# A randomized phase II study of the administration of prebiotics and probiotics during definitive treatment with chemotherapy-radiotherapy for patients with squamous cell carcinoma of the anal canal (BISQUIT)

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## Introduction

Although anal canal squamous cell carcinoma (ACSCC) is rare in developed countries, it has shown an annual increase of 4% in its incidence in Brazil (1), and according to data from the Oncocenter Foundation of São Paulo (FOSP), 2,338 cases were diagnosed in 2000 and 2016.

The standard treatment for localized ACSCC (without distant metastases) is definitive chemo-radiotherapy (Ch-RT) concomitant with administration of a fluoropyrimidine (5FU or capecitabine) combined with mitomycin or cisplatin, which provides cure rates of 60-80 % depending on the staging (2). When there is no complete remission, surgical rescue through anal amputation is the only potentially curative modality. However, this strategy is associated with great morbidity, besides negative emotional and social impacts, with consequent reduction of quality of life. Therefore, interventions that may increase the chance of cure in ACSCC should be investigated.

The main risk factors for ACSCC are human papillomavirus (HPV) infections and immunosuppression, including human immunodeficiency virus (HIV) infection. Chronic HPV infection and HIV-induced immunosuppression point to research strategies that

strengthen the immune system to reduce the risk of developing ACSCC. In the metastatic setting, the use of immune checkpoint inhibitors, such as anti-programmed death protein-1 (PD1) antibodies, were shown to be promising in ACSCC patients, promoting response rates of approximately 25% (3). However, there is no evidence of modulation interventions of the immune system in patients with localized ACSCC.

Recently, studies have shown that the composition of the intestinal microbiota influences the onset of colorectal cancer (4), and may even disrupt the effects of chemotherapy in this neoplasm. A preclinical study in animal model showed that E. coli impaired the antitumor effect of fluoropyrimidines, drug used in colorectal cancer and ACSCC (5). The intestinal microbiota also participates in a large set of metabolic processes (such as reduction, hydrolysis, dehydroxylation, etc.) involved in drug metabolism. For example, some intestinal bacteria have β-glucuronidases that cleave glucuronide from the inactive metabolite of irinotecan (SN-38G), a drug used in gastrointestinal tumors, releasing active metabolite (SN38) in the intestine, causing diarrhea and colitis (6). Ciprofloxacin has been shown to inhibit this enzyme by suppressing the diarrhea associated with irinotecan in an experimental model of mice (6). Mycoplasma hyorhinis encodes a thymidine phosphorylase that strongly restricts the cytostatic activity of pyrimidine nucleoside analogues (6).

On the other hand, the replacement of the intestinal microbiota "carcinogenic" (Fusobacterium spp and Bacteriodes fragilis) (7) by a protective microbiota (Bifidobacterium and Lactobacillus) (8) has been the reason of numerous investigations with prebiotics and probiotics. According to the International Scientific Association of Probiotics and Prebiotics, probiotics are composed of living organisms which, when administered, promote health benefits, such as antimicrobial action against intestinal pathogens, modulation of the immune system, reduction of cholesterol levels, reduction of colitis and prevention of colorectal cancer (7). Kefir is an example of probiotic. Already prebiotics are inert ingredients that promote alteration in the composition or activity of the gastrointestinal microflora, conferring health benefits (7). Example of prebiotic is polysaccharide inulin. Studies with these compounds have been conducted, showing promising results. A small placebo-controlled trial using B. breve breve (Yakut®) in children undergoing chemotherapy for a variety of neoplasms has shown that this group had fewer episodes of fever and less frequency of use of intravenous antibiotics compared to controls (6). There are also studies that suggest that the alteration of the intestinal flora can increase the effectiveness of immunotherapy as a form of modulation of the immune system in several animal models of colorectal cancer (9). In addition, the use of this strategy could have a modulatory effect on local and systemic toxicity of the treatment, possibly reducing the morbidity of the treatment, as already suggested by studies in cervical carcinomas (10).

Despite the strong scientific rationale, there are no studies that have evaluated the use of probiotics or prebiotics in order to increase the effectiveness of conventional Ch-RT treatment in ACSCC. Therefore, based on the assumptions that (1) there is a need for research that increases the cure rates of the definitive treatment of Ch-RT in ACSCC; (2) ACSCC is a virus-associated tumor in many cases and therefore potentially immunogenic; (3) immunotherapy is a promising strategy in ACSCC; and (4) that pre-

and probiotics can stimulate the immune system through modulation of the intestinal microbiota, and improve oncological outcomes, we propose a randomized phase II study of the use of pre-probiotics during definitive treatment of Ch-RT for patients with ACSCC located.

The primary hypothesis of this study is that addition of pre- and probiotics increases the proportion of patients with complete clinical and radiological response after Ch-RT to ACSCC. Secondary hypotheses are that pre- and probiotics increase the metabolic response measured by positron emission computed tomography (PET-CT) with 18F-2-fluoro-2-deoxy-D-glucose fluorodeoxyglucose (18-FDG) and promote greater control of local disease after Ch-RT; and reduce local and systemic toxicity of treatment.

## Hypotheses:

*Primary:* The addition of pre- and probiotics increases the proportion of patients with complete clinical and radiological response after Ch-RT to ACSCC.

*Secondary:* Pre- and probiotics increase the metabolic response measured by PET-CT 18-FDG and promote greater control of local disease after Ch-RT; and reduce local and systemic toxicity of treatment.

## Objectives:

*Primary:* Evaluate whether the addition of pre- and probiotics during Ch-RT increases the post-Ch-RT complete clinical and radiological response rate for ACSCC.

*Secondary:* To evaluate if the addition of pre- and probiotics influences the metabolic response by PET-CT 18-FDG; the complete clinical and radiological response rate at 6 months; colostomy free survival at 12 months and disease-free survival; toxicities of Ch-RT treatment are reduced; to assess whether the presence of HPV in the tumor tissue correlates with clinical outcomes; and promotes variation of systemic immune parameters (11).

## Outcomes:

*Primary:* Complete clinical and radiological response rate at 6-8 weeks post-Ch-RT, defined as absence of disease visible on clinical examination and magnetic resonance imaging (MRI) of the pelvis (or pelvic tomography if contraindicated to MRI) and without disease at a distance, through tomography of the chest and abdomen. This moment was chosen because it is when we evaluate response to Ch-RT in the routine, according to national and international guidelines.

*Secondary:*

*(1*) Metabolic response by 18-FDG PET-CT, comparing the mean pre-and post-Ch-RT volume-capture measurements of each patient at 6-8 weeks post Ch-RT. As there are no studies evaluating ACSCC volume-uptake measures, the volume-withdrawal value

(s) of the volume-uptake measures that is most associated with the complete clinical and radiological response at 6-8 weeks post-Ch-RT will be determined a posteriori.

(2) Complete clinical and radiological response rate at 6 months, defined as absence of disease visible to the clinical and pelvic MRI (or pelvic tomography) and without disease at a distance, through tomography of the chest and abdomen;

(3) Progression / disease free survival, defined as the time from day 1 of the Ch-RT treatment to local or remote relapse, or death from any cause, whichever occurs first. Individuals with no evidence of disease at the time of study analysis will be censored at that point in the analysis.

(4) Proportion of patients without colostomy 12 months after termination of Ch-RT.

(5) Toxicity frequency: Adverse events of grade 2 or higher by the Common Toxicity Criteria for Adverse Events (CTCAE) version 4.0, through study completion.

(6) Incidence of positivity to HPV in tumor tissue through genotyping, through study completion.

(7) Variation of systemic immune parameters, defined by variation in the total number of lymphocytes, neutrophil / lymphocyte ratio (NLR) and lymphocyte / monocyte ratio (RLM) evaluated by comparison of values before pre-and probiotic use and collection values of blood during treatment, already performed in the routine, through study completion.

*Exploratory*

(1) Collection of plasma, leukocytes, faeces and anal swab before and after the use of pre-and probiotic for subsequent analysis of ctDNA (mutation profile and gene fusions), activation profile of immune cells, dosage of cytokines and intestinal microbiota. These biological materials will be stored for future studies, after completeness of the proposed study.

(2) Evaluation of tumor microbiota of paraffin-shaped anatomo-pathological material from ACSCC biopsies filed at the service. This analysis will be carried out in the future, as part of future studies, after completeness of the proposed study.

# Methods:

*Design:*

A randomized prospective, open-label, phase II study of oral prebiotics and probiotics during definitive treatment of Ch-RT for patients with localized ACSCC versus same treatment without the addition of pre-or probiotics. Treatment of Ch-RT (using fluoropyrimidine [5FU or capecitabine] associated with cisplatin or mitomycin as a chemotherapy protocol) will occur according to institutional protocol and no change in standard treatment is planned in this protocol.

Eligible patients who consent to participate in this study will be randomized 1: 1 in two groups:

- Prebiotics and probiotics group, which will receive standard nutritional guidance from the institutional routine and prebiotics in combination with probiotics, starting one (1) week prior to initiation of Ch-RT and daily throughout the treatment up to 6 to 8 weeks post-Ch- moment of response evaluation (primary outcome).
- Control group that will receive standard nutritional guidance only prior to initiating Ch-RT.

Randomization will be simple and conducted independently by clinical trial support staff who are not participating in the study. The random sequence will be generated by computer program.

Both groups will receive nutritional orientation by nutritionist of the institution before randomization, according to institutional routine.

Both groups will be evaluated for: (1) food inventory, which will be conducted by the study research team through food questionnaire (12); (2) assessment of response to treatment as per routine; (3) assessment of metabolic response to the study; (4) assessment of adverse treatment events, as per routine; (4) kinetics of systemic immune parameters (NLR and MLR), according to the analysis of hemograms collected in the routine; (5) HPV tumor screening; (6) collection of biological materials (blood, plasma, feces, anal / rectal swab) that will be stored for future studies. Therefore, the interventions of this study, besides the use of pre- and probiotics, are items (3), (5) and (6).

The following clinical data will be collected prospectively and structured in an electronic database: sex, age at start of use of pre-and probiotics, clinical staging (according to institutional routine), HIV status, smoking, alcoholism, comorbidities such as any clinical condition requiring pharmacological therapy), concomitant medications of chronic use, use of antibiotics and / or anti-inflammatories within 30 days or during the study, body mass index prior to the use of pre-probiotics, laboratory parameters (blood count, performance status, symptoms associated with ACSCC, QT regimen, type of RT technique (IMRT vs 3D), adverse events during Ch-RT, results of symptomatic, clinical, radiological and metabolic responses and food questionnaire before, during week 3 of Ch-RT and at the end of the study.

## Study population: eligibility criteria

Patients will be recruited from Clinical Oncology, Coloproctology or Radiotherapy outpatient clinics and must meet all the criteria below Inclusion and none of Exclusion:

*Inclusion criteria*

- Patients older than 18 years;

- Confirmed histological diagnosis of squamous cell carcinoma / squamous cell carcinoma of the anal canal (ACSCC);
- Patients with localized ACSCC (≥ T2N0M0, according to American Joint Committee on cancer 8th edition) staged by conventional imaging methods according to institutional routine;
- Indication of starting definitive treatment with Ch-RT in the institution. HIV-positive patients may be included;
- Free informed consent signed by the patient or legal representative.

*Exclusion Criteria*

- Perianal squamous cell carcinomas;
- Clinical condition leading to difficulty in swallowing;
- Patients with a contraindication to receiving Ch-RT, ie receiving only radiotherapy or not receiving polychemotherapy;
- Clinical condition that, due to the investigator's judgment, prevents adherence to the study
- Active infection requiring antibiotic therapy.

## Intervention

Patients in group A (experimental) will receive Symbioflora®, a food supplement containing probiotics represented by high amounts (109) of bacteria of the genus Bifidobacterium and Lactobacillus and prebiotics composed of non-digestible fruit-oligosaccharides (inulin). This food compound is already used in the A.C. Camargo Cancer Center by the Nutrition team.

Both will be administered orally, in the form of sachets, dissolved in half a glass of water, once a day, preferably at a fixed time, from 7 days (+/- 2 days) before the day 1 cycle 1 of Ch-RT, during the whole treatment of Ch-RT, until 6 to 8 weeks after termination of Ch-RT, when the response evaluation (primary outcome) will be performed. The last day of pre-and probiotic will be the day of MRI (or tomography) of the pelvis.

The investigators will provide dispensing of the pre- and probiotic to start the study, with sufficient amount until the next medical visit, when new dispensation will occur. The evaluation of adherence to the use of pre-and probiotic will be made through questioning during the medical visits and counted in the medical record.

Adverse events associated with the use of Symbioflora® are infrequent and include flatulence, abdominal discomfort and rarely, diarrhea. Patients with severe immunosuppression (not the case of patients who are treated by Ch-RT for ACSCC) may develop intestinal infection by the probiotic component.

Patients may discontinue pre-and probiotic use for any of the following reasons: disease progression, death, adverse event (s) leading to permanent interruption of Ch-RT, pre-and probiotic intolerance, withdrawal of patient consent (or legal representative) or need for new systemic oncologic therapy or surgery.

Patients in both groups will receive nutritional counseling, with a low residue diet, according to standard routine and prior to randomization (Group A) or before Ch-RT (Group B).

*Concomitant medications*

In general, concomitant medications considered necessary for the treatment and safety of the patient are allowed, and their use is documented in the patient's medical records and in the clinical file.

Concomitant administration of investigational medicinal products is not permitted. The use of growth factors is allowed at the discretion of the investigator for patients with neutropenia, according to institutional routine. The use of antibiotics during the study should be avoided as long as it does not interfere with patient safety.

# Evaluation of Outcomes

## Efficiency Outcomes

*Primary Outcome:*

Patients will perform imaging examinations with chest and abdomen CT scans and pelvic NRMs preferably within 21 days (+/- 3 days) of pre-and probiotic use (group A) or isolated standard nutrient orientation (Group B) and in 6-8 weeks after completion of Ch-RT and will be assessed clinically by complete physical examination, including anal inspection and rectal examination, at the same times. Regarding the evaluation of response, patients will be classified, in the evaluation of the primary outcome, with or without complete response (absence of tumor in the clinical examination and by pelvic MRI); however, it will be noted if there is partial response (any reduction), stable disease (same measures) and progression (any growth); we will not use the RECIST criteria because they are not reliable in the primary ACSCC assessment. Patients with contraindication to pelvic MRI may perform pelvic tomography for response evaluations.

Assessment of symptoms that may be related to disease progression will be monitored at each medical visit.

*Secondary Outcomes:*

In addition to evaluating tumor response according to conventional imaging and physical examination already used in the routine, both groups will also be evaluated by metabolic response with 18-FDG PET-CT. PET-CT scans will be performed prior to the

use of pre-and probiotic (within 15 days) and after 6-8 weeks after termination of Ch-RT. The evaluation of metabolic response by PET-CT will be done by a method that takes into account the volume of the capturing lesions and their intensity of uptake, total lesion glycolysis (TLG) (13).

The methodology will be according to a study published by the researcher: 14 TLG is a measure that takes into account the volume and intensity of the uptake, being produced by multiplying the volume of the disease by the average Standardize Uptake Value (SUV) within that volume. It will be calculated using AW Volume Share 5 (General Electric, Waukesha, WI). The minimum values of SUVs used to define the limits of the lesion (s) will be determined by visual analysis and the total volume of interest (VOI) that circumscribes the primary tumor and lymph nodes will be calculated automatically by the switch program. The VOI will be multiplied by the average SUV within that volume. Regions of physiological or inflammatory uptake above the SUV boundaries will be excluded from the images. The TLG value will be calculated for both PET-CT 18-FDG tests and according to published methodology (13). The variation of TLG values between the two examinations will be calculated by subtracting the value of the second TLG from the first one and dividing the result by the value of the first TLG. In patients with no initial uptake of 18-FDG PET-CT (TLG = 0), we will use the symbolic value of TLG = 0.1 to allow calculations of TLG variation.

Patients will also be evaluated for complete clinical and radiological response within 6 months after the termination of Ch-RT and from that moment on, they will initiate routine follow-up (described below) to evaluate disease-free survival outcomes and colostomy at 12 months .

**Toxicities**

For evaluation of adverse events, patients will be seen before the start of the study, within 72h of the pre- and probiotic dispensing (group A) or within 7 days of the initiation of Ch-RT (group B), every 15 days (+/- 3 days) during Ch-RT, according to institutional routine, and at the end of the study (in the evaluation of primary outcomes, 6-8 weeks post Ch-RT) with complete clinical examination and routine laboratory tests (blood count, urea, creatinine and others, if clinically indicated).

Patients who present grade 2 intolerable or grade 3 toxicity will have the treatment dose reduced and / or radiotherapy interrupted as routine. All adverse effects will be graded and evaluated whether or not they are related to treatment. Patients requiring hospitalization should be immediately identified by the Principal Investigator of the study.

As a dietary supplement, we do not expect to see serious adverse events or to lead to intolerance. However, if there is intolerance, the amount of pre-and probiotic will not be allowed to be reduced, but we will recommend discontinuation. In this case, the patient is still in the study, if he wishes.

Adverse events will be assessed according to the Common Adverse Event Toxicity Criteria (CTCAE) version 4.0. An adverse event for the purposes of this protocol is the

appearance of (or worsening of the pre-existing) signal (s), symptom (s) or undesirable medical condition (s) occurring after signature of free and informed consent, even if the event is not considered to be related to the study drug (s). The occurrence of adverse events should be obtained through direct questions to the patient or when they are voluntarily reported by the patient during or between visits or through physical examination or laboratory tests.

Whenever possible, each adverse event should be evaluated to determine:

1. Severity (mild, moderate, severe and life threatening) or (CTCAE grade 4.0)

2. Their relationship to each study drug (suspected / suspected)

3. Its duration (starting and ending dates or continuing in the final exam)

4. Conduct adopted (no dosage adopted, dosage of study drug adjusted / temporarily discontinued, study drug permanently discontinued as a result of said adverse event, concomitant medication given, non-medicated therapy, hospitalization / extended hospitalization).

## Follow-up:

Follow-up begins after evaluation of the primary endpoint, 6 to 8 weeks post Ch-RT, and will occur according to institutional routine, with clinical examination, including anal region and rectal examination, every 8 weeks (+/- 5 days) during the first year and every 6 months (+/- 5 days) from the second year to the fifth year. Abdominal and chest tomography imaging and pelvic resonance imaging will be performed every 8 weeks (+/- 5 days) in the first year and every 6 months (+/- 5 days) in the second and third years, and annual fourth to fifth year.

Cases of persistent disease at 6 months or post Ch-RT recurrence will be discussed, as routine, at Tumor Board meetings.

**Methodology for HPV screening in tumor tissue through genotyping**

The Roche LINEAR ARRAY HPV test will be used, according to the routine of the service. This is an in vitro qualitative test for the detection of Human Papilloma Virus. Thirty-seven HPV genotypes are detected by this method, including high and low risk types [6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52 (MM7), 83 (MM7), 53, 54, 55, 56, 58, 61, 62, 64, 66, 67, 68, 69, 70, 71, 84 (MM8), IS39 and CP6108]. HPVs 33, 35, 52 and 58 make up the region of the so-called high-risk HPV POOL.

The LINEAR ARRAY HPV Genotyping Test is based on four main processes: sample preparation; polymerase chain reaction (PCR) amplification of target DNA using primers for HPV; hybridization of the amplified products with oligonucleotide probes; and detection of the products amplified and connected to the probe, by colorimetric determination.

**Sample Preparation**

HPV DNA is isolated by lysis of epithelial cell samples by denaturation at elevated temperatures. Lysis is performed in the presence of proteinase K, chaotropic agent and detergent. There is isolation and purification of DNA on a column and elution with sigma water. The human β-globin gene is isolated simultaneously and evaluates cell fitness, extraction and amplification of each individually processed sample.

PCR amplification

- Target Selection

The LINEAR ARRAY HPV Genotyping Test uses biotinylated primers to define a nucleotide sequence within the L1 polymorphic region of the HPV genome, which has approximately 450 base pairs. A group of HPV primers present in the Master Mix is designed to amplify HPV DNA from 37 genotypes, including 14 high-risk genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 , 59, 66 and 68). The uptake probe sequences are located in polymorphic regions of L1 bound by these primers. An additional pair of primers targets the human β-globin gene to provide cell fitness control, extraction and amplification.

- Target Amplification

AmpliTaq® DNA Polymerase is used for "hot start" amplification of HPV target DNA and ß-globin control. First, the PCR reaction mixture is heated to activate DNA Polymerase AmpliTaq® Gold, to denature viral DNA and human genomic DNA and to expose the target sequences to the primer. As the mixture cools, the primers bind to the target DNA. AmpliTaq Gold DNA polymerase, in the presence of Mg2 + and excess dNTPs, elongates the primers attached throughout the target templates to produce a target, double-stranded HPV DNA molecule of about 450 base pairs or a molecule of B-globin DNA, 268 base pairs, termed "amplicon". This process is repeated for a number of cycles. The amplification occurs only in the region of the HPV genome or the β-globin gene that is between the primer pair. The whole genome is not amplified.

In the LINEAR ARRAY HPV Genotyping Test, the selective amplification of the target nucleic acid of the sample is achieved by the use of an enzyme AmpErase (uracil-N-glycosylase) and deoxyuridine triphosphate (dUTP). The AmpErase enzyme recognizes and catalyzes the destruction of DNA chains containing deoxyuridine15, but not of deoxythymidine-containing DNA strands. Deoxyuridine is not found in nature DNA but is always present in the amplicon due to the use of deoxyuridine triphosphate in addition to the deoxythymidine triphosphate in the Master Mix reagent; therefore, the amplicon is the only one containing deoxyuridine. Deoxyuridine makes the contaminating amplicon susceptible to destruction by the enzyme AmpErase prior to amplification of the target DNA. The enzyme AmpErase, which is in the Master Mix reagent, catalyzes cleavage of deoxyuridine-containing DNA into deoxyuridine residues by opening the deoxyribose chain at the C1 position. When heated, in the first step of thermal amplification, at the alkaline pH of the Master Mix, the amplicon DNA strand breaks at the deoxyuridine position, thereby rendering the DNA non-amplifiable. The AmpErase enzyme is inactivated at temperatures above 55øC, i.e., during the thermal amplification steps, there is no destruction of the target amplicon. After amplification,

any residual enzyme is denatured by the addition of the Denaturation Solution, thereby preventing the degradation of any target amplicon.

Hybridization Reaction

After PCR amplification, HPV and β-globin amplicons are chemically denatured to form a single stranded DNA, by the addition of Denaturation Solution. The denatured amplicon aliquots are transferred to the appropriate tray of the typing tray, which contains hybridization buffer and a single LINEAR ARRAY HPV Genotyping Strip, which is coated with probes bands for the HPV and β-globin types. The biotin labeled amplicon will only hybridize to the oligonucleotide probes if the amplicon contains the corresponding sequence of the complementary probe.

Detection Reaction

Upon completion of the hybridization reaction, the LINEAR ARRAY HPV Genotyping Strip is thoroughly washed to remove any unbound material. Streptavidin-Horseradish Peroxidase Conjugate is added to the strip. The Streptavidin-Horseradish Peroxidase Conjugate binds to the biotin-labeled amplicon hybridized with the oligonucleotide probes, which are present in the strand. The strip is washed to remove Streptavidin-Uncoated Strontium Peroxidase Conjugate and a substrate solution containing hydrogen peroxide and 3,3 ', 5,5'-tetramethylbenzidine (TMB) is added to each strip. In the presence of hydrogen peroxide, streptavidin-horseradish peroxidase catalyzes the oxidation of TMB to form a blue staining complex, which precipitates at the positions of the probe where hybridization occurs. The LINEAR ARRAY HPV Genotyping Strip is visually read by comparing the blue stripe pattern with the LINEAR ARRAY HPV Genotyping Test Reference Guide.

**Exploratory Studies**

As part of this project, we will collect the following biological materials for further studies, after this study complete the recruitment and evaluation of the primary outcome of all patients included.

1. We will collect a sample of 10 ml of peripheral blood pre-study, before the start of the use of pre- and probiotics, in the evaluation of response of 6-8 weeks and in 6 months post-Ch-RT. This collection will configure a liquid biopsy that will investigate the mutation and gene fusions and mutational load (TMB) profile in the samples. TMB analysis will be performed on circulating tumor DNA and also on pre-treatment paraffin-shaped tumor material (biopsy of the diagnostic routine). Both the mutation profile and the gene fusions profile and the BMR will be correlated with the clinical outcomes.

2. Collection of anal / rectal swabs and feces for microbiota analysis at the same times as blood collections, with subsequent comparison with tumor microflora of stored paraffin wax pretreatment. We will also compare the pre- and post-use microbiota of group A pre-and probiotics, as well as microbiota variations in group B. We will evaluate if the anal and intestinal microbiota profile correlates with clinical endpoints and systemic immune parameters (NLR and MLR ). This study will be

important to evaluate if the engraftment of the microbiota of the probiotics correlates with the outcome of the patients.

3. Collection of 5 ml of blood at the same times as (1) for plasma separation for analysis of inflammatory cytokines and leukocytes for immunophenotyping by flow cytometry. The findings will also be correlated with clinical outcomes.

## Statistical Considerations

*Statistical plan*

Descriptive statistics will be used to report the results of categorical and continuous variables. The time-to-event variables will be reported in medians and Kaplan-Meier curves. The degrees of toxicity will be tabulated. The number of patients who discontinue treatment for any reason will be presented.

Efficacy and toxicity analyzes will be performed by intention to treat. Patients receiving at least one pre-and probiotic dose in addition to day 1 cycle 1 of Ch-RT in group A and at least day 1 cycle 1 of Ch-RT in group B will be evaluated for toxicity and efficacy.

Inferential analyzes will be performed according to the type of variable of interest and if the comparison is intra- or intergroup. For the analysis of the primary endpoint, complete response rate at 6-8 weeks, as well as comparisons between groups of 6-month response rates and 12-month colostomy-free survival and frequency of toxicities, we will use the Chi-square test . For comparative analyzes between groups regarding disease-free survival, we will use a non-parametric log-rank test. For intra-group comparisons, for example, the kinetics of NLR and MLR, we will use the ANOVA test. For the metabolic response analyzes, the TLGs of each pre- and post-intervention patient will be compared by paired T test and unpaired T test, when the TLG drop comparison is intergroup. The TLG drop values most associated with complete clinical and radiological response at 6-8 weeks and at 6 months post Ch-RT will be presented by percentage drop of TLG and will be determined by ROC curves.

In order to minimize possible effects of imbalances between groups on prognostic characteristics that may influence the outcome of the primary endpoint, we will conduct univariate and multivariate logistic regression analyzes, adjusting the analysis of the primary endpoint for other prognostic factors in addition to the pre-e probiotics. These factors will be: clinical stage (T2N0 vs T3 / 4 or N +), sex, HIV status, age at diagnosis, presence of comorbidities, need for interruption of radiotherapy, and depending on the number of negative cases, HPV status. Values of $p < 0.20$ in the univariate analysis will be considered significant for the variable to enter the multivariate model.

For all inferential analyzes, bicaudate values of $p < 0.05$ will be considered statistically significant.

*Sample Size Calculation*

The study sample will be 75 patients. For this calculation, we considered H0 of complete clinical and radiological response at 6-8 weeks post-Ch-RT of 70%, based on literature data (3), H1 as 90%, type I bicaudate error of 10 %, 80% power and loss of follow-up / non-adherence of 10%.

## Recruitment and Study Duration

In the A. C. Camargo Cancer Center, 30 to 35 patients with localized ACSCC are treated with Ch-RT annually. Therefore, the estimated recruitment time is 3 years, with an average of 2 patients per month, plus an 8-week follow-up for each patient to evaluate the outcome. Thus, it is expected to complete the study in 3.5 years from the first included patient. The patients included will be followed, according to institutional routine, for up to 5 years, with collection of clinical data for disease-free or colostomy-free survival outcomes.

## Ethical Considerations

The study will be conducted in accordance with the protocol, Good Clinical Practice guidelines of the International Conference on Harmonization (ICH GCP) and applicable local laws and regulatory requirements. The Free and Informed Consent Term (ICF) must be in accordance with ICH GCP guidelines, local regulatory regulations, and legal requirements.

The investigator should ensure that each study patient, or his / her legal representative, is fully informed about the nature and objectives of the study and possible risks associated with participation. The investigator will obtain the written consent of each patient before any specific study activity is performed. The investigator will retain one path of each consent form signed by the patient. It will be emphasized that participation is voluntary and that the patient has the right to refuse participation or to leave in the middle of the study whenever he wishes. This will not impair subsequent care to the patient. Female patients will be instructed not to become pregnant during the treatment of Ch-RT, being advised to use contraceptive methods if of childbearing age, as already done in routine. There is a risk of loss of confidentiality if patient data are identified, however, all appropriate measures will be taken to prevent this from happening.

The protocol was approved by the Local Ethics Committee. The study will be enrolled in the ClinicalTrial.gov international clinical trial bank.

## Funding and Sponsorship of the Study

The patient will not incur any costs in relation to the use of pre-and probiotics or the 18-FDG PET-CT, HPV research in biopsies or collections of biological samples. These will be funded by development. All of the rest (Ch-RT, laboratory tests, conventional imaging, medical appointments and nutritional counseling) is not part of this protocol because it is already part of the ACSCC patient care routine at the AC Camargo Cancer Center, paid by the paying source, that is, the patient's medical covenant or the Unified Health System.

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**A randomized phase II study of the administration of prebiotics and probiotics during definitive treatment with chemotherapy-radiotherapy for patients with squamous cell carcinoma of the anal canal (BISQUIT). ID: 2625-18**

## INFORMED CONSENT FORM

## IDENTIFICATION DATA FOR THE SUBJECT OF THE RESEARCH OR LEGAL ENTITY

**1**.NAME.:..... .....

IDENTITY DOCUMENT Nº: ..... SEX: M □ F □

BIRTH DATE: ...../...../.....

ADRESS ..... Nº .....

APT: .....

DISTRICT:..... CITY.....

ZIP:..... PHONE: (.....) .....

**2**.LEGAL RESPONSIBLE .....

TYPE (degree of kinship, tutor, healer, etc.) .....

IDENTITY DOCUMENT Nº:.....SEX: M □ F □

BIRTH DATE.: ...../...../.....

ADRESS: ..... Nº .....

APT: .....

DISTRICT: .....

CITY: .....

ZIP: .....

PHONE: (.....).....

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March, 05, 2019

## RESEARCH DATA

1. **TITLE OF THE RESEARCH PROTOCOL** "Randomized phase II study of prebiotic and probiotic administration during definitive treatment with chemoradiotherapy of patients with squamous cell carcinoma of the anal canal."
2. **RESEARCHER:** RACHEL SIMÕES PIMENTA RIECHELMANN

POSITION / FUNCTION: Director of the Clinical Oncology Department of the AC Camargo Cancer Center REGIONAL COUNCIL INSCRIPTION Nº 98131

**3. ARESEARCH RISK ASSESSMENT:**

| | | | |
|---|---|---|---|
| MINIMUM RISK | □ | AVERAGE RISK | □ |
| LOW RISK | X | HIGH RISK | □ |

**4. RESEARCH DURATION:** 3,5 YEARS

**1 – Study design and objective (s)**

You are being invited to participate in this clinical study to be performed at AC Camargo Cancer Center, São Paulo.

You have the diagnosis of localized anal cancer (without involvement of other organs); this tumor is located in the region of the anus and can cause pain, difficulty to evacuate as well as bleeding.

The treatment best considered for localized anal cancer is chemotherapy along with radiation therapy. Chemotherapy is performed with the application of drugs into the vein (cisplatin or mitomycin on the first day and 5-fluorouracil from the first to the fourth day), in the first and last week of radiotherapy. It may be used in place of 5-fluorouracil capecitabine, chemotherapy tablets taken every day that radiotherapy is done. Radiation therapy applies a dose of radiation daily into the anus and basin region by on average for 6/7 weeks. This treatment is standard in this and in several institutions in the country and the world for being the one that offers greater chance of cure. However, approximately 20 to 25% of patients are not cured with chemo-radiotherapy and therefore, research is important to understand about anal cancer.

The reason for this study is to evaluate whether some microorganisms, given as dietary supplements, can improve the response in the treatment of anal cancer and thus its cure. These supplements are called prebiotics and probiotics.

Probiotics are beneficial bacteria that live in the gut and improve the overall health of the body, bringing benefits, such as facilitating digestion and absorption of nutrients, and strengthen the immune system. When the intestinal flora is in imbalance, the intestine ends up being populated by pathogenic or "bad" bacteria, which compromise the immune system and leave the organism susceptible to diseases. Prebiotics are nondigestible foods and fibers that have the function of stimulating growth and / or activating the metabolism of some beneficial gut bacteria. In this way, prebiotics constitute the "food" of probiotic bacteria.

In this study for which you are being invited to participate voluntarily, we aim to evaluate the benefit (fewer side effects, better treatment response and cure), the use of prebiotics and probiotics given during chemo- radiotherapy for anal cancer. We idealized this study because, despite the scientific logic, we do not know if probiotics prebiotics benefit anal cancer patients.

**2 – Procedures to be carried out and their purposes**

All patients who agree to participate in the study will receive, in addition to standard chemo-radiotherapy treatment, nutritional counseling and will be randomly selected to receive prebiotics and probiotics.

You will be in one of the groups below:

Prebiotics and probiotics group: They will receive standard nutritional guidance from the institutional routine and prebiotics in combination with probiotics. Both will be administered orally as a sachet dissolved in water once a day, preferably on a fixed schedule, starting 7 days before the first day of chemo-radiotherapy, throughout the

chemo-radiotherapy treatment, and up to 6 at 8 weeks after completion of chemo-radiotherapy; therefore, total time of approximately 3 months.

Control group: Patients will not receive probiotics or prebiotics, only standard nutritional guidance.

To evaluate response to treatment, blood and imaging tests with chest and abdomen tomography and pelvic resonance imaging, PET-CT 18-FDG scan, will be performed prior to the start of treatment and at 6 to 8 weeks post-completion of chemo-radiotherapy; blood tests, CT scans and MRI scans are already part of the AC Camargo Cancer Center routine.

The PET-CT scan will be done for this study. The PET-CT scan is a safe examination that is used in the routine treatment of various types of tumors and evaluates the whole body for the presence of tumor (s). We believe this test to be helpful in assessing the location of your tumor and also how much it decreases after conventional chemoradiotherapy treatment.

We will also collect samples and blood, feces and secretion from the intestine and anus at three times, and HPV virus research on the biopsy material that you have already done. The purpose of these collections is to further study the effects of this dietary supplement on the chance of cure of anal cancer and to understand more about the origin of anal cancer, that is, because some people develop this tumor and others do not, and because some heal and some don't.

**3 – List of study procedures (out of routine) and how they are performed**

- Prebiotics and probiotics (group A only): a sachet dissolved in a glass of water once a day, starting one week before chemo-radiotherapy, during chemo-radiotherapy, and for 6 to 8 weeks after chemotherapy, in total approximately 3 months.
- Blood test with approximately 10 ml (2 tablespoons) of blood withdrawn per arm vein puncture before treatment started at 6 to 8 weeks and at 6 months after chemoradiotherapy.

In these blood samples, we will investigate the profile of tumor mutations, inflammatory cells and hormones to better understand anal cancer

- Anal swab examination (collection of swab secretion) and stool collection before treatment started 6 to 8 weeks and 6 months after the end of chemo-radiotherapy

In this analysis, in the future, we will investigate the composition of the bacteria in your gut and whether this helps us to identify reasons why some patients are not cured.

- PET-CT scan 18-FDG scan: before treatment begins and 6 to 8 weeks after chemoradiotherapy termination.

The PET-CT scan will assess the location and presence of metastases (tumor spread to other organs) and also the response to chemo-radiotherapy treatment (tumor reduction).

**4 – Description of the discomforts and risks expected in the procedures;**

The risks associated with treatment will be described below.

Prebiotics and probiotics (Group A): Since these compounds are dietary supplements, the risks of using them are minimal.

- Common (> 10%): flatus enlargement, colic / abdominal discomfort, abdominal distension

- Uncommon or rare (<10%): diarrhea, intestinal infection, allergic reaction

PET-CT scan 18-FDG

- Image examination that is already used routinely for patients with other tumors; as it requires intravenous contrast, some patients may have allergic reactions. Tell your doctor about any allergies you may have experienced in the past.

**5 – Risks related to pregnancy:**

Female patients of childbearing age will be advised not to become pregnant during the study. For them, a pregnancy test will be done before the start of treatment. If you become pregnant during your study participation, you should notify the study doctor immediately.

**6 – Benefits for the participant**

The hypothesis of the study is that the use of prebiotics and probiotics increases the chance of cure and improves the side effects of chemo-radiotherapy. This may or may not happen. PET-CT scanning may be useful for assessing tumor extension. Regardless of the results of this study, other patients in the future may benefit from the information obtained here.

**7 – Alternative Treatments**

There are no alternative treatments to the use of prebiotics or probiotics.

**8- Payment to research subject**

Participation in this study will not incur any additional cost to you and no payment will be made if you agree to participate in this study. You are entitled to damages if you incur damages associated with the study.

**9 – Voluntary participation / Study discontinuation**

Participation in this study is entirely voluntary (you decide whether you want to be a part or not). Even if you decide to participate in the study, you can leave it at any time, without giving explanations for it, and may even refuse to publish data collected about you. If this occurs the doctors no longer have collected the data about you, but may publish non-personal information collected before the cancellation. This decision will not affect your future medical treatment in any way.

The study doctor may also remove you from this study if you feel that this is in your best interest after discussing and explaining the reasons to you, or in case the study is interrupted earlier than planned because it is considered unsafe.

**10 – Confidentiality**

If you choose to participate in this study, your health information and registration of your participation will be kept confidential and confidential. Researchers will identify you through a unique number and through the initials of your name (not using your full

name). A copy of this informed consent will be filed in your AC Camargo Cancer Center medical record. However, there is a risk of loss of confidentiality; we will take all possible steps to prevent this from happening.

**11 – Access Guarantee**

Questions about procedures should be directed directly to the researchers listed at the end of this consent form.

**12 – Signatures**

I confirm that I read the Term of Free and Informed Consent and had the opportunity to clarify all my doubts related to this study. I understand that if I have any additional questions in the future regarding the study or my participation in it, I may contact the telephone 11- 2189-5000

Through my signature, I agree to participate in this study as a volunteer. I received a copy of this Informed Consent Form.

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Name of Participant (letter of form) Date ___/___/___

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Signature of Participant

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Name of Investigator (letter of form) Date ___/___/___

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Signature of the Investigator

If witness or legal representative is required:

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Signature of witness / legal representative Date ___/___/___

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Signature of witness / legal representative

**Contact with researchers:**

AC Camargo Cancer Center
Rua Professor Antonio Prudente n 211, São Paulo-SP, Phone - +55(11) 2189-5000.