Helicobacter Pylori Eradication Therapy in Portugal: Prospective, randomized, blind and multicentre trial on the efficacy of quadruple therapies and their clinical impact, and immunological and gut microbiota changes

Study Protocol and Statistical Analysis Plan

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Keywords: Helicobacter pylori, Eradication therapy, Gut microbiota, Immunology

Abstract

Introduction: Helicobacter pylori (H. pylori) infection remains a major public health problem, with an estimated prevalence of over 50% worldwide and 60-86% for Portugal. H. pylori is associated with significant morbidity and mortality from peptic ulcerative disease to gastric cancer, whose eradication therapy has proven to be effective in preventing these complications. Factors involved in the development of these conditions include H. pylori virulence, host genetic factors and gut microbiota. Given the increasing pattern of antibiotic resistance evidenced by this bacterium and the scarcity of available antibiotic therapy, both in Portugal and worldwide, there is not enough evidence on the best eradication strategy. Regarding the uncertainties about the potential negative impact of indiscriminate use of eradication therapy on gut microbiota, either by proton pump inhibitors or by antibiotics per se, there is an overriding need for evidence about the real impact of this therapy on gut flora and possible clinical consequences in immunological, metabolic, nutritional and oncological terms. Objectives: Comparative evaluation of the efficacy of the different quadruple therapy regimens recommended for the H. pylori eradication. Comparative evaluation of the safety profile in terms of clinical, immunological and gut microbiota impact of the different therapies for the H. pylori eradication. Methods: Prospective longitudinal multicentre study of total of patients with gastric infection by H. pylori, diagnosed by 13C-urea breath test or histological analysis of gastric biopsies and clinical indication for its eradication, referred to the different participating Portuguese hospital units and a blind randomized controlled clinical trial of the efficacy and safety of the different quadruple therapy regimes recommended for the H. pylori eradication. This study will be carried out in 4 phases: Phase 1 - Recruitment and randomization of patients by the different quadruple eradication schemes with and without bismuth (5 parallel arms); Phase 2 - H. pylori eradication with evaluation of the efficacy and safety rates at 1 month and the absence of reinfection at 12 months after treatment and collection of stool samples before and after the eradication therapy for evaluation of changes in gut microbiota; Phase 3 - Analysis of richness, diversity and uniformity of gut microbiota by DNA sequencing using the hypervariable region of the ribosomal 16S bacteria gene as a taxonomic identification marker and their clinical impact on immunology, metabolism and nutrition at 12 months after the H. pylori eradication therapy; and Phase 4 - Analysis of immunological changes through the study of cell populations by flow cytometry (CD4+, CD8+, B-cell, T-cell, natural killer cells, cells ratio) and cytokines, chemokines and growth factors by xMAP/Luminex before and 12 months after the H. pylori eradication therapy. Expected results, impact and scientific outputs: Given the high rate of triple therapy inefficacy, high antibiotic resistance and the scarcity and controversy of existing literature on quadruple regimens, there may be relevant differences in the approved quadruple regimens for the H. pylori eradication, being necessary to define which is the most effective and safe in Portugal, decreasing the rate of ineffectiveness and exposure to multiple antibiotics. The homeostasis of gut microbiota is significantly changed after H. pylori eradication and this modification may be

substantially different according to the therapeutic scheme used, with clinical implications on immunology, metabolism and nutrition. Thus, a randomized trial to compare quadruple regimens is need, allowing in the future, an individualized selection of the H. pylori eradication regimen, taking into account the higher efficacy and safety and lower gut dysbiosis and its systemic consequences, in short and long term. Modulating gut microbiota therapies, including prebiotics, probiotics, symbiotics, fecal microbiota transplantation and perhaps targeted-immunotherapy may be beneficial as adjuvant therapy to existing H. pylori eradication regimens, in a systematic way or for some therapeutic regimes or risk groups.

Literature Review

Helicobacter pylori (H. pylori) infection remains a major public health problem, affecting over 50% of the worldwide population and 60-86.4% of the Portuguese population.1,2 This bacterium is responsible for chronic active gastritis, likely to develop into peptic ulcerative disease, atrophic gastritis, gastric adenocarcinoma and mucosal-associated lymphoid tissue lymphoma.3,4 Factors involved in the development of these conditions include bacterial virulence, host genetic factors and gut microbiota.3,4 H. pylori eradication therapy has been shown to be effective in preventing the development or recurrence of these complications.3,5 However, the increase in primary and secondary resistance of this bacterium to most antibiotics has reduced the efficacy of this therapeutic strategy. As in the rest of Europe, overall resistance to multiple antibiotics is high in Portugal, with a prevalence of 42-50% for clarithromycin, 25-34.4% for metronidazole, 18-33.9% for levofloxacin, 0.2-0.6% for tetracycline, 0.1-0.6% for amoxicillin and 20% for clarithromycin and metronidazole.6-8 Taking into account the pattern of H. pylori antibiotic resistance in Portugal and the selection flowchart proposed by [GrLeWu14],9 the currently recommended therapeutic regimens include quadruple therapies: sequential, hybrid, concomitant with bismuth and concomitant bismuth-free.3,5,7,10-12 However, some studies including quadruple schemes have shown unsatisfactory results, not exceeding 90% considered as the minimum efficacy rate.12,13 Given the high rate of resistance of H. pylori to nitroimidazoles registered in Portugal, it would be worth considering therapeutic optimization with metronidazole 500mg tid and not only bid.3 Thus, at present, there are no solid data that allow us to state the best therapeutic option in this country, being essential to conduct a randomized study to definitively resolve this uncertain. Animal and human studies have shown that H. pylori results in changes in gut microbiota.4,14,15 Human gut microbiota represents a dynamic and complex ecosystem in close linking with the immune system, regulating gastrointestinal homeostasis. It is composed mostly of Gram-negative anaerobic bacilli, mainly Bacteroidetes and Fusobacteria. Gut dysbiosis has been associated with immune, metabolic, nutritional and oncological changes, such as the development of several gastrointestinal and systemic conditions such as inflammatory bowel disease, obesity, diabetes mellitus, metabolic syndrome, dyslipidemia, asthma and allergies.16-18 However, the interactions between H. pylori, gut microbiota and host pathophysiology are still far from being understood. Similarly, there are also uncertainties regarding the potential negative impact of indiscriminate use of H. pylori eradication therapy on the gut microbiota, mediated by either proton pump inhibitors or antibiotherapy.17,19 In some recent studies, eradication of H. pylori has been associated with a decrease in butyrate-producing bacteria (such as Faecalibacterium prausnitzii), Proteobacteria, Actinobacteria and Firmicutes, and an increase in several potentially harmful bacteria such as Bacteroidetes and the Bacteroidetes:Firmicutes ratio.4,19 These changes persist until 12-18 months after H. pylori eradication therapy,16 although in one study changes in gut microbiota

were documented up to 4 years after eradication.20 Given that only approximately 20% of H. pylori infected individuals will develop complications21 and the potentially harmful effects of antibiotherapy use on the gut microbiota, further studies are needed to understand the complex interactions between the H. pylori-gut microbiota-host axis in order to identify individuals at increased risk of long-term sequelae.19 H. pylori gastric infection can induces humoral and cellular immune responses. Some studies showed an increase of Th1 and Th17 cells into the gastric mucosa and activation of humoral immunity, Th2 polarization and some cytokines such as $IL1_{\beta}$, Il4 and IL5 in the peripheral blood, during H. pylori infection.22-26 Data on immunological changes after eradication of H. pylori are very scarce, with one study showing a downregulation of the Th1 cytokines, a prolonged Th2 response with persistent raised IL4 levels and markedly reduction of the T-regulatory response.27 To date, there is a lack of evidence on the impact of H. pylori eradication therapy, as well as the comparative impact of different therapeutic regimens used in gut microbiota, immunological changes and their implication in clinical practice.

Plan and Methods

A. Research hypothesis

The authors hypothesize that there are relevant differences in the quadruple schemes that can be used in the H. pylori eradication therapy, being necessary to define which of them is the most effective in Portugal, in order to decrease the failure rate and subsequent exposure to potentially harmful effects of antibiotics. Additionally, the authors assume that homeostasis of gut microbiota is significantly changed after H. pylori eradication therapy and these alterations may not be similar among the different recommended therapeutic regimes. Likewise, these alterations may have clinical implications in the immunological, metabolic and nutritional status of individuals submitted to this therapy. Hypothesis: There is a quadruple H. pylori eradication therapy associated with a higher efficacy and safety than other currently approved quadruple regimens in terms of clinical, immunological and microbiota changes in H. pylori gastric infection.

B. Objectives

• To determine and compare the efficacy of the different therapeutic regimens recommended as H. pylori eradication therapy;

• Comparative evaluation of the safety profile in terms of clinical, immunological and gut microbiota impact of the different recommended therapies for the H. pylori eradication:

• To evaluate the changes in gut microbiota after the H. pylori eradication therapy;

• Compare the changes in gut microbiota induced by the different therapeutic regimens for H. pylori eradication and evaluate their clinical implications on immunology, nutrition and metabolism;

• To evaluate the immunological changes after the H. pylori eradication therapy;

• Compare the immunological changes induced by the different therapeutic regimens for H. pylori eradication and evaluate their clinical implications.

C. Expected results, study clinical impact and scientific outputs

Given the high rate of triple therapy inefficacy, high antibiotic resistance and the scarcity and controversy of existing literature on quadruple regimens, there may be relevant differences in the approved quadruple regimens for the H. pylori eradication, being necessary to define which is the most effective and safe in Portugal, decreasing the rate of ineffectiveness and exposure to multiple antibiotics. The homeostasis of gut microbiota is significantly changed after H. pylori eradication and this modification may be substantially different according to the therapeutic scheme used, with clinical implications on immunology, metabolism and nutrition. Thus, a randomized trial to compare quadruple regimens is need, allowing in the future, an individualized selection of the H. pylori eradication regimen, taking into account the higher efficacy and safety and lower gut dysbiosis and its systemic consequences, in short and long term. Modulating gut microbiota therapies, including prebiotics, probiotics, symbiotics, fecal microbiota transplantation and perhaps targeted-immunotherapy may be beneficial as adjuvant therapy to existing H. pylori eradication regimens, in a systematic way or for some therapeutic regimes or risk groups.

D. Methodology and Phases of project development

D.1 Type of study

Prospective longitudinal multicentre study and a blind randomized controlled trial involving several hospital units in Portugal. Regarding the evaluation of gut microbiota and immunological changes, the study will be blinded, since the researchers responsible for sequencing and immunological analysis will not know the therapeutic scheme applied. The trial will be registered on the https://clinicaltrials.gov platform.

D.2 Study population

Patients with gastric infection by H. pylori, diagnosed by 13C-urea breath test or histological analysis of gastric biopsies and clinical indication for its eradication.

D.3 Study design

This project will be carried out in 4 distinct phases:

Phase 1. Recruitment and randomization of patients by the different H. pylori eradication schemes (5 parallel arms)

A total of patients with gastric infection by H. pylori and clinical indication for its eradication, who have never had previous H. pylori eradication therapy, referred to the Gastroenterology Departments of the different participating Portuguese hospital centres (Centro Hospitalar e Universitário de Coimbra, Francisco Gentil Portuguese Oncology Institute of Coimbra and Leiria Hospital Centre), will be included in the study. Patients' selection will taking into account the inclusion and exclusion criteria and the minimum number of patients required in order to obtain statistical power. Inclusion criteria: Gastric infection by H. pylori. H. pylori status will be determined by histological examination of gastric biopsies or carbon 13-labeled urea breath test. The breath test will be considered positive if [13CO2] exceeds the baseline value by > 4 parts per thousand (> 0.4%) after 30 minutes. Histological examination for H. pylori will be considered positive when this bacterium is detected in ≥1 biopsies (staining with hematoxylin-eosin, modified Giemsa and/or Warthin-Starry). Exclusion criteria: Age < 18 years; Pregnant, breast-feeding or women of childbearing age who do not comply with effective anticonception measures; History of allergy, hypersensitivity or contraindication to the use of H. pylori eradication drugs (antibiotics or proton pump inhibitors); History of previous gastrointestinal

surgery or neoplasia; Previous H. pylori eradication therapies; Antibiotic or probiotic therapies in the month prior to recruitment; Use of proton pump inhibitors, other antacids or gastric mucosal protection agents in the 2 weeks prior to recruitment; Corticosteroids or immunomodulatory therapy in the month prior to recruitment; Immunodeficiency; Insulin-treated diabetes mellitus; Obesity (Body mass index ≥30Kg/m2); Use of laxative therapy in the 15 days prior to recruitment; Decompensated heart, liver, kidney or respiratory diseases and; Refusal or inability to give informed consent. H. pylori eradication schemes: Each patient will randomly follow one of the five H. pylori eradication schemes (5 parallel arms): sequential, hybrid, concomitant with bismuth and two concomitants bismuth-free. Sample size calculation: Assuming the estimated prevalence of H. pylori gastric infection in Portugal in adults is 86.4%1, it was determined that for a 95% confidence level and a 10% sampling error it will be necessary to include 46 patients in each of the 5 arms of the study, making a total of 230 patients.

Phase 2. Eradication of H. pylori and collection of stool an blood samples

Stool samples shall be collected from patients and analyzed on 3 occasions: before (baseline), after stopping treatment (Day 10 or 14) and 1 month after treatment (Day 40 or 44). Blood samples will be collected and analyzed on 2 occasions: before (baseline) and 12 months after treatment. Efficacy and safety rates of H. pylori eradication will be determined for each therapeutic regimen. Carbon 13-labeled urea breath test or upper gastrointestinal endoscopy with biopsies will be performed at 1 month (D40 or D44) and 12 months after treatment. The clinical data, adherence and therapy-related adverse events will be recorded at baseline and during follow-up at 1 and 12 months post-eradication.

Phase 3. Analysis of gut microbiota changes by DNA sequencing after H. pylori eradication therapy and their clinical impact on immunology, metabolism and nutrition

The impact of H. pylori eradication therapy on gut microbiota will be studied by next generation genome sequencing techniques. A comparative analysis will be performed for each therapeutic regimen used. Microbiota changes found will be compared with clinical and analytical data in order to establish their impact on immunology, metabolism and nutrition status at 1 and 12 months post-eradication. Phase 4. Analysis of immunological changes of cell populations, cytokines, chemokines and growth factors after H. pylori eradication therapy and their clinical impact The impact of H. pylori eradication therapy on humoral and cellular immunity will be studied by flow cytometry and xAMP/Luminex. A comparative analysis will be performed for each therapeutic regimen used. Immunological changes found will be compared with clinical and analytical data in order to establish their clinical impact at 12 months post-eradication.

D.4 Statistical Analysis

Using the Statistical Package for the Social Sciences (SPSS) version 22.0 (IL, USA). The level of statistical significance will be for a p-value < 0.05. The normality of data distribution will be evaluated by Kolmogorov-Smirnov or Shapiro-Wilk tests. Continuous variables will be expressed as mean and standard deviation or median and interquartile range and categorical variables as frequency and percentage. The comparison of continuous variables will be made using the Kruskal-Wallis test or Anova test and categorical variables using the Qui-squared test. For the comparison of paired data the Wilcoxon test or Friedman test and the t-Student or Anova test will be used. Further statistical analysis of H. pylori eradication efficacy, gut microbiota and immunological changes is described in Tasks section. The genome sequences will be grouped in OTU according to their similarity. The alpha taxonomic richness and diversity of fecal samples will be expressed in observed species, Sobs index and Shannon index. The composition of fecal

microbiota will be expressed in rarefaction curves and the comparison of the differences in OTU richness with the t-Welch test. Immunological findings will be expressed by radar charts and representative FACS dot-plots. Additional statistical analyses will be defined throughout the study.

D.5 Ethical aspects

The project will be subject to the standards of good clinical practice and will at all times comply with the ethical precepts in the World Medical Association Helsinki's Declaration and updates, including the Oviedo agreement. This study was approved by Ethical Committee of University of Coimbra (ref.n.º CE-001/2019). Written informed consent will be obtained from all patients. The confidentiality of the data will be respected at all times, by means of the anonymity of the data in the database, in accordance with General Data Protection (UE 2016/679) and updates.

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