. Introduction

Over a century ago (1901), English chemist Frederick Gowland Hopkins made a significant contribution to the field of nutrition by discovering the essential amino acid Tryptophan (Trp) from casein, a milk protein. As an essential amino acid, Trp can only be obtained through diet, mainly meat, dairy, and seeds. Trp is required for protein and niacin biosynthesis and is a precursor of serotonin and melatonin. Apart from these fundamental functions, Trp is appreciated for its influence on both host and microbial metabolism via two distinct pathways: the Kynurenine (Kyn) Pathway and Indolic Pathway. These pathways metabolize Trp into metabolic and neuroactive compounds that influence microbial composition and host physiology in an enzyme-dependent manner. Indole has a bicyclic ring formed by benzene and pyrrole groups. This indole structure is part of Trp and makes it unique in its structure and function. Indole can be kept intact or cleaved from the Trp structure, generating bioactive molecules [1]. Specific taxa among the microbiota also take advantage of this unique property of Trp to produce indoles for signaling and defense [2]. Microbial and dietary indoles can also trigger or suppress immune function thereby maintaining a symbiotic relationship with the host [3,4]. However, both Kyn and indolic processes can become dysfunctional in the pathogenesis of colon cancer.

In this review, we begin by describing the different metabolic pathways of Trp and their impact on the host physiology. We then discuss the alterations that occur in these pathways during colon cancer. Next, we introduce the significance of the microbial degradation of Trp in healthy conditions, along with the repercussions of microbial alterations in

carcinogenesis. We conclude by highlighting the gaps in knowledge regarding microbial-host Trp metabolism and propose dietary, microbial and drug targets that could have therapeutic applications.

2. The Four Main Pathways of Human Trp Metabolism

There are four known metabolic pathways that Trp can enter once in the upper gastrointestinal (GI) tract; Kyn pathway, serotonin pathway, indolic pathway (bacterial degradation) and tryptamine pathway [5]. These distinct pathways compete for the available free Trp pool and specifically convert Trp into Kyn, serotonin, indoles, and tryptamine as the end products, respectively [6]. About 90–95% [7,8] of Trp is converted into Kyn and downstream metabolites (Figure 1) via the Kyn pathway. These metabolites include L-kynurine acid, 3-hydroxykynurine, anthranilic acid, quinolinic acid, 3-hydroxyanthranilic acid, picolinic acid, most of which produce NAD⁺ and ATP in host cells, and play a vital role in inflammation, immune tolerance, and neurotransmission [5,9–11].

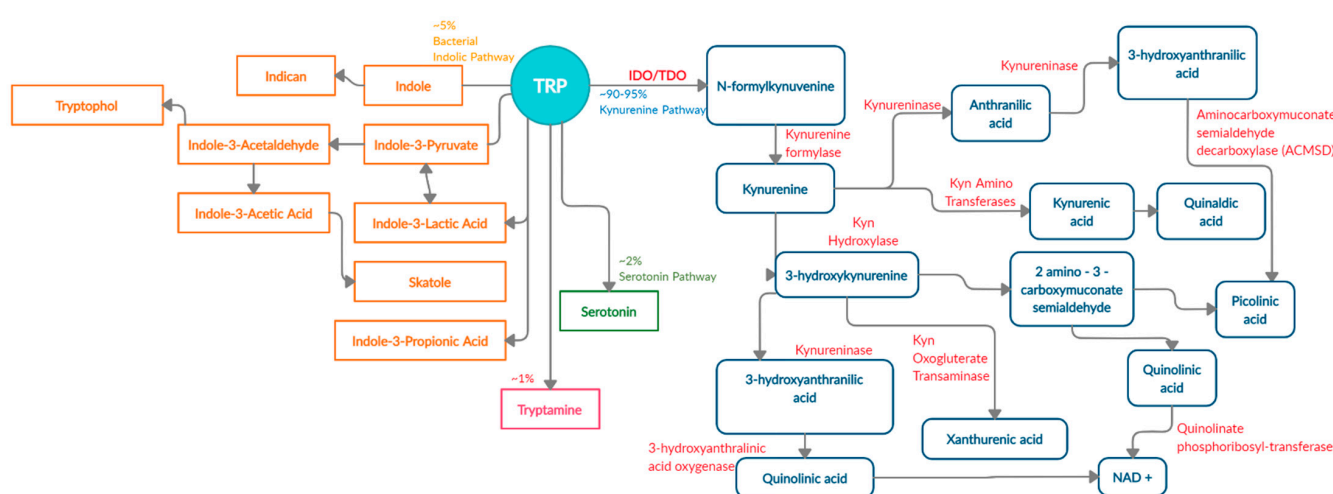


Figure 1. Schematic representation of enzymes and metabolites in the Trp-Kyn pathway.

About 4–6% of unabsorbed Trp then moves along the gastrointestinal tract and is metabolized by the microbiome into indole and indolic compounds including indole-3 pyruvate, indole-3-acetamide, indole-3-acetaldehyde, indole-3-acetic acid, indole-3-lactic acid, and indole-3-propionic acid [8,12–16]. These indolic compounds can bind to pregnane X receptors (PXR) and aryl hydrocarbon receptor (AhR) to promote intestinal homeostasis, enhance barrier function and tight junctions, reduce permeability, regulate intestinal immune tolerance [5,13,17,18]. Additionally, indoles can act as antioxidants and neuroprotective compounds [19,20]. Overall, indole production has an important impact on host health as a result of Trp metabolism by the microbiota [5].

In addition to Kyn pathway and microbial degradation, 1–2% of Trp can be converted into 5-hydroxytryptamine (serotonin) in the enterochromaffin cells of the intestinal mucosa. Serotonin is primarily involved in regulating gut motility, vasodilation, and maintenance of mood [21]. A minor contribution of Trp (about 1%) is towards the production of tryptamine, which can be initiated by both the host and microbiota [22]. Microbial tryptamine, a ligand for serotonin receptors, can also affect gut motility and transit time, given the abundance of such receptors in the GI tract [23]. Contribution of the gut microbiota towards serotonin and tryptamine production is an understudied field of research and will need further investigation in the context of carcinogenesis.

3. The Kynurenine Pathway Is the Major Tryptophan Degradation Pathway

The Kyn pathway (90–95% of ingested Trp) is the major Trp degradation pathway that produces Kyn and other neuroactive metabolites either in the liver or in the extrahepatic tissue. In addition to neuroactive metabolites, the Kyn pathway also regulates systemic Trp

levels, availability of Trp for serotonin synthesis, vitamin B3, and hepatic heme synthesis [9]. About 90% of Trp metabolized via the Kyn pathway takes place in the liver where all the enzymes for complete transformation of Trp into NAD⁺ are present. The hepatic Kyn pathway is mainly induced by signaling from glucocorticoids, as well as estrogen and glucagon [5,24,25]. The remaining 10% of the Trp transformation takes place via the Kyn pathway in the extrahepatic tissues, and is induced by cytokines (Interferon- γ (IFN- γ), Interleukin-1 (IL-1), Interleukin-6 (IL-6)), lipopolysaccharides, prostaglandins and amyloid peptides [7]. However, the metabolites produced in the extrahepatic tissues do not contain all the enzymes for complete conversion of Trp into NAD⁺. Availability, or lack thereof, of all enzymes to completely transform Trp determines the intermediates that are produced, ultimately affecting the functional outcome [9,26–28].

The array of metabolites produced via the Kyn pathway have led many researchers to investigate Trp and the role of these metabolically active metabolites in immune function, behavioral disorders including mood, anxiety and depression, inflammatory conditions, gut homeostasis, inflammatory bowel disease (IBD), neurodegenerative disorders such as Alzheimer's disease, and cancer. All these disease states appear to have an altered or amplified Kyn pathway activity [1,10,29–34]. Under inflammatory conditions, common to most of these disease states, Trp is shunted into the Kyn pathway, especially in the extrahepatic tissue (mostly immune cells), while the hepatic pathway activity is reduced [9,25,35]. This shunting of Trp into Kyn pathway, reduces Trp availability for other pathways especially serotonin and indolic pathways; the two pathways which have shown to be beneficial for host health in the presence of Trp [34,36,37]. Furthermore, reduced Trp and increased Kyn, alters the activation and balance of innate and adaptive immune cells towards a tolerogenic milieu [37]. Therefore, Kyn pathway activity is amplified under inflammatory conditions, in part due to an NAD⁺ requirement for increased energy demands in the immune cells and to implement immunomodulatory functions [35].

3.1. Kynurenine Pathway and the First-Rate Limiting Enzyme IDO1

The first and rate-limiting enzymes in Trp catabolism are Tryptophan 2,3-dioxygenase (TDO) and Indoleamine 2,3-dioxygenase (IDO1), which are present in the liver and in the extrahepatic tissues, respectively. TDO regulates systemic levels of Trp. Under normal physiological conditions, the majority of Trp is metabolized in the liver to produce Kyn, C-reactive proteins, haptoglobin, and fibrinogen [25]. IDO1 is expressed by various different tissue and cells types including lungs, placenta, GI tract, immune cells (e.g., monocytes, macrophages, dendritic cells, antigen-presenting cells) and epithelial cells. The function of IDO1 in extrahepatic cells is the same, which is the conversion of Trp into Kyn [38].

Under normal conditions, IDO1 expression regulates T cell proliferation to prevent tissue damage and reduce oxidative stress [25]. During inflammation, the expression of TDO is reduced and IDO1 expression is increased. Once IDO1 is activated upon signaling from cytokines such as IFN- γ , TNF α , prostaglandins and lipopolysaccharides, it converts tryptophan into N-formylkynurenine, followed by a rapid transformation into Kyn, the first stable catabolite in the pathway [5,39,40]. Kyn is then acted upon by various enzymes in a tissue-dependent manner and produces downstream neuroactive and immunoactive metabolites which regulate immune cell activity [41].

Extrahepatic Kyn production governs immune homeostasis [42]. This action occurs by reduction of activated T-cells, dendritic cells and, natural killer cells and induction of Th1 cell apoptosis to control excessive inflammation [11,40]. Each downstream Kyn metabolites perform specific functions. Kynurenic acid elicits an anti-inflammatory response through its anti-oxidant properties, while picolinic acid exhibits anti-tumor activity by suppressing T-cell and c-Myc activation [11,43–45]. Furthermore, 3-hydroxyanthralinic acid and quino-
linic acid can act as neurotoxins in certain disease states including chronic brain injury, osteoporosis, coronary heart disease, Huntington's disease, stroke, depression, and colon cancer [40,46,47]. Collectively, these downstream metabolites modulate immune home-

ostasis in part by activating the ligand-dependent transcription factor aryl hydrocarbon receptor (AhR).

3.2. Kynurenine Pathway and AhR Activation

AhR is a transcription factor that belongs to the family of basic helix-loop-helix transcription factors that control genes containing xenobiotic response elements (XREs), as well as through non-XRE response elements; estrogen receptor and retinoic acid receptors [48]. In an inactive state, AhR is bound to chaperone proteins. Upon ligand binding, the chaperone protein disassociates with the complex and cytoplasmic AhR translocate into the nucleus heterodimerizing with AhR nuclear translocator (ARNT), leading to transcription of CYP1A1, CYP1B1, IDO1, TDO, IL-22, GSTA and AhRR (Table 1) [49,50]. Several of these genes control metabolism of xenobiotic and environmental chemicals including dioxins, polycyclic aromatic hydrocarbons, benzathracens and halogenated aromatic hydrocarbons through regulation of cytochrome P-450 [51–55]. Furthermore, AhR can interact with additional transcription factors, including pro-inflammatory nuclear factor- κ B, and epigenetic regulators, as well as, acting as an E3 ubiquitin ligase. In the intestinal epithelial cells, AhR works to control immune homeostasis, epithelial barrier function, and symbiosis with the microbiota [48].

Table 1. Classes of AhR ligands.

Class of Molecule	Metabolites	Genes Activated	Effects (Ligand Dependent)
Tryptophan Metabolites	Kynurenine		
	Kynurenic Acid		
	Xanthurenic Acid		
	Anthralinic Acid		
	Quinolinic Acid		
	Pinolinic Acid		
	Cinnabarinic Acid		
Dioxins	3-hydroxykynurenine		Neurotoxic Effects
	3-hydroxyanthralinic acid		B-cell & T-cell differentiation
	Polycyclic Aromatic hydrocarbons		Development of
	Benzathracenes	CYP1A1/CYP1B1	intraepithelial lymphocytes
	Halogenated aromatic hydrocarbons	AHRR	Immune tolerance
Dietary compounds	3-3-diindolemethane (DIM)	IL-6	Enhanced epithelial barrier function
	Indole-3-carbinol	VEGFA	Anti-inflammatory
	Indole-3-acetonitrile	PTGS2	Intestinal homeostasis
	Indole [3,2-b] carbazole	IL-22	Gut motility
	2-(indole-3-ylmethyl)-3,3-diindolymethane	IDO1	Microbial composition
	Herbs- Ginseng, ginkgo biloba, licorice	TDO	Antimicrobial affects
	Indole		Mucosal barrier function
Microbial metabolites	3-hydroxyindole		Serotonin modulation
	Indolealdehyde		
	Tryptamine		
	Indole acetic acid		
	Tryptanthrin		
	3-methyl indole		
	Indirubin		
Photo-oxidative Trp metabolite	Indigo		
	Indole sulphate		
	Malassezin		
	6-formylindole [3,2-b] Carbazole		
	(FICZ)		

AhR activation is a crucial mediator for intestinal immunity and intestinal homeostasis through the development and regulation of intraepithelial lymphocytes and innate lymphoid cells [13,56–60]. AhR activation can be induced by distinct classes of endogenous ligands including Trp metabolites, dietary compounds, microbial Trp metabolites, bilirubin, arachidonic acid, prostaglandins, and cytokines (Table 1). Depending upon the ligand, AhR activation can result in different molecular and physiological responses including histone modification, lipid synthesis, energy, xenobiotic metabolism, immune function, epithelial barrier function and cell migration [56–61]. Therefore, AhR is involved

in multiple pathways that regulate endogenous and exogenous signals that controls the immune response.

Several reports have shown the importance of AhR ligands in modulating gastrointestinal homeostasis via mucosal immune cells. However, the exact mechanism of this modulation is unclear due to the multitude of different ligands and transcriptional factors involved in controlling physiological effects [50]. AhR-modulated intestinal homeostasis can be achieved in one of two ways 1) it can exert an anti-inflammatory response through the activation of Tregs; 2) it can enhance intestinal mucosal integrity through the activation of Th17 cells and induction of IL-22 [13,50,62,63]. Therefore, AhR activation is critical in maintaining intestinal homeostasis. Thus, disruption in AhR expression or activity can lead to altered intestinal homeostasis and carcinogenesis.

4. Contribution of Host Trp Metabolism in Colon Carcinogenesis

The alterations in Trp metabolism begin early on in colon carcinogenesis and permits immune evasion in tumor microenvironment (Figure 2). Recently, the oncogene c-MYC was shown to contribute to this immune evasion through alteration of Trp metabolism. Specifically, c-MYC accelerated Trp uptake by upregulating Trp transporters SLC7A5 and SLC1A5 in colon cancer cells and tissues [43]. The study also showed an increase in the cytoplasmic levels of IDO1, arylformamidase (hydrolyzes N-formyl-L-Kyn to L-Kyn), and Kyn, which drive T cell inactivation and increase protein synthesis, in pathogenesis of colorectal cancer (CRC). Other studies have also demonstrated increased levels of Kyn in colon cancer patients when compared to healthy individuals [11]. Interestingly, the levels of enzymes involved in further degradation of Kyn into downstream metabolites were not elevated, suggesting Kyn to be the predominant metabolite elevated in CRC [43]. Our data (unpublished) also indicate an alteration in Trp metabolism in the microbiome of individuals with CRC compared to healthy individuals, which creates additional complexity in the relationship with the host Kyn pathway. Thus, understanding the contribution of the microbial Trp-Kyn pathway to colon carcinogenesis will be important for future drug discovery and treatment.

Excessive Kyn levels in the tumor microenvironment is one of the adaptive mechanism for the tumors to escape immune surveillance and metastasize via T-cell inactivation (Figure 3) [11,64,65]. Kyn drives tumor escape by acting as a ligand and constitutively activating AhR to express genes involved in cell-growth. Collectively, the alterations in Trp transporters and degrading enzymes leads to an increased Kyn pool within the tumor microenvironment. The excessive Kyn levels aid in tumorigenesis in two ways; 1), a portion of the Kyn produced can directly induce T-cell inactivation and apoptosis leading to immune evasion; 2) the remaining Kyn can constitutively activate AhR, transcribing genes for not only tumor escape but also for proliferation and metastasis [26,43,53,66,67]. Thus, the Kyn-AhR signaling pathway is one of the major contributors to colon cancer [33,34,36,38,40,43,57].

Over stimulation of the Kyn pathway can also be induced by stress and inflammation [68]. Recent evidence indicates that pro-inflammatory cytokines, including IFN- γ , can induce IDO1 gene expression due to presence of IFN response element. Increased IDO1 expression, especially in immune cells can enhance the activity of Tregs and suppress effector T-cells leading to immune tolerance [69]. Most individuals with cancer, including hepatocellular carcinoma, colon cancer, kidney cancer lung cancer among other, often exhibit increased Kyn levels and decreased serum Trp levels, or increased kyn/Trp ratio, which in part is explained by excessive inflammatory signaling molecules [11,70–72]. Evidence of this increased kyn/Trp ratio was recently shown in a study of individuals with colon cancer. Fecal metabolites from these patients demonstrated both an increased kyn/Trp ration, but also a decreased indole/Trp ratio (Venkateswaran & Conacci-Sorrell, 2020), suggesting loss of either indole producing microbes and/or an increase in microbes producing kynurenine metabolites. Indeed, *Pseudomonas aeruginosa* produces kynurenic acid and 3-OH-Kyn, which may disrupt the Trp metabolism of the host, and thus the

immune response [73]. Additionally, administration with PEGylated kynureninase, a therapeutic enzyme, has shown to inhibit tumor growth by degradation of Kyn into non-toxic metabolites [74]. Therefore, it is becoming widely known, but not completely understood, that alterations in Trp metabolism is one of the key players in overexpression of IDO1 and Kyn. This alteration further leads to constitutive activation of AhR, perpetuating cell-growth. However, what is less well known is how the gut microbiota and their metabolites contribute to IDO1 expression, AhR activation, and immune cell balance. Though recently, the short chain fatty acid butyrate, produced by several commensal bacteria, was shown to decrease expression of IDO1 through reduction of STAT1 and its histone deacetylases inhibitory activity, indicating that bacterial metabolites are able to control Trp metabolism [75].

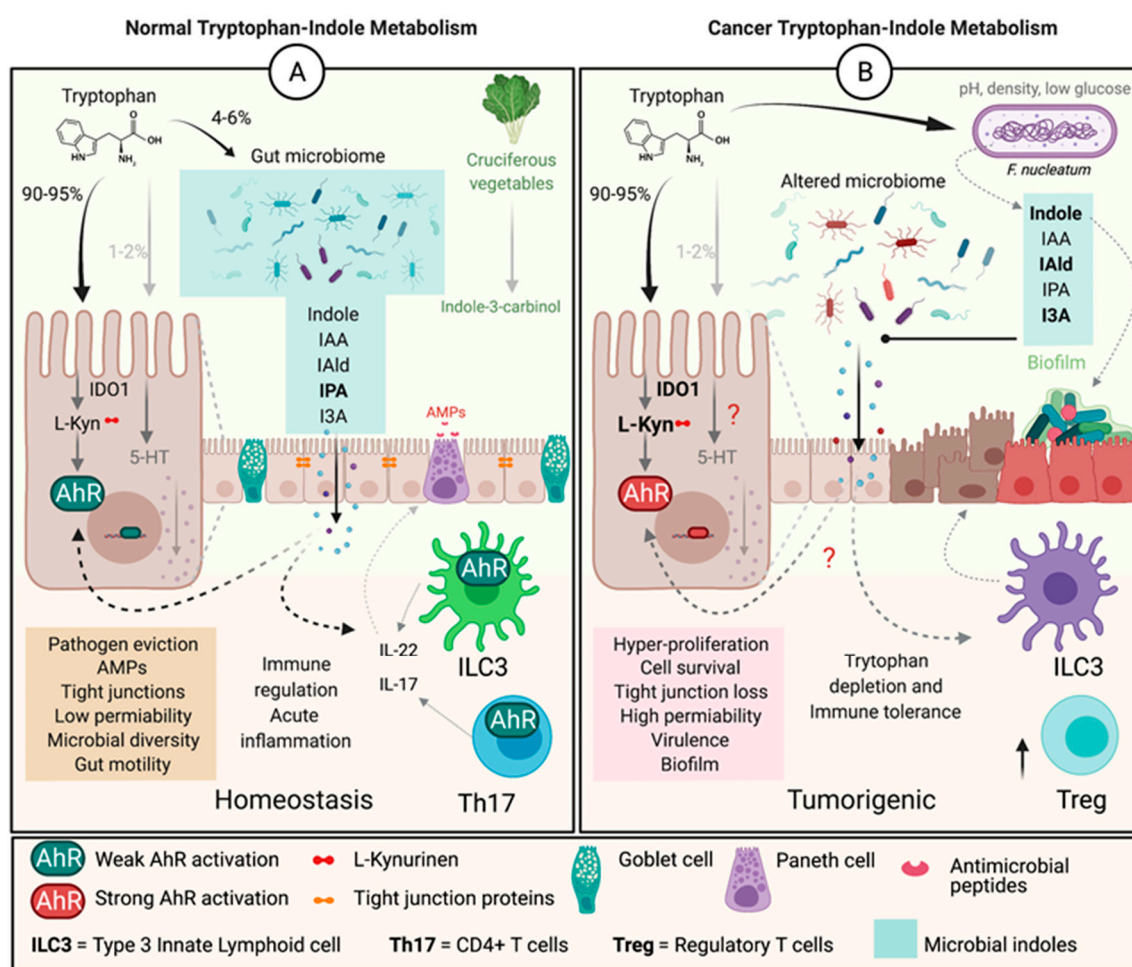


Figure 2. Tryptophan-indole metabolism in normal vs. colon carcinogenesis. (A) Under normal physiological conditions, Trp is mainly metabolised by hepatic or extrahepatic cells by IDO1 into L-Kyn, with the rest being metabolised by bacteria or converted to 5-hydroxytryptamine or serotonin. The bacteria convert Trp to indoles, and together with dietary indoles, IDO1 and L-Kyn are ligands for AhR. Indoles also regulate the production of cytokines in by ILC3 and Th17 cells, which result in production of AMPs and improved barrier function to regulate inflammation and maintain homeostasis. (B) In a tumorigenic environment IDO1 is overexpressed and Trp is depleted driving L-Kyn levels higher and constitutively activating AhR. High L-Kyn leads to potentially stronger AhR activation and overproduction of IL-22/IL-17 and hyperproliferation, along with activation of Treg cells creating an immunotolerant environment permissive for tumor growth. It is unclear (?) how microbial indolic metabolites control epithelial or immune cell metabolism in the context of colon cancer.

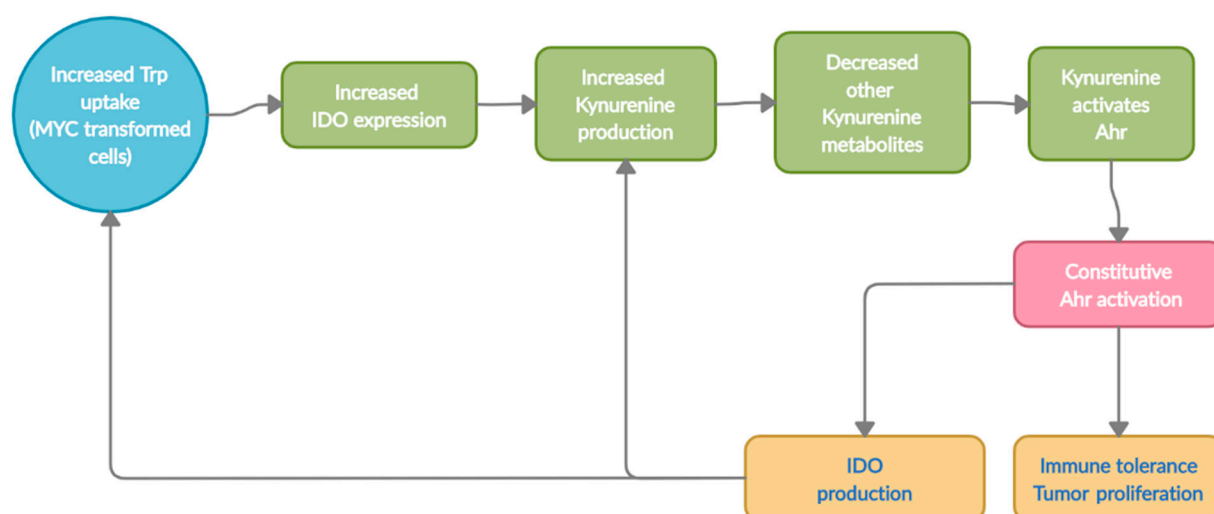


Figure 3. Sequential representation of Trp metabolism in Tumors (host cells).

The versatility of AhR binding has increased the research interest in its ability to regulate the immune system, intestinal homeostasis, and cancer progression. Under normal conditions, AhR activation results in transcription of genes, CYP1A1, CYP1B1 and AhRR (Aryl-Hydrocarbon Receptor Repressor), that control AhR activity in negative feedback loop. However, dysregulation of this feedback loop allows uncontrolled AhR activation, which is correlated with inflammatory processes including IBD, multiple sclerosis, cardiovascular conditions, and allergic responses, and carcinogenesis [76]. The multifaceted role of AhR in carcinogenesis is complex and only beginning to be understood. This, in part, is due to the ability of AhR to bind to many different ligand types, resulting in either enhancing or suppressing carcinogenesis [11,77]. Loss of AhR has been shown to enhance colon carcinogenesis, while re-establishing normal AhR activation inhibits the formation of polyps [48,62]. Reduced AhR expression has been seen in individuals with IBD, colitis, Crohn's disease and CRC [78]. Alterations in Trp metabolism in part explain the reduced AhR availability; a hallmark of inflammatory disease states including CRC. Indeed, Crotti et al. demonstrated that alterations in Trp metabolism occur early in the pathogenesis of colon cancer preceding malignancy [36]. Specifically, it is understood that colon epithelial cells undergo changes in Trp metabolism within the tumor microenvironment as an adaptive mechanism to escape immune surveillance. While under normal physiology the AhR ligands exhibit a protective role by either inducing or by suppressing inflammation, in the context of carcinogenesis, loss of AhR or constitutive activation abrogates this protective function. Therefore, the metabolic end products of the Kyn pathway control the inflammatory process, in part through activation of AhR [36,79–84]. However, the mechanism that leads to these alterations in Trp metabolism and loss or dysfunctional AhR activity in colon carcinogenesis is not well understood.

5. Contribution of the Microbiota in Trp Metabolism

The gut microbiome forms a symbiotic relationship which affects host physiology in several ways. These effects include maintaining gut homeostasis, activating, or suppressing immune function, metabolic and neurological homeostasis. Microbial communities promote host health not only by producing vitamins, degrading bile, and oxalates, but also by synthesizing anti-inflammatory metabolites. These bacterial metabolites can act as neurotransmitters, signaling and secretory molecules, immunoregulators, neurotoxic and antimicrobial molecules directly affecting host physiology. One such metabolite group are indoles, first discovered and isolated by a German scientist Adolf Von Baeyers in 1866 in an indigo reduction process [85]. Indoles are produced through bacterial degradation of dietary Trp by the action of bacterial enzyme tryptophanase (TnaA) or degradation of phenylalanine by phenyllacetate dehydratase (fldAIBC), though other undiscovered

pathways may be involved [86–89]. Over 85 bacterial species express TnaA, with some *Peptostreptococcus* and *Clostridium* species containing fldAIBC gene clusters [2,85,88]. Multiple types of indole derivatives can be produced from Trp due to expression of species-specific enzymes. The most prominent bacterial indoles include indole-3-acetamide, indole-3-acetaldehyde, indole-3-pyruvate, indole-3-aldehyde, indole-3-acetic acid, tryptamine, indole-3-propionic acid, and indole acrylic acid. Indole and indole derivatives play an important signaling role between the host and the microbiota and are a critical component of bacterial function. Within the microbial communities in the gut, indole production affects spore formation, plasmid stability, biofilm formation, antibiotic tolerance, cell division, and virulence [13]. Within the host, indoles activate signaling pathways that result in changes in intestinal epithelial barrier function, reduce permeability, promote immune tolerance, evict pathogens, reduce inflammation, and, control mucin production [12,85,90–95]. Colonization of germ-free mice for 4 weeks with *C. sporogenes* or a mutant (fldC) lacking the an enzyme necessary for indole (indole-3-propionic acid (IPA)) production, resulted in serum concentrations of 80 μ M in mice colonized with wild-type but undetectable levels in mice colonized with the mutant strain. Further, colonization with the fldC mutant increased gut permeability, and lead to higher levels of circulating neutrophils, T cells (CD4+ and CD8+) and sIgA (lumen) [86]. Together, these results indicate that microbiota are capable of producing biologically relevant levels of indoles, and inducing changes in innate and adaptive immune response that may be important in colon cancer pathogenesis.

Indolic compounds can also act as AhR ligands to promote both pro- and anti-inflammatory effects [17,96]. Indoles can reduce expression of pro-inflammatory signals, IL-8 and NF- κ B, and promote expression of anti-inflammatory cytokines including IL-10 [95]. In addition, indolic compounds regulate intestinal homeostasis through induction of IL-22, which improves barrier function, however, in the context of cancer (later stages) IL-22 production can promote tumor progression [97,98]. Microbial indole metabolites are normally a consistent source of host AhR ligands, unlike dietary sources that are transient [99]. The majority of indoles and indole derivatives have demonstrated a protective effect against inflammatory diseases including CRC, with the exception of indoxyl sulfate, a uremic toxin involved in chronic kidney and vascular diseases [100]. Tight epithelial junctions, adequate mucin production and anti-microbial defenses are indispensable for a healthy host physiology; functions all governed via AhR activation by indoles [4,19,90]. Though a more thorough investigation of microbial indoles in early vs. later stages of colon carcinogenesis is warranted given the complex effects on downstream signaling pathways the can both suppress and promote carcinogenesis.

Multiple species of bacteria can produce AhR ligands. *Lactobacillus reuteri* and *acidophilus* produce 3IAld, which stimulates innate lymphoid cells to release AMP's to defend against pathogens [57]. *Clostridium Sporogenes* can produce IPA, inhibiting the action of pro-inflammatory signaling molecules, NF- κ B and TNF- α , and free radicals [94]. *Clostridium sporogenes*, *Peptostreptococcus russellii*, *Peptostreptococcus anaerobius*, and *Peptostreptococcus stomatis* can produce indoleacrylic acid (IA), shown to attenuate inflammatory response and improve barrier function by mucus production and goblet cell differentiation, potentially via AhR activation [88,101]. *Bacteroides ovatus*, *Bacteroides fragilis*, *Bifidobacterium pseudolongum*, *Clostridium bartlettii*, *Clostridium difficile*, and *Clostridium lituseburense*, among others can produce IA which aids in strengthening intestinal mucosal integrity, possible by activating AhR, and production of IL-22, by mechanisms not well understood [101,102]. Besides indolic ligands, bacteria are also capable of producing non-indole AhR ligands. *Pseudomonas aeruginosa* and mycobacterium tuberculosis are capable of activating AhR by releasing virulence factors including phenazines and phthiocol resulting in pathogen clearance [99,103]. Therefore, bacterial contribution to the pool of AhR ligands should not be overlooked. The mechanistic explanation to the microbial contribution may be limited, but the functional outcome of this contribution is significant, and is evident in the attention the field has received in the recent years.

6. Alterations in Microbial Trp Metabolism in Colon Carcinogenesis

As a result of disturbances in host physiology and microbial composition during carcinogenesis, the commensals can take on a pathogenic behavior resulting in an increased inflammation in the host as well as biofilm formation [104–106]. About 16–20% of all cancers are the result of pathogenic infections [107]. Patients with Crohn's disease harbor pathogenic *Escherichia coli* that contribute to a pro-inflammatory state [108]. This pathogenic behavior can directly or indirectly influence changes in host IDO1 expression and cell proliferation [109]. Increases in *Enterococcus faecalis* and *Escherichia coli* enhance the production of intestinal inflammatory signaling molecules, IFN- γ and IL-4, capable of inducing increased expression of IDO1 and altering Trp metabolism [99,110,111]. Another species associated with CRC and IBD, is *Fusobacterium nucleatum*. *F. nucleatum* species isolated from patients with IBD are more invasive and more frequently found in adenomas and adenocarcinomas of the colon [112,113]. Further *F. nucleatum* elicits substantially greater amounts of pro-inflammatory TNF- α gene expression, which can lead to increased IDO1 activation [99,106,114,115]. Both *F. Nucleatum* and *Peptostreptococcus anaerobius* can attach to the cancer cells through cell surface attachment proteins, activating the PI3k-Akt pathway leading to cell proliferation [116,117]. Intriguingly, both of the species can also produce high levels of indoles from Trp metabolism. Therefore, the microbiome is a key contributor to inflammation through altering Trp metabolism [11] which is an important contributing step in colon carcinogenesis.

Changes in microbial indole production is also a characteristic of colon cancer. A reduction in indolic pathway activity was observed in the fecal samples of individuals with CRC. The study demonstrated a lower indole/Trp ratio in CRC when compared to healthy individuals, as well as a higher kyn/Trp ratio [109]. This alteration in the indolic pathway can lead to an increased inflammatory response via TNF α , IL-1 β , and IL-6 in colon carcinogenesis, subsequently altering AhR activity [109]. AhR activation is reduced in diseases including IBD, liver disease, metabolic syndrome, autoimmune disease, and cancer, suggesting that alteration in Trp metabolism or indole production may contribute to AhR dysfunction [39,54,55,79]. Fecal samples from individuals with metabolic syndrome, obesity, Type-2 diabetes, and chronic intestinal inflammation also have significantly lower levels of microbial Trp metabolites, potentially contributing to reduced AhR activation. Specifically, individuals with obesity have a significantly higher concentration of Kyn and IDO1 in adipose tissue, which is associated with an imbalance in Th17/Treg cells; suggesting a mechanistic link between altered tryptophan metabolism, obesity and CRC pathogenesis [52,118]. Additionally, administration of microbial indolic metabolites, diindolylmethane or I3C (AhR ligands), reduces tumor formation in *Apc*^{Min/+}*AhR*^{+/+} and *Apc*^{Min/+}*AhR*^{+/-} mice indicating that specific microbial indoles are protective against carcinogenesis [119]. Treatment with 6-Formylindolo [3,2-b] carbazole (FICZ), a Trp derived AhR ligand, is also effective at restoring intestinal integrity [52]. Intriguingly, *Lactobacillus reuteri* is capable of differentiating T cells into Tregs through AhR activation via indolic compounds, implying additional mechanism to reestablish epithelial barrier function [120]. Similarly, restoration of AhR activation, using *L. reuteri*, reestablished AhR activity in murine model of impaired metabolic syndrome by increasing AhR ligand availability [52]. Collectively, it is implied that a disrupted microbial Trp-indole-AhR pathway significantly contributes to the pathogenesis of CRC. Therefore, identifying the effects of bacterial indoles in the pathogenesis of colon carcinogenesis is important in understanding the impact of the microbiome and potentially developing therapeutics.

7. The Contribution of Serotonin (5-HT) in the CRC Microenvironment

Apart from the Kyn and the indolic pathway, 1–2% of Trp can undergo hydroxylation to produce serotonin via the serotonin pathway. As a potent signaling molecule, having 14 classes of receptors, serotonin exhibit diverse effects on the immune function, nervous and endocrine system, psychological processes, blood clotting, metabolic homeostasis, bone metabolism, hematopoiesis, and epigenetic control [121,122]. While serotonin is well

recognized as a neurotransmitter of the brain, about 95% of the serotonin is synthesized and released by the enterochromaffin cells of the intestines, the largest neuroendocrine organ. With majority of serotonin produced in the gut, it plays an important role in maintaining normal gut function, including gut motility, absorption, and vasodilation [121,123]. Therefore, the role of serotonin in a healthy physiology is indispensable.

While serotonin is essential in healthy physiology, studies have demonstrated both protective and detrimental role of serotonin in colon carcinogenesis [124], where a detrimental role is taken over in response to an altered or impaired serotonin activity [125]. Serotonin exhibits both pro- and anti-inflammatory signaling activity in the intestines via activation of 5-HT₇ and 5-HT₄ receptors, respectively [121]. Studies have also confirmed the role of serotonin in tumor growth, cell invasion, angiogenesis, and metastasis in CRC [126–128]. A β -catenin mutation (CtnnB1) occurring during early phases of stem cell differentiation into enteroendocrine cells, has shown to induce tumors expressing serotonin [127,129]. Activation of serotonin receptors, 5-HT_{1B} and 5-HT_{2B} stimulate tumor angiogenesis and cell proliferation [130,131]. Receptor 5-HT_{1D}, by activating Axin1/ β -catenin/MMP-7 pathway, also promote tumor cell invasion. The same study demonstrated the inhibition of tumor metastasis by administering 5-HT_{1D} antagonist, GR127935, in a mouse model [125]. Early interventions with Mirtazapine, inhibitor of serotonin 2 C receptor (HTR_{2C}), has shown to reduce tumor growth and prolong survival rate in tumor-bearing mice. This reduction in tumor growth was due to activation of immune response and restoration of serotonin levels [132]. The above evidence supports the dual role of serotonin in tumor prevention and proliferation, due in part to altered serotonin pathway, mutations, and inflammation.

The microbiota plays a significant role in regulating serotonin levels, synthesized and released by the enterochromaffin cells of the intestines [121]. Short chain fatty acids produced by the microbiota in response to saccharides in the diet, stimulate the enterochromaffin cells to produce and release serotonin [133]. Spore-forming bacterial have shown to produce metabolites that promote serotonin synthesis, mechanistically unknown. This microbiota-induced serotonin production can affect gut motility and intestinal homeostasis, suggesting a therapeutic intervention [134]. Several microbial species including *Corynebacterium* spp., *Escherichia coli*, and *Streptococcus* spp., can also produce serotonin [134]. Serotonin-like molecules are also secreted by *Rhodospirillum rubrum*, *Bacillus cereus*, *Enterococcus faecalis*, and *Staphylococcus aureus* [135]. Collectively, the intestinal and microbial serotonin production is involved in several functions including gut motility, platelet function, and immune response under normal physiological conditions [134,136]. However, serotonin uptake and production can become dysregulated in pathogenesis of several diseases including colon cancer [124,134].

Serotonin is essential in protecting against cellular damage and disease development. Serotonin activity protects the intestines from DNA damage [21,127] and induces signaling (HTR₄ receptor; effect-gut motility) that aids in reducing the development of colitis and controlling the development of early CRC [137]. Under the conditions of immune homeostasis and a diverse microbiota, serotonin exerts a protective role [133,134,138]. A disruption in the balance of the immune system and the microbial composition allows serotonin to instead promote cell proliferation and angiogenesis [124,127,128]. Specifically decreased serotonin activity is associated with DNA damage, apoptosis, and constricted arterioles in CRC leading to a promotion of carcinogenesis [127,139,140]. A study comparing the metabolomic profile of cancer and normal tissues demonstrated that Trp metabolites; serotonin and 5-Hydroxy-3-indoleacetic acid [5-HIAA]) were only detected in normal tissue and absent in tumor tissue, indicating that the tumor microenvironment was actively metabolizing serotonin [141]. Collectively, the following evidence demonstrates the dual role of serotonin in promoting intestinal homeostasis and accelerating carcinogenesis.

8. The Contribution of Tryptamine in CRC Microenvironment

Decarboxylation of Trp leads to production of tryptamine, a monoamine alkaloid with an indole ring in its structure [142]. Tryptamine is mainly found in the central nervous

system, but can also be produced by bacterial, fungi, and plants [143]. Under normal physiology, tryptamine acts as a neurotransmitter, vasoconstrictor and vasodilator, anti-oxidant and antibacterial agent [144]. Monoamine oxidase (MAO)-mediated tryptamine metabolism also provides an endogenous source of AhR ligand, thereby inducing CYP1A1 expression [143]. Tryptamine can also induce the enterochromaffin cells to release serotonin, fluctuations of which have shown to play a significant role in pathology of inflammatory disease, a risk factor for colon carcinogenesis [145].

Tryptamine is also produced by the microbiome, which aids in colonic fluid secretion via activation of serotonin receptor HT-4, subsequently affecting colonic transit in a mouse model. Mechanism by which tryptamine increases colonic fluid secretion remains unclear, due in part to the structural similarities of tryptamine to serotonin and ligand-binding ability of tryptamine to serotonin receptors [23]. Given that ~50% of all patients undergoing chemotherapy for colon cancer experience chemotherapy-induced diarrhea, understanding the impact of microbial tryptamine production would be important to abrogate this serious side effect. Specific bacteria have been identified that can perform decarboxylation of Trp into tryptamine, a common practice in plants, but rare among the bacterial species [146]. These bacteria include *Xenorhabdus nematophilus* and *Bacillus atrophaeus*. *Lactobacillus bulgaricus* can also excrete tryptamine [147,148]. Therefore, engineered tryptamine producing bacteria may be a potential therapeutic intervention to increase serotonin levels and AhR ligand availability, but may have negative effects in the context of colon carcinogenesis and treatment.

9. Tryptophan Metabolism and Bacterial Indoles as Therapeutic Targets

Both host and bacterial-Trp metabolites play a significant role in colon cancer initiation and development. Increased expression of Kyn and IDO1, and reduced production of indoles have been detected in variety of cancers including CRC [149,150]. Increased activity of IDO1 in conjunction with Trp depletion, leads to cell cycle arrest in activated T lymphocytes. This increased IDO1 activity further leads to apoptotic T-cell death [151–153]. Together, evidence indicates that increased IDO1 activity and Trp depletion promotes immunosuppression in the tumor microenvironment, making IDO1 a promising target for therapy in combination with traditional chemotherapeutics [154,155]. Additionally, evidence demonstrates that engineered microbes targeting the IDO1 pathway have therapeutic benefits in animal models of colon cancer [156,157]. Therefore, small molecule compounds with immunoregulatory effects, including both chemical and microbial IDO1 inhibitors, seem hopeful as therapeutics. Yet, it is unknown whether microbial indole production is protective or deleterious in the context of increased IDO1 or AhR expression in colon cancer. We present the following therapeutic modalities to be considered for study as interventions among individuals with CRC and high IDO1 or AhR expression, and discuss the current understanding and opportunities for future research around Trp metabolism and indole production for therapeutic intervention in CRC.

9.1. Therapeutic Targeting of Indoleamine 2,3-Dioxygenase (IDO1)

The overexpression of IDO1 is not merely a consequence of tumorigenesis but is seen as an early hallmark of inflammatory bowel disease and colitis indicating that it is in part driving tumorigenesis [158–161]. Loss of tumor suppressor gene bridging integrator 1 (BIN1) and overexpression of pro-inflammatory enzyme, COX-2 are also associated with an increased IDO1 expression, along with signals from pro-inflammatory cytokines, especially IFN γ [162]. IDO1 overexpression is also observed in colon cancer cell lines (HCT-116 and HT-29) in absence of an inflammatory environment, suggesting that genetic mutations or epigenetic modification of IDO1 could also be driving colon carcinogenesis [32,163,164]. Evidence from several seminal studies have clarified the carcinogenic role of IDO1, the blocking of which inhibits tumorigenesis and proliferation [164,165]. Not only has IDO1 inhibition been protective against chronic inflammation, it has been successful at altering

microbial composition, resulting in increased indolic metabolites and reestablishing gut permeability and intestinal homeostasis [13,95,161].

Based on the role of IDO1 in cancer immunity and development, several small molecule drugs have been developed to inhibit IDO1 for many types of cancer including CRC [166]. Recently Liu et al. showed that I-L-MT, an IDO1 inhibitor, decreased expression of cell cycle gene CDC20 resulting in G2/M arrest in HCT-116 and HT-29 cells. I-L-MT also induced mitochondrial injury and apoptosis in cancer cells [167]. Other effector IDO1 inhibitors in clinical trials include Indoximod (trial completed), Epacadostat (trial completed), Navoximod (trial completed), BMS-986205 (Recruiting), and PF-06840003 (trial not active) [166]. Most recently, Shen et al., constructed a liposome delivery system containing oxaliplatin and NLG919 (IDO1 inhibitor), to test the efficacy of this treatment in a mouse model of CRC, proposing an effective therapeutic approach for reversing the immunosuppressed tumor environment [168]. Ipilimumab, a CTLA-4 blocking antibody can also inhibit IDO1 expression [168]. These drugs were capable of not only inducing immunogenic cell death, but also reducing the conversion of Trp into Kyn through IDO1 inhibition. In addition to the clinical trials for IDO1 inhibitors, there have been clinical trials for vaccination using IDO1 peptides [169]. In lieu of the above treatment trials, there is an ample body of literature to support further investigate the oncoenzyme, IDO1 and IDO1 blockade in host cells. This is especially important for colon cancer therapy which appears to be more refractory to immunotherapy as compared to other types of cancer and thus would benefit from drugs that enhance the T cell response.

In addition to studying IDO1 blockade using traditional drug target methods, bacterial species are being used or engineered to block IDO1 expression. Specifically, *Lactobacillus johnsonii* decreased IDO1 activity (47%) in HT-29 intestinal epithelial cells by producing hydrogen peroxide (H₂O₂) which can inhibit IDO1 activity [157]. Additionally, *Bifidobacterium Infantis* decreased IDO1 activity and Kyn-Trp ratio, which reduced inflammation in murine model of depression. In contrast, *B. Infantis* can enhance levels of kynurenic acid, a protective metabolite, which inhibits colon cancer in a murine model of inflammation [7,170]. The ability of *Lactobacillus reuteri* to reduce pro-inflammatory cytokines is an important step in reducing IDO1 expression, which is tightly regulated by INF- γ [171]. *L. reuteri*, which can reduce NF- κ B activity, inhibit cell proliferation, and promote apoptosis of colon cancer cells [172] has been used in clinical trials (NCT03501082) to improve gut microbiome function. Additionally, Phan et al. investigated the efficacy of *Salmonella typhimurium* delivered shRNA targeting IDO1 inhibition (shIDO1-ST) which resulted in reduced tumor growth in two mouse models of CRC [173]. Bacterial species capable of producing short chain fatty acids, especially butyrate and propionate, can inhibit INF- γ -induced IDO1 expression via downregulation of STAT-1 and reduced HDAC inhibition activity [75]. Interestingly, Zelante et al. demonstrated that in the absence of IDO1 expression (IDO1 knock out), *Lactobacilli* were increased leading to an increased production of AhR-activating indolic compounds including indole-3-lactic acid and indole-3-aldehyde, suggesting that IDO1 expression also controls microbial composition and indole production. Further, the AhR activation via indolic compounds increased transcription of IL-22 which stimulated the epithelial immune response, and reduced pathogen abundance via recruitment of neutrophils and macrophages [13,174,175]. Therefore, the utility of *L. reuteri*, *B. Infantis*, and *L. johnsonii*, as well as engineered bacteria, as therapeutics in CRC is of great interest. The above evidence suggests a significant role of the microbiome in manipulating IDO1 expression and visa versa. Therefore, not only are drug targets of IDO1 promising therapeutics for CRC, but also are several microbial species.

9.2. Therapeutic Targeting of Aryl Hydrocarbon Receptor (AhR)

AhR plays a significant role in intestinal inflammation and colon carcinogenesis. Several studies have proposed AhR as a target for anticancer therapy [26,176]. Under inflammatory conditions, including CRC, there can be either partial or complete loss of AhR, whereas xenobiotic induced AhR activation has shown to reduce colitis in experimental models [177]. Therapeutically targeting intestinal AhR activation is problematic

due to the multiple AhR ligands, and plethora of downstream AhR target genes that have disparate effects [13,177,178]. Despite these complexities, the role of AhR in therapeutics for tumor suppression is under intense investigation. Several AhR ligands were recently in clinical trials for renal and breast cancer, autoimmune diseases, and multiple sclerosis [179–182]. Recently, however, a trial using an AhR inhibitory drug (BAY2416964) was initiated for treatment of advanced colon cancer (NCT04069026). Results from this trial will be important to understand the effect of AhR modulation in colon cancer progression.

Bacterial indoles, as a class of AhR ligands, are also being studied extensively in colon carcinogenesis. Depending on the concentration and structure of the indole compound, they can act as either agonists or antagonists of AhR [61]. Bacterial indoles, indole-3-acetate, tryptamine, IA, and indole-3-pyruvic acid can act as AhR agonists, while indole and IPA are AhR antagonists [177,183]. Both dietary and bacterial indoles exhibit promising therapeutic targets for abrogating carcinogenesis. [17,95,184]. However, more research is required as indoles demonstrate concentration dependent and tissue specific outcomes [83,185].

9.3. Therapeutic Targeting of Oncogene, c-Myc, to Control Trp Metabolism

It is well established that overexpression of oncogene, c-Myc, which is evident in about 70% of all colon cancer cases [186–188], is an important metabolic reprogramming in colon cancer initiation and progression [189,190]. The oncogene c-Myc dysregulates the vital cellular functions including survival, apoptosis, differentiation and proliferation, and promotes angiogenesis, metastasis, and immune evasion [188,190–193]. Most important, overexpression of c-Myc increases Trp uptake and conversion to Kyn stimulating proliferation of colon cancer, indicating Myc as a promising therapy in colon cancer to reduce Trp uptake [43].

Several strategies have been deployed to target c-Myc directly or indirectly including inhibiting c-Myc transcription, translation, activity, stability, gene targets, and interactions via different pathways including PI3K-mTOR to control CRC progression [194]. Wiegering et al. demonstrated silvestrol inhibited c-Myc expression reducing proliferation of colon cancer cells in vivo [195]. Unfortunately, targeting c-Myc has not been as successful in the trials due to several concerns. These reasons include the essential role of c-Myc in tissue homeostasis, and presence of partially redundant transcription factors (MYC, MYCN, MYCL), which would need to be targeted together for efficient therapeutics [196]. Despite these challenges, c-Myc continues to be an interesting and promising target for colon cancer, especially due to its upregulation of Trp metabolism, the blocking of which has been successful at reducing tumor proliferation.

9.4. Trp Metabolizing Bacteria as a Therapeutic Target

Apart from drug compounds, engineered bacterial species may be another avenue for therapeutics modulation of Trp metabolism in colon cancer. Several bacterial species carry the gene for tryptophanase (TnaA), an enzyme that degrades Trp to indoles, including *Escherichia coli*, *Proteus vulgaris*, *Bacillus alvei*, *Porphyromonas gingivalis*, *Haemophilus influenzae*, *Proteus rettgeri*, and *Aeromonas liquefaciens* [197]. Another gene cluster was identified by Wlodarska et al. with the capacity to metabolize phenylalanine to indole compounds including IPA and IA. The gene cluster, *fldAIBC*, was identified in *Peptostreptococcus russellii*, *Peptostreptococcus anaerobius* and *Peptostreptococcus stomatis*, which demonstrated the ability to utilize mucins, and produce indoles that inhibited inflammatory response [88]. Similarly, *Clostridium sprogenes* can metabolize Trp into IPA, protecting against gut permeability and inflammation [198]. Several other Trp-metabolizing phyla including Actinobacteria, Firmicutes, Bacteroidetes, Fusobacteria, Proteobacteria, and genera including *Burkholderia*, *Streptomyces*, and *Pseudomonas* have been recognized to possess this function [199]. Kaur et al. suggested Fusobacteria as one of the highest indole producing phyla with about 72% of the Fusobacteria strains containing genes associated with indolic pathways [199]. Interestingly, *Fusobacterium nucleatum* is one of the most abundant bacteria in colon adenomas and in colon adenocarcinomas and is associated with colon cancer promotion [200].

Additionally, biofilm formation is a key hallmark of adenomas and colon carcinomas. It is intriguing that *Fusobacterium nucleatum* is one of the strongest producers of indole, which is also known to promote biofilm formation. Yet, bacterial indole production is a strong anti-inflammatory factor in the host. Thus, an outstanding question in the field of colon cancer is whether *F. nucleatum*, or similar indole producing bacteria, is contributing to biofilm formation through indole production, and whether indole is acting in a protective manner or not in the context of colon cancer?

9.5. Dietary Indoles as Therapeutic Targets

In addition to indoles and Trp metabolites, AhR activation can be achieved by dietary compounds, collectively called brassica metabolites. These include 3-3-diindolylmethane (DIM), Indole-3-carbinol (I3C), Indole-3-acetonitrile (I3ACN) and, Indole[3,2b] carbazole (ICZ), which can be found in cruciferous vegetables including cabbage, broccoli, kale, collards, turnip greens, mustard, radish, rapeseed, kohlrabi and brussels sprouts.

Cruciferous vegetables are widely studied in cancer prevention, including colon cancer [3,201,202]. These vegetables are a good source of glucosinolate, predominantly, glucobrassicin, which can be broken down into indolic compounds. The enzymatic cleavage of glucobrassicin by myrosinases produce indolic compounds including I3C and I3ACN, which can be further reduced to DIM and ICZ. Collectively, these indolic compounds can activate AhR, and have demonstrated a reduced cancer risk [13,80,203–207]. However, it is unclear how liver metabolism of dietary indoles contributes to negative effects through formation of indoxyl sulfate (aka. indican) that can cause oxidative stress.

Specifically, I3C is emerging as an anti-carcinogenic molecule, exhibiting anti-proliferative effects in several colon cancer cell line including HT29, Colo320, Caco-2, HCT-116, and WS480 [3,208,209]. The protective role of I3C is not only seen in cancer but also evident as a preventive factor in inflammatory diseases [210–212]. Mechanistically, I3C reduces inflammation and carcinogenesis through suppression of NF- κ B signaling pathway, and induces cell cycle arrest, and apoptosis [183,213–216]. Additionally, I3C has demonstrated beneficial effects on the microbiome by promoting the production of antimicrobial factors that inhibit pathogenic biofilm formation. However, higher doses of I3C increased colonic lesions and tumor initiation due to overactivation of AhR in a murine model of hepatocarcinogenesis [13,217,218]. Therefore, the anti-cancer properties of dietary indole compounds may overcome the pathogenic indole production and reduce colon carcinogenesis in a dose and context dependent manner; however, much more research will be required to precisely determine the dietary vs. bacterial contribution to carcinogenesis.

10. Conclusions

Trp metabolism and indoles, both dietary and bacterial, are key factors in maintaining normal host physiology, including immune homeostasis, gut barrier function and microbial composition. However, under inflammatory conditions such as colon cancer, a series of alterations that affect host and microbial Trp metabolism occur. One key alteration includes overexpression of IDO1 leading to an increase production of Kyn. Kyn can constitutively activate AhR to enhance Treg activity, promoting immune escape. In contrast, Trp metabolites, including indoles, can activate intestinal AhR to suppress colonic stem and progenitor cell growth and reduce tumorigenesis in vivo; potentially ameliorating cancer stem cell proliferation. Furthermore, the changes in the gut microbiome during colon carcinogenesis also result in a paucity of indoles from Trp metabolizing bacteria. Subsequently, the reduced availability of protective indolic metabolites disrupt the symbiosis among microbial communities leading to an enhanced inflammation response. This heightened inflammatory condition further increases IDO1 activity driving a vicious cycle of tumor escape, proliferation, and metastasis. As such, increasing dietary indoles or promoting the growth of Trp metabolizing bacteria (e.g., *Lactobacillus*) to produce indoles may act to reduce the availability of Trp to cancer cells.

Harnessing the Trp-indole pathways in bacteria and the host hold the potential to attack the tumor cells through several different facets; Trp starvation, IDO1 inhibition, and indolic AhR activation. Further investigation is required to identify specific ligands that control AhR activation and mechanisms that reduce IDO1 expression or activity. Both dietary and microbial indoles hold promise as therapeutic adjuvants to target these pathways to inhibit colon carcinogenesis.

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