MEDICAL UNIVERSITY OF VIENNA

THESIS PROPOSAL

for the Doctoral Program at the
Medical University of Vienna

Intestinal Biofilms in Gastrointestinal Disease

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Life Sciences Call – Linking Research and Patient's Needs /LS18-053
Targeting mucosal biofilms in patients with gastrointestinal disorders

Date: 22.11.2019
month, year

Max Baumgartner
Signature of the applicant

Summary and aim (minimum 200 words)

Inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) are chronic gastrointestinal disorders which affect 10-15% of the Western population. These disorders drastically lower life quality and result in substantial socio-economic costs. The molecular mechanisms underlying the pathogenesis of these diseases are poorly understood and currently there is only symptomatic treatment.

Recently, we have observed endoscopically visible biofilms in the gastrointestinal tract of IBD and IBS patients, but their disease relevance, function and composition are unknown. This project aims to (i) establish sound epidemiologic data on ileal and colonic biofilms (N=800 consecutive colonoscopies at the Vienna general hospital, department of gastroenterology) (ii) apply a multi-omics approach on biofilm-biopsies, control-biopsies and stool samples to identify biomarkers and gain mechanistic insight in biofilm formation (iii) use fluorescent in-situ hybridization analysis of gastrointestinal biofilms to analyze biofilm composition and (patho)physiology

This project aims to improve treatment for IBD and IBS patients and pave the way towards precision medicine. Innovative aspects include: establishing prevalence, location and appearance of ileal and colonic biofilms in the healthy population, IBS and IBD patients and correlation of ileal and colonic biofilms to IBD/IBS phenotype and severity, understanding of biofilm composition and function, thereby identifying biofilm- / disease-specific biomarkers that can be used for diagnosis and treatment guidance. Expected outcomes include novel information on biofilm location, morphology and disease association, biomarkers to aid diagnosis and treatment, that could ultimately lead to novel therapeutic avenues for IBD and IBS patients.

List of abbreviations

boston bowel prep score	BBPS
Crohn's disease	CD
fluorescence in-situ hybridization	FISH
gastrointestinal	GI
inflammatory bowel disease	IBD
irritable bowel syndrome	IBS
IBS with predominant constipation	IBS-C
IBS with predominant diarrhea	IBS-D
IBS, mixed-type	IBS-M
permutational multivariate analysis of variance	perMANOVA
ulcerative colitis	UC

Background (minimum 350 words)

The gastrointestinal (GI) disorders inflammatory bowel disease (IBD, including Crohn's disease (CD) and ulcerative colitis (UC)) and irritable bowel syndrome (IBS) are increasingly prevalent in the whole world. Patients with these multifactorial GI disorders suffer from limited treatment options due to incomplete understanding of disease pathogenesis[1, 2]. Western lifestyle, frequent antibiotic therapy, food additives and microbiome-altering medications have been implicated in disease etiology[3, 4]. Today 15 % of the Western population are affected by IBD/IBS which leads to increasing health care costs of over €100 billion in Europe[1, 5]. A better understanding of disease pathophysiology and development of novel diagnostics, prevention strategies and therapies are thus imperative.

IBS is an umbrella term for a variety of GI-related symptoms[6]. The global IBS-burden is on the rise and according to recent studies the prevalence is as high as 10-20 %, depending on geographic region[7]. Woman seem to be more at risk to develop IBS than man. The sub-classification of IBS is based on symptoms and comprises of IBS with predominant diarrhea (IBS-D), with predominant constipation (IBS-C) or mixed-type (IBS-M). As the symptoms of IBS are diverse, the etiologies of the disease still remain elusive.

Associations between gut-specific or systemic diseases and alterations in microbiota are reported for IBD, IBS, *Clostridium difficile* infection[8], diabetes, obesity, gastric -, and colorectal cancer[9].. The intestinal microbiota of IBS, UC and CD patients is reduced in species richness, diversity and stability, both in fecal and biopsy-associated communities and they appear to be sensitive to disease severity[10]. Also, more bacteria are penetrating the intestinal mucosa. Fecal microbiota transplantation has been shown to be safe and effective in IBD and IBS patients, underlining the importance of microbiota in the pathogenesis of GI disease[11, 12]. Due to advances in sequencing technology microbiome research has been focused on taxonomic composition, neglecting spatial bacterial composition in the human gut.

Microbial biofilms are a distinct form of bacterial growth where microbial communities synthesize extracellular matrix to form a protective microenvironment. Biofilms play a causative role in a variety of human diseases. In preliminary studies, we have found mucosal biofilms (Figure 1) in IBD (UC) and IBS patients, but rarely in healthy individuals.

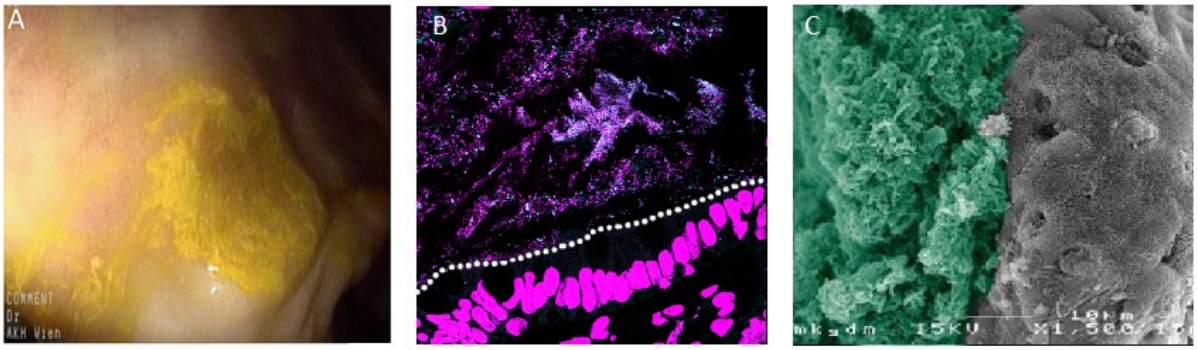


Figure 1. *(**A**) Colonoscopic image of a colonic biofilm, a dense layer sticking to the mucosal wall. (**B**) Fluorescence in situ hybridization with Eub338 (turquise) to detect all bacteria residing in the biofilm of a patient with ulcerative colitis. DAPI (pink) was used to visualize DNA; the border of the epithelium is marked by a dotted line*. **(C)**

Biofilms and the correlation to human disease is well-established for dental plaques. Formation of biofilms on intravascular and urinary catheters and prosthetic implants are a threat for regional or systemic inflammation, as they might build an extracellular matrix and antibiotic resistance. It is suggested that a vast proportion of human bacterial infections might be related to biofilm formation (e.g. cystic fibrosis and Pseudomonas aeruginosa, ulcers,

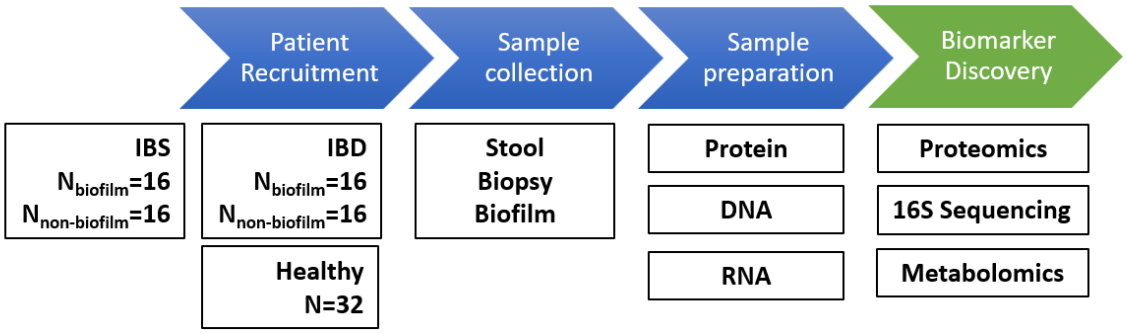
urinary tract infections)[13]. Only recently it was shown that a high percentage of right-sided surgically resected colorectal cancers are associated with bacterial biofilms, which were also found in distant normal mucosa[14]. Recent studies revealed a causal relationship of bacterial biofilms and carcinogenesis[15]. Biofilm formation in IBD was already reported more than ten years ago, with Bacteroides fragilis as the major residing bacteria within the biofilm, analyzed from intestinal biopsies[16]. Bacterial biofilm formation in UC can be suppressed by antibiotic treatment, with a dramatic rebound effect on bacterial regrowth[17]. However, the prevalence, composition, or stability of biofilm-forming bacterial communities in healthy populations and GI diseases is unknown. Until now biofilms have not been described in IBS. Despite the link between the intestinal microbiome and a growing number of pathological conditions, very little is known about the exact nature and functional relevance of these biofilms in the pathogenesis of IBD and IBS.[2] A more defined and systematic approach is thus required to establish their functional relevance which might ultimately lead towards novel therapeutic avenues.

Thus, the following hypothesis emerges:

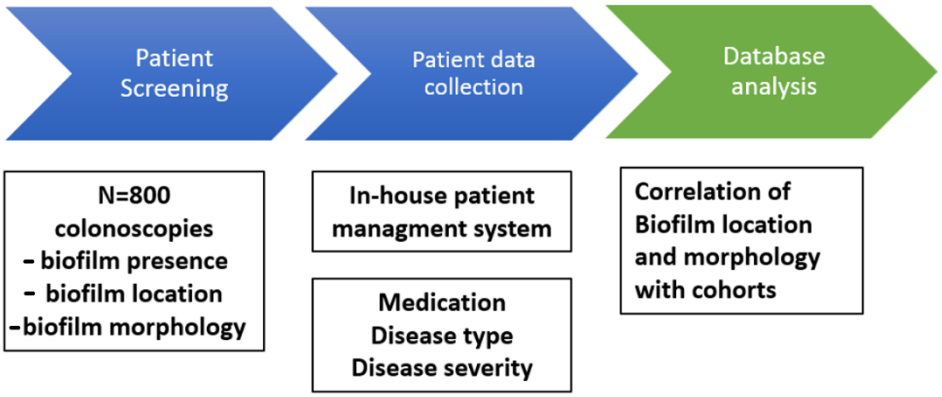
Endoscopically visible Bacterial biofilms reflect a disturbed microbiome and induce and support symptoms in IBS and IBD

Operational objectives: (minimum 300 words)

1st year – Sample collection and epidemiology of intestinal biofilms



Informed consent will be obtained from IBD (ulcerative colitis), IBS patients and healthy individuals undergoing screening colonoscopy. Inclusion criteria are defined as >18 years of age, inactive/active ulcerative colitis, symptomatic IBS-D/IBS-M or healthy, with no current or previous colorectal cancer, colectomy, or any other serious concomitant disease; no intake of antibiotics in the previous three months. Biofilm (ileal and colonic) and normal mucosa samples (biopsy/brush) and stool samples will be collected during colonoscopy. Disease type and severity will be determined using the Mayo endoscopic score[18] and Rome IV criteria[19] for IBD and IBS patients, respectively.



Biofilm surveillance will be performed at the endoscopy unit of the Vienna General Hospital, one of Europe's biggest hospitals. We aim to screen 800 consecutive colonoscopies. Doctors and study nurses will be educated about biofilm scoring and fill out report sheets at every colonoscopy they perform. Biofilms are defined as cohesive mass that sticks to the epithelium of the gut, detaching in a film-like manor when flushed off with the endoscopic endowasher. Pictures of Biofilms will be taken and Biofilms will be scored from one to three in regards of extension (1=small and patchy, 2=medium extent of the gut wall covered, 3=large extent of the gut wall covered) and resistance towards detachment with the endowasher (1=readily detachable with the endowasher, 2=resisting detachment, 3=hard to detach even with highest setting of the endowasher). Additionally, the preparation of each patient will be assessed using the boston bowel prep score (BBPS)[20]. Each region of the colon receives a "segment score" from 0 to 3 and these segment scores are summed for a total BBPS score ranging from 0 to 9. Doctors on the endoscopic unit will prospectively report biofilm scores together with information on biofilm location and BBPS while performing endoscopy. To counteract the possibility of false-positive cases, patients with a BBPS<6 and biofilms which are readily detachable with the endowasher (resistance<2) will be excluded from the analysis. In parallel, information on patient medication history and laboratory values

will be collected retrospectively from the in-house patient management system of the Vienna General Hospital (AKIM). Disease type and severity will be determined using the Mayo endoscopic score[18] and Rome IV criteria[19] for IBD and IBS patients, respectively. Biofilm location and morphological appearance will be correlated to disease phenotype, severity and medication history.

Expected outcomes: The first year will deliver a detailed high-quality patient database that can be used to test if our hypothesis holds true in a correlative sense. Additionally, the database will be used to proceed with the second year of the study.

Operational objectives: (minimum 300 words)

2nd year – Multi omics analysis of intestinal bioflims

Metabolomic and proteomic measurements will be performed at Cemm (Research center for molecular medicine of the Austrian academy of science, Vienna). 16S rRNA amplicon sequencing will be performed by the joint microbiome facility (JMF, Vienna). Additionally, faecal bile acid profiles will be analysed via HPLC-MS/MS. Data will be processed using established pipelines and bioinformatic workflows[21]. Data will be compared to healthy individuals using relative abundances of bacteria, proteomics, metabolomics and bile acid profiles. Healthy profiles will be subtracted, from biofilm profiles and a detection threshold applied to identify biomarker leads that are present in at least 80% of the IBD or IBS cohorts. The identified biomarkers will be correlated to disease type and severity to provide novel insights for disease relevance.

Preliminary data: Biofilm sampling and 16S rDNA sequence analysis have been established from mucosal and biofilm biopsies and faecal samples from patients with IBS or IBD and healthy controls. Fluorescence in situ hybridization of biofilm biopsies showed tightly packed bacteria within the biofilm close to the epithelium (Figure 1B). Preliminary 16S rDNA sequencing analysis from the biofilms suggests an enrichment of specific genera (Bacteriodes) or families (Lachnospiraceae) within biofilm biopsies and flushes compared to the normal mucosa of patients with no biofilms (Figure 2). Also, the diversity is lower in biofilm samples than in the normal mucosa of patients with no biofilm (Figure 2B).

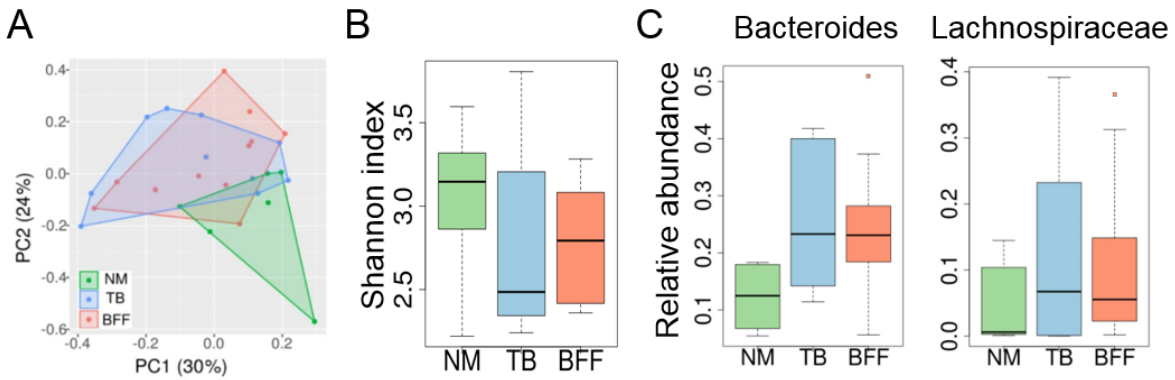
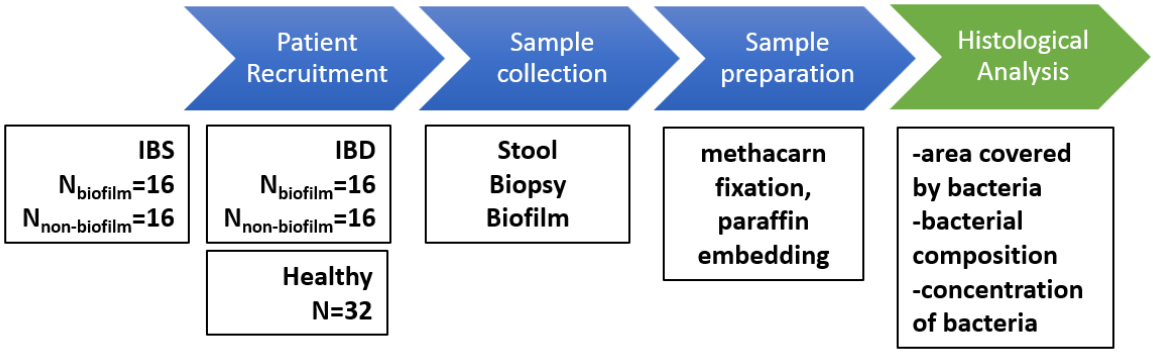


Figure 2. *Colonic biofilm analysis using 16S rDNA sequencing.* ***(A)*** *Principal component (PC) analysis of biofilm biopsies (TB, blue, n=10), biofilm flush (BFF, red, n=11) and "normal mucosa" biopsies of biofilm negative patients (NM, green, n=6) revealed distinct clusters for TB/BFF and NM.* ***(B)*** *Species richness / evenness is lower in TB and BFF than in NM.* ***(C)*** *Relative abundance plots of Bacteroides and Lachnospiraceae, a genus and family enriched in biofilm-presenting pathological states.*

Expected outcomes: The second year will deliver microbial and molecular biomarker leads that will be correlated to disease type and severity. It will provide novel and fundamental insights into biofilm composition and biodiversity, which will help to advance our understanding of the role of biofilms in IBD and IBS.

Operational objectives: (minimum 300 words)

3rd year - Biofilm histological analysis



Biofilms exist of complex multi-taxon communities, but the knowledge about structural organization and co-localization of intestinal microorganisms on the micrometer scale is limited due to limitation in sample collection, labelling and imaging technologies. Approaches exist to simultaneously stain for and image up to 15 different taxa in biofilms (dental plaque) by Combinatorial Labelling and Spectral Imaging fluorescence in-situ hybridization (FISH) with genus and family-specific probes[22].

Mucosal biopsy samples (collected in the ileum and caecum) will be fixed in methacarn solution (fixative preserving bacteria and the structural integrity of the mucus layer, composed of 60 % ethanol, 30 % chloroform and 10 % glacial acetic acid) for 2 h at 4° C and subsequently washed in PBS. If not processed immediately, samples will be stored in 70 % ethanol/30 % PBS at -20° C until embedding. Finally, 8-12 µm serial sections will be performed for subsequent FISH analysis.

Based on the results of the 16S rRNA gene-targeted sequencing analysis we will develop specific 16S rRNA-targeted probe sets for microbial biomarkers of UC for pioneering systems-level FISH analyses. Probes for known fecal microbial biomarkers of health state[23] and newly identified microbes will be developed as described by Valm et al[22].. Briefly, probes will be either identified in the literature or designed with the ARB program. Oligonucleotides will be conjugated to one or two different fluorophore combinations (e.g. Alexa Fluor dyes). FISH and confocal laser scanning microscopy (CLSM, TCS SP8 X,Leica) will be carried out in cooperation with David Berry and Alexander Loy (Department of Microbiology and Ecosystem Science, University of Vienna). Bacterial abundances of specific genera/species will be quantified in relation to a universal bacterial probe (EUB338) and spatial organization to mucosal wall will be determined.

Additionally, area covered by bacteria per sample section and concentration (density) of bacteria will be analyzed using ImageJ. Area covered by bacteria and bacterial density will be correlated to biofilm positivity, location and morphology as well as disease cohort.

Expected outcomes: The third year will deliver important insights into biofilm pathophysiology and establish the crucial link between biofilms visible by high definition white light endoscopy and histological findings.

Statistical Analysis Plan (minimum 150 words).

The proportion of biofilm positive subjects between different cohorts (Crohn's disease Ulcerative colitis, irritable bowel syndrome, transplant patients, portal hypertension, cancer, screening colonoscopy) will be compared using Pearson's chi square test. Bonferroni correction will be used for multiple comparisons.

To analyze similarity of microbial profiles generalized UniFrac distances will be used. Significant separation of groups will be assessed with permutational multivariate analysis of variance (perMANOVA) and DESeq. To compare bacterial abundances Kruskal−Wallis for overall and Wilcoxon Rank Sum Test for pairwise comparison will be used. Metabolome and proteome data will be analyzed using established workflows[24].

Sample size calculation:

Patient sample size for multi-omics analysis must be at least 16 per group per disease type for sufficient statistical power. The calculation is based on the observed differences in abundance of selected bacteria (represented by the Shannon's index and % operational taxonomic unit abundance, respectively) within biofilm samples and non-biofilm samples and supported by similar studies that found significant differences between colonic biofilms and normal tissue using similar sample sizes[14]. Our preliminary data indicated an enrichment of the Bacteroides genus (25.3±12.7% in biofilm vs 12.8±6.6% in control). Thus, our hypothesis is: H0, Bacteroides abundance is not different between biofilm and normal mucosa or fecal matter; H1, Bacteroides species are enriched in biofilm. H0/H1: assuming data parametricity (paired t-test), alpha(one-sided) =0.05 and power=0.8, 16 biofilm-positive and 16 -negative patients should be included. We therefore have decided to aim for 16 patients per cohort.

Working plan

1st year (minimum 200 words)

Months 1-6

In the months one till six, a screening sheet for routine colonoscopy patients will be designed and introduced into the clinics. (general hospital of Vienna, 7i internal medicine III, department of gastroenterology and hepatology, Vienna) The sheet will cover colonoscopy indication, bowel preparation score, presence of biofilms, location of biofilms and morphological information of biofilms. Doctors and nurses will be educated about endoscopically visible biofilms and the screening sheet will be implemented in the colonoscopy workflow. Patient enrollment for molecular and histological biofilm analysis will be startet.

Months 7-12

Continuing throughout the year screening sheets will be digitalized and put into a database. Patient information regarding disease cohorts (CD, UC; IBS-M, IBS-C, IBS-D, transplant patients (liver, kidney, heart), liver diseases (cirrhosis, portal hypertension, non-alcoholic fatty liver disease, alcoholic fatty liver disease, primary sclerosing cholangitis), GI-cancer, polyps) will be determined via the inhouse patient management system. (AKIM, general hospital of Vienna). Data on biofilm presence, location and morphology will be correlated with different disease cohorts. Reaching high patient numbers and statistical significance is highly feasible considering that 25 patients (for each of our disease cohorts) undergo colonoscopy at our Department of Internal Medicine III (Vienna General Hospital) each week. Ethics are already obtained (EK-Nr: 1780/2019), and our team covers the required expertise.

2nd year (minimum 200 words)

Months 13-18

In the months 13 till 18, 16S rRNA amplicon sequencing workflow, protein and metabolite extraction will be established and optimized for mucosal biopsies (i.e. sampling procedure, DNA/protein/metabolite extraction, 16S amplification, library preparation). Patients will be recruited throughout the whole year. Samples will be pooled and submitted to sequencing in cooperation with the joint microbiome facility (medical university of Vienna and general university of Vienna). Metabolomic and proteomic measurements will be performed at Cemm (Research center for molecular medicine of the Austrian academy of science, Vienna). Additionally, endoscopic screening sheets will be collected from patients which undergo 16S sequencing to boost sample size for the epidemiological part of the study.

Months 19-24

In the months 19 till 24, 16S sequencing data will be analyzed using state of the art methodology (DADA2, SINA and DESeq2). Difference in microbial composition and diversity indices will be determined between biofilm positive and negative patients in different disease cohorts. Supervised and unsupervised clustering methods will be applied to the microbial, protein and metabolite profiles to determine if biofilms are correlated with alterations in the microbiome. Our laboratory has extensive experience in patient recruitment and biofilm sampling. Preliminary data look promising to identify biofilm-specific biomarkers and align well with literature regarding normal mucosa bacteria.27 Biofilm sampling ethics (EK-Nr: 1910/2019) is already accepted, and our team covers the required expertise and is furthermore supported by state-of-the-art equipment and facilities (i.e. Biomedical Sequencing Facility).

3rd year (minimum 200 words)

Months 25-30

In the months 25 till 30, FISH methodology will be established and data analysis from months 19 till 24 will be continued. Different pretreatments (Triton X-100, 0.1 M HCl, lysozyme, and proteinase K) will be applied to enhance binding efficacy of FISH probes and thereby increasing signal intensity during confocal laser microscopy. Bacteria that are associated with different disease cohorts or GI biofilms will be selected and FISH probes of specific genera/species which were correlated to biofilm positivity, disease cohort, biofilm morphology and biofilm location in the 16S analysis will be designed using established methodology. All samples will be analyzed for area covered by bacteria and bacterial density. These measurements will then be correlated to disease cohorts, biofilm positivity, biofilm location and biofilm morphology. Sequencing and patient recruitment form the second year will be continued if necessary.

Months 31-36

In the moths 32 till 36, FISH data analysis will be finalized. Additionally, final 16S sequencing analysis of combined data from the previous years will be performed. Publication quality graphs will be generated for the correlation of biofilms to different diseases (Objective I), 16S sequencing data and FISH analysis. The data will be compiled into a manuscript and submitted for publication.

Alternative strategies (minimum 100 words)

FISH probe binding efficacy can be diminished due to certain IBD related medications (5-ASA). If FISH analysis does not work, we will use DAPI staining for the quantification of bacteria. (area covered by bacteria per section, bacterial density). It could be difficult to amplify bacterial DNA from mucosal biopsies as the majority of DNA will be human. In case the 16S sequencing posses' difficulties we will apply a commercial kit, which enriches bacterial DNA and selectively depresses human DNA. (Qiagen) If we encounter difficulties during any of the aims, we have strong collaborations which are experts in the proposed methodologies. (i.e. Prof. David Berry, Department of Microbial Ecology, university of Vienna)

References (maximum 50)

1. Molodecky NA, Soon IS, Rabi DM, et al. Increasing Incidence and Prevalence of the Inflammatory Bowel Diseases With Time, Based on Systematic Review. YGAST 2012;142:46-54.e42.
2. von Rosenvinge EC, O'May GA, MacFarlane S, et al. Microbial biofilms and gastrointestinal diseases. Pathogens and disease 2013;67:25-38.
3. Chassaing B, Koren O, Goodrich JK, et al. Dietary emulsifiers impact the mouse gut microbiota promoting colitis and metabolic syndrome. Nature 2015;519:92-96.
4. Ananthakrishnan AN. Epidemiology and risk factors for IBD. Nature reviews. Gastroenterology & hepatology 2015;12:205-217.
5. Canavan C, West J, Card T. Review article: the economic impact of the irritable bowel syndrome. Aliment Pharmacol Ther 2014;40:1023-34.
6. Ford AC, Lacy BE, Talley NJ. Irritable Bowel Syndrome. New England Journal of Medicine 2017;376:2566-2578.
7. Lovell RM, Ford AC. Global prevalence of and risk factors for irritable bowel syndrome: a meta-analysis. Clin Gastroenterol Hepatol 2012;10:712-721 e4.
8. van Nood E, Vrieze A, Nieuwdorp M, et al. Duodenal infusion of donor feces for recurrent Clostridium difficile. N Engl J Med 2013;368:407-415.
9. Abreu MT, Peek RM, Jr. Gastrointestinal malignancy and the microbiome. Gastroenterology 2014;146:1534-1546 e3.
10. Lloyd-Price J, Arze C, Ananthakrishnan AN, et al. Multi-omics of the gut microbial ecosystem in inflammatory bowel diseases. Nature 2019;569:655-662.
11. Johnsen PH, Hilpüsch F, Cavanagh JP, et al. Faecal microbiota transplantation versus placebo for moderate-to-severe irritable bowel syndrome: a double-blind, randomised, placebo-controlled, parallel-group, single-centre trial. The Lancet Gastroenterology and Hepatology 2018;3:17-24.
12. Rossen NG, Fuentes S, Van Der Spek MJ, et al. Findings From a Randomized Controlled Trial of Fecal Transplantation for Patients With Ulcerative Colitis. Gastroenterology 2015;149:110-118.
13. Hung CS, Henderson JP. Emerging concepts of biofilms in infectious diseases. Mo Med 2009;106:292-6.
14. Dejea CM, Wick EC, Hechenbleikner EM, et al. Microbiota organization is a distinct feature of proximal colorectal cancers. Proceedings of the National Academy of Sciences of the United States of America 2014;111:18321-6.
15. Dejea CM, Fathi P, Craig JM, et al. Patients with familial adenomatous polyposis harbor colonic biofilms containing tumorigenic bacteria. Science 2018;359:592-597.
16. Nava GM, Friedrichsen HJ, Stappenbeck TS. Spatial organization of intestinal microbiota in the mouse ascending colon. The ISME journal 2011;5:627-38.
17. Swidsinski A, Loening-Baucke V, Bengmark S, et al. Bacterial Biofilm Suppression with Antibiotics for Ulcerative and Indeterminate Colitis: Consequences of Aggressive Treatment. Archives of Medical Research 2008;39:198-204.
18. Lai EJ, Calderwood AH, Doros G, et al. The Boston bowel preparation scale: a valid and reliable instrument for colonoscopy-oriented research. Gastrointest Endosc 2009;69:620-5.
19. Rutgeerts P, Sandborn WJ, Feagan BG, et al. Infliximab for induction and maintenance therapy for ulcerative colitis. N Engl J Med 2005;353:2462-76.
20. Mearin F, Lacy BE, Chang L, et al. Bowel Disorders. Gastroenterology 2016.

21. Lang M, Berry D, Passecker K, et al. HuR Small-Molecule Inhibitor Elicits Differential Effects in Adenomatosis Polyposis and Colorectal Carcinogenesis. Cancer Res 2017;77:2424-2438.
22. Valm AM, Mark Welch JL, Rieken CW, et al. Systems-level analysis of microbial community organization through combinatorial labeling and spectral imaging. Proc Natl Acad Sci U S A 2011;108:4152-7.
23. Berry D, Reinisch W. Intestinal microbiota: A source of novel biomarkers in inflammatory bowel diseases? Best Practice & Research Clinical Gastroenterology 2013;27:47-58.

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Timelines

	1st year		2nd year		3rd year	
	Months 1-6	Months 7-12	Months 13-18	Months 19-24	Months 25-30	Months 31-36
-development of clinical biofilm screening sheet -implementation of biofilm screening sheet in colonoscopy ward						
-generation of patient database -gathering of patient diagnoses from in house patient management system						
-establishing 16S workflow (sampling, DNA extraction, 16S amplification, sequencing)						
-sequencing of biopsies and stool samples -16S data analysis						
-establishing FISH staining and histological analysis of biopsy samples						
-FISH staining and histological analysis -data analysis (16S, histological data and patient database)						

1. Molodecky NA, Soon IS, Rabi DM, et al. Increasing Incidence and Prevalence of the Inflammatory Bowel Diseases With Time, Based on Systematic Review. YGAST 2012;142:46-54.e42.
2. von Rosenvinge EC, O'May GA, MacFarlane S, et al. Microbial biofilms and gastrointestinal diseases. Pathogens and disease 2013;67:25-38.
3. Chassaing B, Koren O, Goodrich JK, et al. Dietary emulsifiers impact the mouse gut microbiota promoting colitis and metabolic syndrome. Nature 2015;519:92-96.
4. Ananthakrishnan AN. Epidemiology and risk factors for IBD. Nature reviews. Gastroenterology & hepatology 2015;12:205-217.
5. Canavan C, West J, Card T. Review article: the economic impact of the irritable bowel syndrome. Aliment Pharmacol Ther 2014;40:1023-34.
6. Ford AC, Lacy BE, Talley NJ. Irritable Bowel Syndrome. New England Journal of Medicine 2017;376:2566-2578.
7. Lovell RM, Ford AC. Global prevalence of and risk factors for irritable bowel syndrome: a meta-analysis. Clin Gastroenterol Hepatol 2012;10:712-721 e4.
8. van Nood E, Vrieze A, Nieuwdorp M, et al. Duodenal infusion of donor feces for recurrent Clostridium difficile. N Engl J Med 2013;368:407-415.
9. Abreu MT, Peek RM, Jr. Gastrointestinal malignancy and the microbiome. Gastroenterology 2014;146:1534-1546 e3.
10. Lloyd-Price J, Arze C, Ananthakrishnan AN, et al. Multi-omics of the gut microbial ecosystem in inflammatory bowel diseases. Nature 2019;569:655-662.
11. Johnsen PH, Hilpüsch F, Cavanagh JP, et al. Faecal microbiota transplantation versus placebo for moderate-to-severe irritable bowel syndrome: a double-blind, randomised, placebo-controlled, parallel-group, single-centre trial. The Lancet Gastroenterology and Hepatology 2018;3:17-24.
12. Rossen NG, Fuentes S, Van Der Spek MJ, et al. Findings From a Randomized Controlled Trial of Fecal Transplantation for Patients With Ulcerative Colitis. Gastroenterology 2015;149:110-118.
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14. Dejea CM, Wick EC, Hechenbleikner EM, et al. Microbiota organization is a distinct feature of proximal colorectal cancers. Proceedings of the National Academy of Sciences of the United States of America 2014;111:18321-6.
15. Dejea CM, Fathi P, Craig JM, et al. Patients with familial adenomatous polyposis harbor colonic biofilms containing tumorigenic bacteria. Science 2018;359:592-597.
16. Nava GM, Friedrichsen HJ, Stappenbeck TS. Spatial organization of intestinal microbiota in the mouse ascending colon. The ISME journal 2011;5:627-38.
17. Swidsinski A, Loening-Baucke V, Bengmark S, et al. Bacterial Biofilm Suppression with Antibiotics for Ulcerative and Indeterminate Colitis: Consequences of Aggressive Treatment. Archives of Medical Research 2008;39:198-204.

18. Rutgeerts P, Sandborn WJ, Feagan BG, et al. Infliximab for induction and maintenance therapy for ulcerative colitis. N Engl J Med 2005;353:2462-76.
19. Mearin F, Lacy BE, Chang L, et al. Bowel Disorders. Gastroenterology 2016.
20. Lai EJ, Calderwood AH, Doros G, et al. The Boston bowel preparation scale: a valid and reliable instrument for colonoscopy-oriented research. Gastrointest Endosc 2009;69:620-5.
21. Anders S, Huber W. Differential expression analysis for sequence count data. Genome Biol 2010;11:R106.
22. Valm AM, Mark Welch JL, Rieken CW, et al. Systems-level analysis of microbial community organization through combinatorial labeling and spectral imaging. Proc Natl Acad Sci U S A 2011;108:4152-7.
23. Berry D, Reinisch W. Intestinal microbiota: A source of novel biomarkers in inflammatory bowel diseases? Best Practice & Research Clinical Gastroenterology 2013;27:47-58.
24. Chong J, Xia J. Using MetaboAnalyst 4.0 for Metabolomics Data Analysis, Interpretation, and Integration with Other Omics Data. Methods Mol Biol 2020;2104:337-360.