STOP HPV study protocol: a nationwide case–control study of the association between oropharyngeal cancer and human papillomavirus (HPV) infection in Brazil

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ABSTRACT

Introduction Human papillomavirus (HPV) is the most common sexually transmitted infection and is associated with several types of cancer. The number of cases of HPV-associated head and neck squamous cell carcinomas (HNSCCs), especially oropharyngeal carcinomas, has increased significantly in recent years despite decreased tobacco smoking rates. Currently, no data concerning the risk factors and prevalence of HPV in HNSCC patients in all regions of Brazil are available, making it difficult to promote advances in this field of public health. Therefore, our goal is to determine the impact of infection by HPV, including HPVs with different genotypes, on head and neck cancer and the risk factors associated with the development of head and neck cancer in all regions of Brazil.

Methods and analysis This is a case–control study that will include 622 patients and 622 controls from all regions of Brazil. A questionnaire will be applied to gather information on sociodemographic, behavioural and health factors. Oral, cervical or penile/scrotal, and anal specimens and serum samples will be collected from all participants. Formalin-fixed paraffin-embedded tissue from tumour biopsies will be analysed only in the case group. Molecular and serological analyses will be performed to evaluate the presence and role of HPV in the development of head and neck cancer.

Ethics and dissemination This project was approved by the research ethical committee of the proposing institution (Hospital Moinhos de Vento, number 2.852.060). Ethical approval from the collaborators is currently under evaluation and is not yet complete. The results of this study will be presented at meetings with the Brazilian Ministry of Health through technical reports and to the scientific community at national and international events, with subsequent publication of scientific articles.

INTRODUCTION

It is estimated that viral infections are responsible for 15% of all human cancers. Human papillomavirus (HPV) has received attention in this field due its identification as the causative agent of several types of cancer, such as cervical, penile, vulvar and anal cancer as well as head and neck squamous cell carcinomas (HNSCCs), specifically oropharyngeal cancers.

Even with the overall reduction in cigarette smoking and alcohol consumption, the number of HPV-associated head and neck cancer cases has increased by approximately 5% per year, reaching epidemic levels, especially in developed countries and in young men. In the USA, oropharyngeal squamous cell carcinoma is the most common HPV-associated cancer, and it is estimated that by 2020, the number of oropharyngeal cancer cases will exceed those of uterine and cervical cancer cases. The development of HNSCC is a multifactorial process that results in

Strengths and limitations of this study

► An evaluation of the association between human papillomavirus (HPV) and head and neck cancer in all geographical regions of Brazil will be conducted, and different sites as well as tissue and serum antibodies will be analysed.

► A broad evaluation of the different risk factors associated with head and neck cancer will be conducted.

► An evaluation of sexual partners will allow the identification of HPV transmission pathways.

► Some data, such as data on diet and sexual behaviour, will be gathered retrospectively and will thus be prone to recall bias.

► Although we are including patients from the entire country, we cannot exclude selection bias due to overexposure to certain risk factors.

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distinct clinical–pathological characteristics in tumours associated or not associated with HPV.

The increased incidence of this type of HPV-induced cancer has been associated with changes in sexual behaviour, with a fourfold increase in the incidence rate among men.7 Since HPV is the most common sexually transmitted infection (STI), contamination of the oropharyngeal region can occur through vaginal, anal or oral sexual contact.8,9

In some individuals, this infection progresses to cancer through type-specific virus–host interactions.2,10 However, we have a limited understanding of why oncogenic oral HPV infection develops into cancer in some individuals and not in others and its relation with tobacco, which is a recognised risk factor for oropharyngeal cancer, along with diet, sexual behaviour, oral hygiene and other potential risk confounders.11–13

There are no nationwide data concerning head and neck cancer and HPV infection in Brazil. Some studies have described a low prevalence of oral HPV infection in the Brazilian population,14–15 but the results of the Papillomavirus Prevalence Study in Brazil (POP-Brazil) are still pending and may provide more precise data concerning prevalence rates.16 Therefore, we designed this protocol to evaluate the prevalence of HPV among patients with head and neck cancer and their partners to understand the interaction between HPV infection and its different known risk factors.

OBJECTIVES
To evaluate the association between HPV and oropharyngeal cancer in all regions of Brazil.

Secondary objectives
► To determine the prevalence of HPV and its types in tumours of patients with oropharyngeal cancer in Brazil.
► To determine the prevalence of HPV and its types in different body regions, such as the oral, anal and genital regions, in individuals with HPV-induced oropharyngeal cancer as well as in their sexual partners and control participants.
► To determine the impact of HPV seropositivity on the risk of developing oropharyngeal cancer.
► To determine the frequencies of infection types and infections at different sites in patients and their partners.
► To evaluate the associations between oncoproteins in the tumour tissue of patients and HPV-related oropharyngeal cancer.
► To evaluate behavioural and lifestyle factors associated with oropharyngeal cancer.

METHODS AND ANALYSIS
Design
The Study of Oropharyngeal Cancer and HPV in Brazil (STOP) will be a multicentre, nationwide case–control study. This design allows the simultaneous examination of multiple risk factors and is useful for establishing associations as a baseline for future studies.

Recruitment
The project will include participants from throughout the national territory of Brazil by enrolling participants in tertiary hospitals in all regions of Brazil. We will select at least one hospital in each of the five Brazilian geographical regions, and the number of participant centres will be increased until we reach the minimum calculated sample size. The hospital should present a large volume of patients treated with head and neck cancer and should have the physical structure and human resources necessary for the collection of biological material. The evaluation of patients with head and neck cancer in all regions of the country will be representative of the national population and support national public health policy decision-making with the information gathered.

We will consider and invite all eligible individuals who meet the inclusion criteria to participate in the study.

Inclusion criteria
Eligible patients must be over 18 years of age with newly diagnosed squamous cell carcinomas of the head and neck, without chemotherapy or radiotherapy, with no more than 45 days from diagnosis. Cases will consist of those with an International Classification of Disease, 10th revision (ICD-10) diagnosis code of oropharyngeal carcinoma (ICD-10: C01; C02.4; C05.1; C05.2; C09.0-C09.9; C10.0-C10.9, C14.2), oral cavity cancer (ICD-10: C00.3-C00.9, C02.0-C02.3, C03.0-C03.9, C04.0-C04.9, C06.0, C06.1, C06.2, C02.8), laryngeal cancer (ICD-10: C32.0-C32.9), hypopharyngeal cancer (ICD-10: C12, C13.0-C13.2, C13.8-C13.9) and palate cancer (ICD-10: C05.8 and C05.9).

The codes C80 (malignant neoplasm, without localisation specification), C06.8 (malignant neoplasm of other parts and unspecified parts of the mouth, with invasive lesion), C06.9 (malignant neoplasm of mouth, unspecified) and C02.9 (tongue, unspecified) will be included in the pilot phase and assessed for their incidence rates, since the location most frequently associated with HPV infection is the oropharynx. The ICD code C80 will be included initially since a significant number of patients with head and neck cancer in Brazil are classified with this code because of an unknown head and neck primary tumour.

Control participants without a history of cancer will be selected from the same population and from among companions of any patient or patients seen at the same hospital as the case for benign conditions. They will be frequency matched to cases by sex, 5-year age categories and town of residence.

The current sexual partners of all patients will be invited to participate in the study since data collected from this population can provide important information regarding
HPV transmission. Controls and sex partners will be over 18 years of age.

**Exclusion criteria**
Cases and controls undergoing chemotherapy, radiotherapy or immunotherapy or with less than 5 years of treatment for any other type of cancer, excluding non-melanoma skin cancer, will be excluded. We will also exclude pregnant women, patients taking interferon and those who have undergone solid organ transplantation and are undergoing immunosuppression therapy.

**Data collection**
Healthcare professionals will be trained and certified at their location by the team coordinator for data collection. A standardised questionnaire based on validated instruments will evaluate the following:

- **Sociodemographic variables:** Age, gender, race/skin colour, educational level, occupational status and last month’s family income will be analysed. We defined social class according to the Brazilian Association of Research Enterprises (Associação Brasileira de Empresas de Pesquisa) and categorised the participants into four different groups, A, B, C and D–E, according to access to specific goods and services.17

- **Smoking status, alcohol consumption and other drug use:** Questions regarding lifetime smoking habits (including quantity and frequency) as well as alcohol and other drug use (including legal and illegal drugs) will be based on questionnaires from other studies.18–20

- **Sexual and reproductive health:** Sexual preference, number sexual relationships, condom usage, number of sexual partners and sexual practices will be analysed. Reproductive health questions will evaluate information such as age of menarche, number of pregnancies and deliveries, history of abortion, use of contraceptive methods and occurrence of STIs.

- **Oral hygiene:** Questions regarding the number of teeth extracted, use of oral antiseptics, frequency of tooth brushing, frequency of flossing, gingival bleeding and frequency of visits to the dentist will be included.11 18–21

- **Diet and food consumption:** Food consumption frequency, food group consumption and the ingestion of processed/ultraprocessed food will be evaluated through a food frequency questionnaire adapted from other studies, such as the European Prospective Investigation into Cancer and Nutrition and Surveillance System for Risk and Protective Factors for Chronic Diseases by Telephone Survey studies.22 23

- **Aspects related to head and neck cancer:** Questions regarding perceptions of health, access to health services and a family history of cancer (presence of family history of cancer, type of cancer in family members, age of diagnosis and degree of kinship) will be asked.

The evaluation of this information is important for controlling recognised risk factors, such as alcohol and tobacco use, for oropharyngeal cancer and for analysing other potential or not well-established risk factors, such as sexual behaviour, oral hygiene and dietary differences.

**Biological sampling**
Biological samples from the oral, genital and anal regions and serum samples will be collected from all participants including sexual partners; formalin-fixed paraffin-embedded tissue (FFPET) samples from cases will also be analysed (figure 1).

Oral samples will be obtained through 30 s mouthwash and gargle cycles with 10 mL of a standardised commercial mouthwash.24 25 Cervical samples will be collected by healthcare professionals using the Digene HC2 DNA Collection Kit (Qiagen, Halsterweg, Netherlands) following the manufacturer’s instructions. Penile samples will be self-collected using FLOQSwabs (Copan) previously moistened with sterile saline solution. After

![Figure 1](image)
collection, the FLOQSwabs will be stored in 2 mL of eNAT® preservation medium for nucleic acids (Copan). Anal samples will be self-collected using a Digene Female Swab Specimen Collection Kit. The participants will be instructed to insert 3 cm of the swab into the anal canal, rotate it 360° at least twice and gently remove the swab afterwards.26 Participants will be instructed by healthcare professionals and will receive illustrated schematic instructions for reference for both types of self-collection (online supplementary file 1). Study staff will remain available for assistance during all procedures. All the biological samples described above will be refrigerated between 2°C and 8°C and transported to a central laboratory in Porto Alegre for specimen processing. The temperature will be monitored with a data logger.

To identify the presence of HPV-specific antibodies, serum will be collected by sampling in microtainer blood collection tubes (SST, Becton Dickinson).14 16 17 Within 30 min of collection, the blood sample will be centrifuged, and the serum will be aliquoted and stored at −20°C until transport to the central laboratory.

FFPET blocks from surgical materials or from diagnostic biopsies will be collected for DNA/RNA isolation and a pathological diagnosis confirmation. For DNA/RNA isolation, the FFPET block will be sectioned into 10 sections at 5 μm each and distributed into two microtubes (five sections per tube). For p16 analysis and H&E staining, four sections at 3 μm each will be distributed on four TOMO Adhesion Microscope Slides (11/90) (Matsunami, Bellingham, USA) (one section per slide). To avoid cross-contamination by nucleic acids during each FFPET block section, the microtome blade will be changed, acetone and ethanol will be used to clean the sectioning area and sections destined for p16 analysis, and H&E staining will be cut from the beginning and end sections of the same block. The FFPET aliquots will be maintained at room temperature (15°C–25°C). All data, including interview, sampling and laboratory processing data, will be gathered in an online platform devoted to data storage and analysis for this project. Through this system, we can control the progress and quality of the study as well as make the results available to health professionals and participants through an external webpage interface.

Specimen processing
At the central laboratory, genital swab samples will be homogenised before being aliquoted and stored in freezer at −80°C. Oral samples will be first centrifuged at 2000 rpm for 10 min; the pellet will then be eluted in 8 mL of 1X phosphate-buffered saline (PBS) and will be centrifuged again. The final pellet will be eluted in 3 mL of 1X PBS. The processed genital, anal and oral sample aliquots will be submitted to automated nucleic acid extraction using magnetic beads for isolation and purification by a MagNA Pure LC 2.0 instrument (Roche Molecular Systems). With the same automated instrument, RNA and DNA will be extracted from FFPET sections using LC RNA III (tissue) and LC DNA II (tissue) isolation kits, respectively.

The presence and genotyping of HPV from the purified DNA from the FFPET section and the oral, genital and anal samples will be analysed with a Novaplex II HPV28 Detection Kit (Seegene, Seoul, South Korea) and the CFX96 real-time PCR instrument (Bio-Rad, Schönenbuch, Switzerland). This system is based on a multiple real-time PCR assay that is able to differentiate the target nucleic acids of 27 HPV types. The test detects high-risk (HR)-HPV types (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 69, 73 and 82) and other HPV infection types (6, 11, 40, 42, 43, 44, 54, 61 and 70). An endogenous internal control will be used to monitor nucleic acid isolation and check for possible PCR inhibition. The protocol will be performed as recommended by the manufacturer.

We will use the extracted RNA from the FFPET sections for E6/E7 oncogene transcription analyses of HR-HPV strains. These analyses will be performed using multiplex reverse transcriptase-PCR with the xMAP methodology (Luminex).27 From the serum, antibodies against E2, E6 and E7 proteins will be identified from the same HR-HPV types mentioned above using a rapid antigenic protein in situ display ELISA.28 The FFPET slides will be H&E stained to confirm the presence of tumour cells. Immunohistochemistry assays using an E6H4 clone and a CINtec p16 histology kit (Roche Technologies, Basel, Switzerland)29 and a Ventana BenchMark automated slide stainer will be performed to evaluate p16 quality. Morphology data will be analysed in the central laboratory by a trained pathologist.

Data analyses and statistics
The categorical variables will be summarised by absolute frequencies and percentages and the continuous variables by means and SD. X2 and Fisher’s exact tests will be performed to compare proportions and t-tests or non-parametric Mann-Whitney U tests will be performed to analyse the continuous variables. The association between HPV infection and socio-behavioural variables will be estimated by calculating crude and adjusted ORs using logistic regression. Possible effect modifiers will be evaluated using interaction terms. The collinearity of sexual behaviour and habits such as smoking, alcohol consumption and drug use will be investigated through the construction of independent models for the different risk factors. We will compare the risk of cases with that of controls as well as sexual partners. All participants included will provide answers to the same questionnaire and undergo the same biological sampling procedure, in addition to tissue evaluations. We also will calculate the risk attributable to exposure. This is a measure of association that uses the absolute difference between two risk estimates and can be used to assess a possible cause-effect relationship.
Sampling and sample size
To assess the association between oropharyngeal cancer and oral HPV, with a 95% significance level and 90% power, an expected prevalence of 11% among controls and 18.3% among cases was estimated.30 31 We will enrol 622 patients and 622 controls, as a sample loss of 20% was estimated. In addition, all the sexual partners of the patients who are interested in participating in the study will be included. Sampling will be stratified according to cancer incidence rates in each geographical region.

Monitoring and quality control
Standardisation manuals related to monitoring and quality control were established during the project development stage.

The quality control process includes training of the team involved in collecting data in each site, as well as the certification and recertification of the team (through written tests and simulated interviews). We also plan to conduct monitoring visits, interview observations and quantitative data monitoring through periodical reports generated by the system.

A trained researcher from the coordination team will perform a blind second interview by phone for 20% of the participants between 2 and 4 weeks after the inclusion of the participant. The degree of agreement will be analysed through measures such as the kappa coefficient, coefficient of variation and systematic differences between observations.

A pilot study will be performed to identify and correct problems in data collection, logistics and laboratory procedures.

Patient and public involvement
Neither the patients nor the public were involved in the study design and/or protocol. In agreement with the Brazilian Research Ethic’s regulatory body, which requires us to give feedback on examination currently implemented in the country, the genital HPV results will be communicated to the participants by healthcare providers. The results of HPV infection at other sites will not be provided. Patients will be informed about the meaning of the result and receive instructions regarding STI prevention and follow-up according to the national guidelines.

ETHICS AND DISSEMINATION
Ethical approval has been obtained by the ethical committee of the proposing institution (Hospital Moinhos de Vento, number 2.852.060). Ethical approval from the collaborators is currently under evaluation and is not yet complete (online supplementary file 2). The pilot project is ongoing and enrolment is expected to be complete by the end of 2020.

Individuals included in this study will participate voluntarily and must sign an informed consent form. This project complies with Resolution 347/05 and Resolution 466/2012 of the National Health Council. During the study, the biological material will be accessed only by researchers associated with this study. The backup samples will be kept in an appropriate storage location in Porto Alegre, and the informed consent will include specific authorisation for the storage of biological materials. Participants whose biological samples test positive for high-grade genital HPV will be informed of this result by their health facility’s healthcare provider and following the protocol of care for each hospital. The results will be reported through a computerised data system. All biological samples will follow all biosafety procedures with the intention of minimising risk.

The results of this study will be presented at meetings with the Brazilian Ministry of Health through technical reports. The results will be presented to the scientific community at national and international events, followed by publication of scientific articles.

DISCUSSION
Although robust evidence suggests the important aetiological contribution of HPV to the development of head and neck cancer, existing data still vary between different populations and are non-existent for a large number of countries, such as Brazil.3 5 32 33 Notably, since the end of the 1980s, head and neck cancers unrelated to HPV declined in their incidence, whereas tumours related to HPV infection increased.34 35 This increased incidence is most likely related to a rise in the prevalence of HPV infection worldwide in both men and women, especially by type 16 HPV, which is commonly associated with oropharyngeal cancers.36 Head and neck cancer related to HPV infection affects younger patients and has a better prognosis than head and neck cancer not related to HPV. These characteristics have already led to a modification in staging and may possibly lead to a change in treatment based on HPV status in the future.36 The aetiological role of HPV in the development of several cancers exists; however, the role of HPV in the genesis of head and neck cancer in Brazil is not known. In addition, the impact of other risk factors related to head and neck cancer is also unknown. Therefore, different aspects regarding diet, smoking habits, sexual health and oral hygiene may interact to create an environment favourable for the onset of oropharyngeal cancer.

Despite a virus-related epidemic and given the predominance of this disease in recent publications, it is unclear whether the increased incidence of head and neck cancer is related solely to changes in sexual behaviour and increased incidence rates of HPV or to other factors not yet identified.

Another important question relates to the role of vaccination in the primary prevention of this pathology. The data collected in this study can help reinforce the need for gender neutral HPV vaccination coverage as well as help to project the impact of the current coverage on cancer incidence and mortality.
Vaccination against HPV has been advocated as a primary prevention method for cervical cancer. In 2014, the national HPV vaccination programme using the quadrivalent vaccine, which comprises immunisation to HPV types 6, 11, 16 and 18 associated with genital warts and cervical neoplasms was initiated. The results of the POP-Brazil study verified that the young Brazilian population and neck cancer associated with HPV infection. A growing global problem. Int J Appl Basic Med Res 2016;6:84

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