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# Delineation Of Surgical Margins using Immunohistochemistry in mucosal Head and Neck Squamous Cell Carcinoma (DOSMI - HNSCC): Study protocol for a bilateral study in Australia and India



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# **D**elineation **O**f **S**urgical **M**argins using **I**mmunohistochemistry in mucosal **H**ead and **N**eck **S**quamous **C**ell **C**arcinoma (DOSMI - HNSCC): Study protocol for a bilateral study in Australia and India

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## Abstract

### Objectives

Treatment failure and poor five year survival in mucosal Head and Neck Squamous Cell Carcinoma (HNSCC) has remained unchanged for decades mainly due to advanced stage of presentation and high rates of recurrence. Incomplete surgical removal of the tumour, attributed to lack of reliable methods to delineate the surgical margins is a major cause of disease recurrence. The DOSMI – HNSCC study aims to identify the true extent of the tumour at the molecular level by performing immunohistochemistry (IHC) with molecular markers, eukaryotic initiation factor, e IF4E and tumour suppressor gene, p53 on the surgical margins and test the use of Lugol's iodine and fluorescence visualisation prior to the wide local excision.

### Method

DOSMI – HNSCC is a bilateral observational research being conducted in Darwin, Australia and Vellore, India. Individuals diagnosed with HNSCC will undergo the routine wide local excision of the tumour followed by histopathological assessment. Tumours with negative surgical margins will be further stained with e IF4E and p53 antibodies. Results of IHC staining will be correlated with recurrences in an attempt to predict the risk of disease recurrence. Patients in Darwin, will undergo intraoperative staining of the lesion with Lugol's iodine and Fluorescence visualisation to delineate the excision margins and the results will be compared with patients in Vellore where these tests are not done.

### Discussion

We describe the study protocol and the anticipated challenges in obtaining an adequate sample size in both locations. As a translational research the DOSMI – HNSCC study may be effective in intra and post-operative delineation of surgical margins to achieve reduction of recurrence rates, better quality of life and impact survival.

### Study registration

Approved by the institutional ethics committees in Darwin (HREC 13 – 2036) and Vellore (IRB Min. No. 8967).

### Trial Registration number

Australian New Zealand Clinical Trials Registry (ACTRN12616000715471)

**Strengths and Limitations of this study**

- Christian Medical College, Vellore and Royal Darwin hospital patients represent regions with high burden of mucosal head and neck squamous cell carcinoma. Hence the samples will provide sufficient representation of the general population.
- Follow up period of a minimum of 1 year is adequate to capture disease recurrence as most recurrences occur within one year of wide local excision.
- Intraoperative methods of staining with Lugol's iodine and VELscope examination being done only in Darwin allows to test the efficacy of these tests in obtaining tumour free margins.
- Late and aggressive initial clinical presentation may poses a challenge to obtain tumour free margins thus limiting the sample size.
- Patient recruitment for 2 years and 1 year for follow up gives the patients recruited in the first year 2 years of follow up and those in the second year only 1 year of follow up; so the recurrences beyond 1 year in the patients from the second near may be missed.

**Key words:** Head and neck squamous cell carcinoma, surgical margins, immunohistochemistry, e IF4E, p53, vital staining, fluorescence visualisation

## Introduction

Head and neck cancer is the eighth commonest cancer in the world with approximately 650,000 new cases reported annually. The vast majority (more than 90%) are squamous cell carcinomas (HNSCCs) that arise from the epithelium lining the sinonasal tract, oral cavity, pharynx, and larynx. HNSCCs are not homogenous on the contrary their distinctive molecular genetic profiles have shown them to be heterogeneous that differ in risk factors, pathogenesis and clinical behaviour. [1]

Despite aggressive treatment regimens with wide surgical excision, radiotherapy and chemotherapy which are all associated with substantial morbidity, the 5 -year survival rates for head and neck cancer have not significantly changed in the last three to four decades. Much of this is attributed to advanced stage of the disease at presentation, high rates of loco-regional recurrence from inadequate resection ensuing from compromised surgical margins of the tumour and distant metastases. The numerous anatomic sites and the diversity of histologic types in these locations also have a contributory role in treatment outcomes. [2-3]. Hence early diagnosis and complete resection remains the key to prognosis, recurrence and survival in cancer management.

The completeness of tumour resection is assessed by obtaining tumour free margins which is associated with decrease in the rates of recurrence [4]. The intraoperative assessment of the tumour margin has conventionally been by naked eye examination and palpation along with available imaging techniques. Vital staining done by applying Lugol's iodine on the tumour and surrounding area highlights the extent of tumour including premalignant conditions like dysplasia and carcinoma in situ thus elucidating the surgical margin [5-6] which can be completely missed with naked eye observation. The use of VELscope (visually enhanced lesion scope), a simple noninvasive handheld device allows direct visualization of alterations such as dysplasia to tissue fluorescence.[7]

In many institutions the adequacy of surgical resection of the primary tumour is traditionally determined intraoperatively by histopathological diagnosis of haematoxylin and eosin (H&E) stained frozen sections of the surgical margins. The formalin fixed specimens of the excised tumour and remaining frozen section samples of the margins are histologically assessed and have been used as a

potential indicator for recurrences and prognosis. However the predictive ability of histopathological diagnosis alone has proven to be far from satisfactory. [8-9]This has been attributed to the invisible molecular changes that occur within cells in the proximity of the visible tumour as HNSCC is known to develop second tumours that are multifocal in origin. This phenomenon has been explained by Slaughter[10] as "field cancerization" where multiple cell groups independently undergo neoplastic transformation under the stress of regional carcinogenic activity. These genetic alterations may lack the evidence of histopathologic dysplasia and appear to show uninvolved mucosa that account for local recurrence and incomplete surgical resection.[1]

The initiation and progression of HNSCC is a multi-step process that involves progressive acquisition of genetic and epigenetic alterations. Therefore molecular analysis of surgical margins will perhaps play an increasingly important role in establishing tumour free surgical margins.[8, 11] However most markers lack the sensitivity and ease of applicability for effective clinical use. [12] Mutations and overexpression of the tumour suppressor gene p53 are found in 40-60% of HNSCC.[8, 13] The eukaryotic protein synthesis initiation factor, e IF4E ( also known as 4E) has been found to have 100% overexpression in tumours of breast, head and neck and colon [9]. Overexpression of e IF4E in more than 5% of the basal cell layer of histologically tumour free surgical margins of the head and neck squamous cell carcinomas (HNSCC) predicted significant increase in the risk of recurrence. [9, 13] Nathan et al found a strong correlation between tumour recurrence and overexpression of p53 and e IF4E in histologically tumour free margins. They concluded that molecular assessment of margins were more reliable than that with routine haematoxylin and eosin hence has the potential to guide clinicians in obtaining tumour free wide margins for complete excision of the lesion.[13]

Our goal in this study is to confirm the completeness of the excision by immunohistochemical testing of the surgical margins with p53 and e IF4E antibodies. We will also attempt to compare the efficacy of the intraoperative methods of staining the mucosa with Lugol's iodine and using VELscope to demarcate the tumour zone.

## Objective

The aim of the project is to conduct a prospective follow up study of patients with head and neck cancer to:

1. Study the expression of the molecular markers p53 and e IF4E by immunohistochemistry (IHC) on histologically tumour free surgical margins of the excision biopsies of HNSCC in patients from the Royal Darwin Hospital, Northern Territory and Christian Medical College Vellore, India.
2. Determine the correlation of expression of p53 and e IF4E on histological tumour free margins with clinical outcomes such as local recurrence and survival.
3. Determine the sensitivity and specificity of the molecular markers p53 and e IF4E on surgical margins in the assessment of adequacy of surgical excision and predictability of recurrence.
4. Outcomes of intraoperative use of vital staining and fluorescence visualisation
5. Investigate the epidemiological trend in Darwin and Vellore

## Methods/Design

### Study design

The DOSMI study is a prospective bilateral study in two countries Australia and India based at the Royal Darwin Hospital and Christian Medical College and Hospital Vellore.

### Sample size

The average number of patients at Darwin and Vellore are 20 and 70 per year respectively. Most patients present late and obtaining a tumour free margin is a challenge. We anticipate performing IHC on 30 to 40 patients.

### Target population

All patients diagnosed with mucosal HNSCC at RDH and CMC are potential candidates.

### Inclusion criteria

- All patients diagnosed with mucosal head and neck squamous cell carcinoma with negative surgical margins on histopathology at the Royal Darwin Hospital and Christian Medical College Vellore

- **Exclusion criteria**
- Patients diagnosed with any other histological type of mucosal head and neck cancers.
- Patients whose surgical margins showed dysplasia, carcinoma – in – situ and were positive as well as close for invasive tumour on histopathological examination
- Patients with metastatic disease except a single regional lymph node with no extracapsular spread
- Patients with multiple foci of peri-neural invasion
- Patients with previous radiotherapy and chemotherapy
- Patients in whom the margins cannot be defined or with an unknown primary
- Patients under 18 years of age
- Patients who are pregnant

**Patient recruitment**

The patient recruitment in Darwin commenced in November 2013. The two year period ended in November 2015. The patients are currently being followed up until November 2016. In CMC Vellore the two year recruitment period is from September 2014 to September 2016 with a follow up until 2017.

The patients diagnosed to have mucosal HNCs by clinical evaluation, cytology and biopsy at the Royal Darwin hospital (RDH), Darwin, Northern Territory and Christian Medical College (CMC) and hospital; Vellore, India will be initially selected based on the selection criteria for the study and a consent to perform the tests will be procured by the local site investigator. All patients will undergo the relevant imaging (CT and or MRI) tests and an assessment of the eligibility will be made by using the exclusion criteria. (Figure 1)

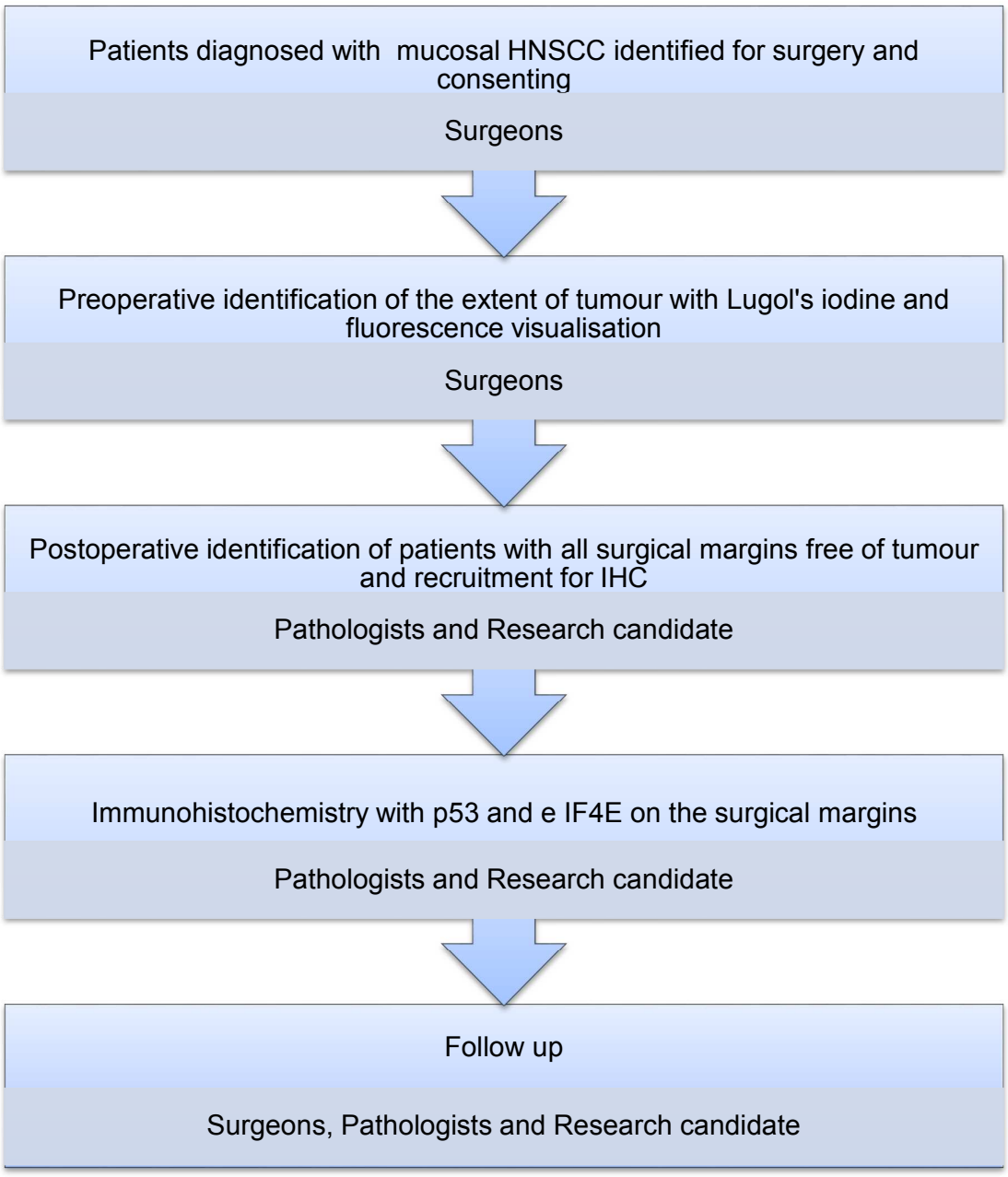


Figure 1 Flow chart of key activity and the involvement of key personnel

**Intraoperative assessment**

Patients in RDH will undergo a VELscope examination and Lugol's iodine staining mark the extent of surgical margins. These test will not be performed in CMC.

**Postoperative assessment**

Five surgical margins of the excised tumour will be colour coded using marking ink, labelled with sutures, numbered and photographed. The surgeons at both sites will mark the margins 1, 2,3,4,5 with black, red, blue, green and yellow respectively.

Paraffin sections from the primary tumour and all the surgical margins will be routinely reported by the resident pathologist at the Royal Darwin Hospital pathology and Department of Pathology at CMC Vellore. The patients with histologically tumour free margins will be finally included for further analysis by immunohistochemistry using p53 and e IF4E antibodies on the mucosal margins.

Immuno-histochemical staining for p53 will be performed using avidin-biotin-peroxidase enzyme complex with a pre-diluted monoclonal anti- p53 antibody ( Ventana ) . A positive p53 staining of the malignant cells will be indicated by an unequivocal brown stain of the nucleus.

Immunohistochemical staining for e IF4E will be carried out with a polyclonal antibody to e IF4E at 1:500 dilution. A brown perinuclear staining of the tumour cells indicates a positive e IF4E stain. The tumour and margins will be graded and scored for both p53 and e IF4E according to the intensity and percentage of cells.
Cases positive will also be evaluated using a 10X objective in at least 10 fields by light microscopy. Areas containing the most uniformly stained tissue will be chosen for evaluation. Immunoexpression will be quantified for (1) percent of immuno-positive neoplastic cells per 10 fields and (2) average intensity of immunostaining in the positive neoplastic cells per 10 fields. The percent positive cells will be graded on scale of 1 through 4 (1= 1% to 25% positive; 2= 26% to 50% positive; 3=51% to 75% positive, and 4=76% to 100% positive). Immuno-staining intensity will be graded 1 through 3 (1=weak; 2=moderate; 3=strong).

Prior to embarking on interpretation, co-authors SJ and GC will come to a consensus on scoring and interpretation of the staining. Subsequently each case will be read by SJ and supervised/counterchecked by GC. The two observers will be blinded to follow up information.

## Follow up

All patients will be followed up and reviewed clinically every 3 months for the first year and at 6 months interval in the second year. In case of any suspicion a biopsy to rule out recurrence will be performed.

## Evaluation of outcomes

The primary outcomes are to 1) list the patients whose surgical margins are reported free of tumour with routine Haematoxylin and Eosin staining that show positive immunohistochemical staining with p53 and / or 4E, 2) list the patients with disease recurrence and metastasis and 3) evaluate the use of Lugol's iodine and VELscope in the patients from Darwin.

The secondary outcomes are to correlate recurrence of disease to positivity with p53 and 4E and correlate metastasis to positivity with p53 and 4E.

During follow up reviews patients will be assessed by local examination, biopsy of a suspicious lesion and MRI scans.

The outcomes will be evaluated based on data collected from patient files with regards to period of tumour free survival, time taken for recurrence and / or metastasis, disease specific survival and overall survival.

## Data Management

The data collection and entry on a excel spreadsheet based on the study proforma will be stored in a password protected computer and a portable external hard drive.

## Statistical analysis

The data on the surgical margins will be analysed statistically with SPSS software. Contingency table and the X2 test will be used to evaluate the association of e IF4E and p53 in the surgical margins with race, sex, stage, lymph node status, histological grade, post-operative radiation and e IF4E and p53 expression in the tumour and margins. A univariate analysis of clinical factors will be performed using Cox model to identify those variables significantly associated with prognosis. Multivariate analysis will be performed to test for simultaneous effect of two or more factors. Event –time distributions for recurrence will be estimated by Kaplan- Meier method and compared by the log rank test to determine the individual and combined effect of

e IF4E and p53 expression in the margins. Similar curves will be performed to determine the effect of nodal status with e IF4E and p53 levels in the margins as nodal status is a significant prognostic factor in HNSCCs.

The consistency of protocol at both the sites will be assessed and the study will be periodically reviewed.

**Discussion**

The DOSMI - HNSCC study is a bilateral higher degree research project in 2 countries that have a huge burden of the disease. Among the states and territories in Australia, Northern Territory has the highest incidence of HNC and the Royal Darwin hospital is the largest public hospital that facilitates the treatment and management of the disease. [13] The actual burden of head and neck cancer in India is much greater than that reflected in the existing literature however it is the commonest malignancy encountered in Indian males.[14] According to the World Health Organisation Lip and oral cancers is the third commonest cancer in India with nearly 68% mortality in 2012.[15]

Head and neck cancer is considered to progress through a multistep process from normal histologic features to hyperplasia, mild dysplasia, moderate dysplasia, severe dysplasia, carcinoma in situ, invasive carcinoma, and metastasis.[3] Malignant transformation in cells are invisible microscopically with H & E stain which may be identified more accurately with molecular markers especially in head and neck cancer, where, as a result of field cancerization, the entire mucosa has often undergone atypical changes.[1,3,9]

A retrospective study conducted in Darwin suggested the efficacy of IHC with e IF4E and p53 antibodies on surgical margins of HNSCC in assessing the completeness of surgery however the sample size was very small for a concrete conclusion. [13] Hence a larger sample and prospective study was warranted to validate the above finding.

The aim in this study is also to evaluate the difference in using vital staining and VELscope. These methods are currently being studied by McCaul et al and Poh et al

respectively. The uniqueness of this project is the ability to study the outcomes and evaluate the efficacy of all three methods put together.

Staining with Lugol's iodine solution has been shown to be effective in intraoperatively delineating the extent and precise border of the cancerous and dysplastic epithelium of the mucosal surface. It is cheap and hence can be used as a cost effective, easy and quick screening test particularly in resource poor countries in detecting premalignant mucosa of individuals who consume tobacco, alcohol and have other lifestyle risk factors.[5-6]
VELscope has up to 55% accuracy in enhancing the direct visualisation of dysplastic mucosa. When combined with Lugol's iodine there is a potential of increasing the accuracy of the screening method. However there is a capital expenditure with purchasing the equipment that may eventually be cost effective in avoiding recurrence. [7]

Molecular analysis by performing immunohistochemistry on surgical margins with e IF4E and p53 has been suggested to predict recurrence in previous studies however the role of p53 is controversial. Besides being a prognostic marker e IF4E can also be targeted for therapeutic intervention.[8, 16, 17]

The TP53 and retinoblastoma pathways are almost universally disrupted in HNSCCs, indicating the importance of these pathways in head and neck tumorigenesis. More than 50% of HNSCC harbor TP53 gene mutations and over 50% demonstrate chromosomal loss at 17p the site where the TP53 gene resides.[1]

The eukaryotic protein synthesis initiation factor e IF4E has been found to be elevated in breast and HNSCCs but not in benign lesions or normal mucosa. Recurrence of HNCs was found to be more common in patients with elevated e IF4E in surgical margins. No other marker has provided evidence for being effective in detecting malignant alteration in cells. Since recurrence in HNSCC usually occurs within the first 2 years the prognostic value of e IF4E can be used in a relatively short follow up time[9].

Since both the institutions receive HNSCC patients representative of sample population the results can be validated to impact. This collaborative trial between two countries has set a precedence to build and continue the partnership for future studies, education and guide protocols in diagnosis and treatment.

**Current status of project**

Open and recruiting

**Completion of project**

December 2017

**List of abbreviations**

IHC Immunohistochemistry
HNSCC Head and neck squamous cell carcinoma
e IF4E Eukaryotic initiation factor 4
RDH Royal Darwin Hospital
CMC Christian Medical College

**Contributors:** Writing committee: Sheela Joseph (SJ),Rajinikanth Janakiraman (RJ),Geeta Chacko (GC), Rama Jayaraj (RaJ) and Mahiban Thomas (MT) made substantial contributions to the conception or design of the study, drafting the manuscript and revising it critically for important intellectual content. All the authors read and approved the version that was submitted.
Data collection and management : SJ

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**Collaborators**: *Australia* - Royal Darwin Hospital – Mahiban Thomas, Cameron Scott, James Badlani. Charles Darwin University – Rama Jayaraj, Sheela Joseph. *India* - Christian Medical College, Vellore – Rajinikanth Janakiraman, Geeta Chacko, Meera Thomas, Sramana Mukhopadhya.

**Competing interests:** None

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SPIRIT
STANDARD PROTOCOL ITEMS: RECOMMENDATIONS FOR INTERVENTIONAL TRIALS

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

| Section/item | Item No | Description |
|---|---|---|
| **Administrative information** | | |
| Title<br>**Page 1** | 1 | Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym |
| Trial registration<br>**Page 2** | 2a | Trial identifier and registry name. If not yet registered, name of intended registry |
| | 2b | All items from the World Health Organization Trial Registration Data Set |
| Protocol version | 3 | Date and version identifier – ~~1/11/16~~ 4/11/16 |
| Funding<br>**Page 13** | 4 | Sources and types of financial, material, and other support |
| Roles and responsibilities | 5a | Names, affiliations, and roles of protocol contributors |
| | 5b | Name and contact information for the trial sponsor |
| **Page 1** | 5c | Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities |
| | 5d | Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee) |
| **Introduction** | | |
| Background and rationale<br>**Page 4 & 5** | 6a | Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention |
| | 6b | Explanation for choice of comparators |
| Objectives<br>**Page 6** | 7 | Specific objectives or hypotheses |

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| Trial design<br>**Page 6** | 8 | Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory) |
| **Methods: Participants, interventions, and outcomes** | | |
| Study setting<br>**Page 6** | 9 | Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained |
| Eligibility criteria<br>**Page 6, 7 & 8** | 10 | Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists) |
| Interventions<br>**Page 8 & 9** | 11a | Interventions for each group with sufficient detail to allow replication, including how and when they will be administered |
| | 11b | Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease) |
| | 11c | Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests) |
| | 11d | Relevant concomitant care and interventions that are permitted or prohibited during the trial |
| Outcomes<br>**Page 10** | 12 | Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended |
| Participant timeline<br>**Page 7 & 9** | 13 | Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure) |
| Sample size<br>**Page 6** | 14 | Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations |
| Recruitment<br>**Page 7** | 15 | Strategies for achieving adequate participant enrolment to reach target sample size |
| **Methods: Assignment of interventions (for controlled trials)** | | |
| Allocation: | | |

| | | |
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| Sequence generation | 16a | Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions |
| Allocation concealment mechanism | 16b | Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned |
| Implementation | 16c | Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions |
| Blinding (masking) | 17a | Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how |
| | 17b | If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial |
| **Methods: Data collection, management, and analysis** | | |
| Data collection methods **Page 9** | 18a | Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol |
| | 18b | Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols |
| Data management **Page 10** | 19 | Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol |
| Statistical methods **Page 10** | 20a | Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol |
| | 20b | Methods for any additional analyses (eg, subgroup and adjusted analyses) |
| | 20c | Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation) |

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| **Methods: Monitoring** | | |
| Data monitoring | 21a | Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed |
| | 21b | Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial |
| Harms | 22 | Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct |
| Auditing | 23 | Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor |
| **Ethics and dissemination** | | |
| Research ethics approval<br>**Page 2** | 24 | Plans for seeking research ethics committee/institutional review board (REC/IRB) approval |
| Protocol amendments | 25 | Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators) |
| Consent or assent<br>**Page 7 & 8** | 26a | Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32) |
| | 26b | Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable |
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| Ancillary and post-trial care | 30 | Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation |

| | | |
|---|---|---|
| Dissemination policy | 31a | Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions |
| | 31b | Authorship eligibility guidelines and any intended use of professional writers |
| | 31c | Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code |
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| Informed consent materials | 32 | Model consent form and other related documentation given to participants and authorised surrogates |
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*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.

BMJ Open

# Predictability of Recurrence using Immunohistochemistry to delineate Surgical Margins in mucosal Head and Neck Squamous Cell Carcinoma (PRISM - HNSCC): Study protocol for a prospective, observational and bilateral study in Australia and India



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| | |

# **P**redictability of **R**ecurrence using **I**mmunohistochemistry to delineate **S**urgical **M**argins in mucosal **H**ead and **N**eck **S**quamous **C**ell **C**arcinoma (PRISM - HNSCC): Study protocol for a prospective, observational and bilateral study in Australia and India

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## Abstract

### Objectives

Treatment failure and poor five-year survival in mucosal Head and Neck Squamous Cell Carcinoma (HNSCC) has remained unchanged for decades mainly due to advanced stage of presentation and high rates of recurrence. Incomplete surgical removal of the tumour, attributed to lack of reliable methods to delineate the surgical margins is a major cause of disease recurrence. The PRISM – HNSCC study aims to redefine margin status by identifying the true extent of the tumour at the molecular level by performing immunohistochemistry (IHC) with molecular markers, eukaryotic initiation factor, e IF4E and tumour suppressor gene, p53 on the surgical margins and test the use of Lugol's iodine and fluorescence visualisation prior to the wide local excision.

### Method and analysis

PRISM – HNSCC is a bilateral observational research being conducted in Darwin, Australia and Vellore, India. Individuals diagnosed with HNSCC will undergo the routine wide local excision of the tumour followed by histopathological assessment. Tumours with clear surgical margins that satisfy the exclusion criteria will be selected for further staining of the margins with e IF4E and p53 antibodies. Results of IHC staining will be correlated with recurrences in an attempt to predict the risk of disease recurrence. Patients in Darwin, will undergo intraoperative staining of the lesion with Lugol's iodine and Fluorescence visualisation to delineate the excision margins while patients in Vellore will not undertake these tests. The outcomes will be analysed.

### Ethics and dissemination

The PRISM – HNSCC study was approved by the institutional ethics committees in Darwin (HREC 13 – 2036) and Vellore (IRB Min. No. 8967). Outcomes will be disseminated through publications in academic journals and presentations at educational meetings and conferences. It will be presented as dissertation at the Charles Darwin University. We will communicate the study results to both participating sites. Participating sites will communicate results with patients who have indicated an interest in knowing the results.

### Trial Registration number

Australian New Zealand Clinical Trials Registry (ACTRN12616000715471)


## Strengths and Limitations of this study

• Christian Medical College, Vellore and Royal Darwin hospital patients represent regions with high burden of mucosal head and neck squamous cell carcinoma thus ensuring external validity of the study.

• The stringent selection criteria ensure internal validity even though it will impact on the sample size at both locations.

• Intraoperative methods of staining with Lugol's iodine and VELscope examination being done only in Darwin allows to test the rigor and efficacy of both these methods.

• Local disease recurrence usually occurs within one year of wide local excision hence the follow up period of a minimum of 1 year is a satisfactory end point to assess this outcome.

• Patients may be lost to follow up in case of death or change of address.

**Key words:** Head and neck squamous cell carcinoma, surgical margins, immunohistochemistry, e IF4E, p53, vital staining, fluorescence visualisation



## Introduction

Head and neck cancer is the eighth commonest cancer in the world with approximately 650,000 new cases reported annually. The vast majority (more than 90%) are squamous cell carcinomas (HNSCCs) that arise from the epithelium lining the sinonasal tract, oral cavity, pharynx, and larynx. HNSCCs are not homogenous, on the contrary their distinctive molecular genetic profiles have shown them to be heterogeneous that differ in risk factors, pathogenesis and clinical behaviour. [1]

Despite aggressive treatment regimens with wide surgical excision, radiotherapy and chemotherapy which are all associated with substantial morbidity, the 5 -year survival rates for head and neck cancer have not significantly changed in the last three to four decades. Much of this is attributed to the advanced stage of the disease at presentation, high rates of loco-regional recurrence from inadequate resection ensuing from compromised surgical margins of the tumour and distant metastases. The numerous anatomic sites and the diversity of histologic types in these locations also have a contributory role in treatment outcomes. [2-3]. Hence early diagnosis and complete resection remain the key to prognosis, recurrence and survival in cancer management.

The completeness of tumour resection is assessed by obtaining tumour free margins which is associated with decrease in the rates of recurrence [4]. The intraoperative assessment of the tumour margin has conventionally been by naked eye examination and palpation along with available imaging techniques. Vital staining done by applying Lugol's iodine on the tumour and surrounding area highlights the extent of tumour including premalignant conditions like dysplasia and carcinoma in situ thus elucidating the surgical margin [5-6] which can be completely missed with naked eye observation. The use of VELscope (visually enhanced lesion scope), a simple noninvasive handheld device allows direct visualisation of alterations such as dysplasia to tissue fluorescence.[7]

In many institutions, the adequacy of surgical resection of the primary tumour is traditionally determined intraoperatively by histopathological diagnosis of Haematoxylin and Eosin (H&E) stained frozen sections of the surgical margins. The formalin fixed specimens of the excised tumour and remaining frozen section samples of the margins are histologically assessed and have been used as a potential indicator for recurrences and prognosis. However the predictive ability of

histopathological diagnosis alone has proven to be far from satisfactory. [8-9]This has been attributed to the undetectable subclinical molecular changes that occur within cells in the proximity of the visible tumour as HNSCC is known to develop second tumours that are multifocal in origin. This phenomenon has been explained by Slaughter[10] as “field cancerization” where multiple cell groups independently undergo neoplastic transformation under the stress of regional carcinogenic activity. These genetic alterations may lack the evidence of histopathologic dysplasia and appear to show uninvolved mucosa that account for local recurrence and incomplete surgical resection.[1]

The initiation and progression of HNSCC is a multi-step process that involves progressive acquisition of genetic and epigenetic alterations. Therefore molecular analysis of surgical margins will perhaps play an increasingly important role in establishing tumour free surgical margins.[8, 11] However most markers lack the sensitivity and ease of applicability for effective clinical use. [12] Mutations and overexpression of the tumour suppressor gene p53 are found in 40-60% of HNSCC.[8, 13] The eukaryotic protein synthesis initiation factor, e IF4E ( also known as 4E) has been found to have 100% overexpression in tumours of breast, head and neck and colon [9]. Overexpression of e IF4E in more than 5% of the basal cell layer of histologically tumour free surgical margins of the head and neck squamous cell carcinomas (HNSCC) predict significant increase in the risk of recurrence. [9, 13] Nathan et al found a strong correlation between tumour recurrence and overexpression of p53 and e IF4E in histologically tumour free margins. They concluded that molecular assessment of margins was more reliable than that with routine haematoxylin and eosin hence has the potential to guide clinicians in obtaining tumour free wide margins for complete excision of the lesion.[13]

**Objective**

The aim of the project is to conduct a prospective follow up study of patients with head and neck cancer to:

1. Study the expression of the molecular markers p53 and e IF4E by immunohistochemistry (IHC) on histologically tumour free surgical margins of the excision biopsies of HNSCC in patients from the Royal Darwin Hospital, Northern Territory, Australia and Christian Medical College Vellore, India.

2. Determine the correlation of expression of p53 and e IF4E on histological tumour free margins with clinical outcomes such as local recurrence and survival.
3. Determine the sensitivity and specificity of the molecular markers p53 and e IF4E on surgical margins in the assessment of adequacy of surgical excision and predictability of recurrence.
4. Outcomes of intraoperative use of vital staining and fluorescence visualisation
5. Determine the epidemiological trend in Darwin and Vellore

## Methods and Analysis

### Study design

The PRISM study is a prospective observational study in two countries Australia and India based at the Royal Darwin Hospital and Christian Medical College and Hospital, Vellore.

### Sample size

The average number of patients at Darwin and Vellore are 20 and 70 per year respectively. Most patients present late and obtaining a tumour free margin is a challenge. We anticipate performing IHC on surgical margins of approximately 50 patients in total – 6-8 from Darwin and 40- 45 from Vellore.

### Target population

All patients diagnosed with mucosal HNSCC at RDH and CMC with a curative intent are potential candidates.

### Inclusion criteria

- All patients at the Royal Darwin Hospital and Christian Medical College Vellore during the recruitment period with a confirmed diagnosis of mucosal head and neck squamous cell carcinoma on initial biopsy.

- Wide local excision biopsy with mucosal surgical margins ≥5mm on histopathological examination.

### Exclusion criteria

- Patients diagnosed with any other histological type of mucosal head and neck cancers.

- Wide local excision biopsy specimens with surgical margins that show dysplasia, carcinoma – in – situ and are positive(< 1mm) and close for invasive tumour ( 1-5mm) on histopathological examination
- Patients with metastatic disease except a single regional lymph node with no extracapsular spread
- Patients with main tumour showing peri-neural and lympho-vascular invasion
- Patients with previous radiotherapy and chemotherapy
- Patients who undergo postoperative radiotherapy
- Patients in whom the margins cannot be defined or with an unknown primary
- Patients under 18 years of age
- Patients who are pregnant at the time of diagnosis

**Patient recruitment**

The patient recruitment period is two years with a follow-up of minimum one year. Recruitment period in Darwin was from November 2013 to November 2015. The follow-up period is until November 2016. In CMC Vellore the two year recruitment period was from September 2014 to September 2016 with a follow up of the enrolled patients until September 2017.

The patients diagnosed to have mucosal HNSCC by clinical evaluation and biopsy at the Royal Darwin Hospital (RDH), Darwin, Northern Territory and Christian Medical College (CMC) and Hospital, Vellore, India will be initially selected based on the selection criteria for the study. All patients will undergo the relevant imaging (CT and or MRI) tests and an assessment of the eligibility will be determined by using the exclusion criteria. Consent to perform the tests on patients being prepared for excision surgery will be procured by the local site investigators MT (Darwin) and JR (Vellore). (Figure 1)

**Intraoperative assessment**

Patients in RDH will undergo a VELscope examination and Lugol's iodine staining to mark the extent of tumour and identify surgical margins. These tests will not be performed in CMC.

**Postoperative assessment**

Five surgical margins of the excised tumour will be colour coded using marking ink, labelled with sutures, numbered and photographed. The surgeons at both sites will mark the margins 1, 2,3,4,5 with black, red, blue, green and yellow respectively.

Paraffin sections from the primary tumour and all the surgical margins will be routinely reported by the resident pathologists at the Royal Darwin Hospital pathology and Department of Pathology at CMC Vellore. The patients with histologically tumour free margins that satisfy the selection criteria will finally be included for further analysis by immunohistochemistry using p53 and e IF4E antibodies on the mucosal margins. An excision margin is free of tumour when it is equal to or more than 5mm away from the tumour. Co-authors SM and/or MeT will counter check the eligibility criteria of the sections selected for IHC.

Immunohistochemical staining for p53 will be performed using avidin-biotin-peroxidase enzyme complex with a pre-diluted monoclonal anti- p53 antibody (Ventana). A positive p53 control (figure 2) standardised in the laboratory will be used in the assessment of the mucosal surgical margins. Positive p53 staining of the malignant cells will be indicated by an unequivocal brown stain of the nucleus.

Immunohistochemical staining for e IF4E will be carried out with a polyclonal antibody to e IF4E at 1:500 dilution. Positive e IF4E control (figure 3) has been standardised on breast tissue with infiltrating duct carcinoma. A brown perinuclear staining of the tumour cells indicates a positive e IF4E stain.

The tumour and margins will be graded and scored for both p53 and e IF4E according to the intensity and percentage of cells. Cases positive will also be evaluated using a 10X objective in at least 10 fields by light microscopy. Areas containing the most uniformly stained tissue will be chosen for evaluation. Immunoexpression will be quantified for (1) percent of immuno-positive neoplastic cells per 10 fields and (2) average intensity of immunostaining in the positive neoplastic cells per 10 fields. The percent positive cells will be graded on scale of 1 through 4 (1= 1% to 25% positive; 2= 26% to 50% positive; 3=51% to 75% positive, and 4=76% to 100% positive). Immuno-staining intensity will be graded 1 through 3 (1=weak; 2=moderate; 3=strong).

Prior to embarking on interpretation, co-authors SJ and GC will come to a consensus on scoring and interpretation of the staining. Subsequently each case will be read by SJ and supervised/counterchecked by GC. The two observers will be blinded to follow up information.

**Follow up**

All patients will be followed up and reviewed clinically every three months for the first year and at six months interval in the second year. In case of any suspicion a biopsy to rule out recurrence will be performed.

**Evaluation of outcomes**

The primary outcomes are to 1) list the patients whose surgical margins are reported free of tumour with routine Haematoxylin and Eosin staining that show positive immunohistochemical staining with p53 and / or 4E, 2) list the patients with disease recurrence and metastasis and 3) evaluate the use of Lugol's iodine and VELscope in the patients from Darwin.

The secondary outcomes are to correlate recurrence of disease to positivity with p53 and 4E and correlate metastasis to positivity with p53 and 4E.

During follow up reviews patients will be assessed by local examination, biopsy of a suspicious lesion and MRI scans.

The outcomes will be evaluated based on data collected from patient files with regards to period of tumour free survival, time taken for recurrence and / or metastasis, disease specific survival and overall survival.

**Data Management**

The data collection and entry on an excel spreadsheet based on the study proforma will be stored by SJ in a password protected computer and a portable external hard drive.

**Statistical analysis**

The data on the surgical margins will be analysed statistically with SPSS software. Contingency table and the X2 test will be used to evaluate the association of e IF4E and p53 in the surgical margins with race, sex, stage, lymph node status, histological grade, postoperative radiation and e IF4E and p53 expression in the tumour and margins. A univariate analysis of clinical factors will be performed using Cox model to identify those variables significantly associated with prognosis. Multivariate

analysis will be performed to test for simultaneous effect of two or more factors. Event –time distributions for recurrence will be estimated by Kaplan-Meier method and compared by the log rank test to determine the individual and combined effect of e IF4E and p53 expression in the margins. Similar curves will be performed to determine the effect of nodal status with e IF4E and p53 levels in the margins as nodal status is a significant prognostic factor in HNSCCs.

The consistency of protocol at both the sites will be assessed and the study will be periodically reviewed.

**Discussion**

The PRISM - HNSCC study is a bilateral research project conducted in 2 countries that have a huge burden of the disease. Among the states and territories in Australia, Northern Territory has the highest incidence of HNSCC and the Royal Darwin hospital is the largest public hospital that facilitates the treatment and management of the disease. [14] The actual burden of head and neck cancer in India is much greater than that reflected in the existing literature however it is the commonest malignancy encountered in Indian males.[15] According to the World Health Organisation Lip and oral cancers is the third commonest cancer in India with nearly 68% mortality in 2012.[16]

Head and neck cancer is considered to progress through a multistep process from normal histologic features to hyperplasia, mild dysplasia, moderate dysplasia, severe dysplasia, carcinoma in situ, invasive carcinoma, and metastasis.[3] Malignant transformation in cells are invisible microscopically with Haematoxylin & Eosin stain which may be identified more accurately with molecular markers especially in head and neck cancer, where, as a result of field cancerization, the entire mucosa has often undergone atypical changes.[1,3,9]

A retrospective study conducted in Darwin suggested the efficacy of IHC with e IF4E and p53 antibodies on surgical margins of HNSCC in assessing the completeness of surgery however the sample size was very small for a concrete conclusion. [14] Hence a larger sample and prospective study was warranted to validate the above finding.

The aim in this study is also to evaluate the use of vital staining and VELscope. These methods are currently being studied by McCaul et al[17] and Poh et al[18] respectively. The uniqueness of this project is the ability to study the outcomes and evaluate the efficacy of all three methods put together.

Staining with Lugol's iodine solution has been shown to be effective in intraoperatively delineating the extent and precise border of the cancerous and dysplastic epithelium of the mucosal surface. It is cheap and hence can be used as a cost effective, easy and quick screening test particularly in resource poor countries in detecting premalignant mucosa of individuals who consume tobacco, alcohol and have other lifestyle risk factors.[5-6]
VELscope has up to 55% accuracy in enhancing the direct visualisation of dysplastic mucosa. When combined with Lugol's iodine there is a potential for increasing the accuracy of the screening method. However there is a capital expenditure with purchasing the equipment that may eventually be cost effective in avoiding recurrence. [7]

Molecular analysis by performing immunohistochemistry on surgical margins with e IF4E and p53 has been suggested to predict recurrence in previous studies however the role of p53 is controversial. Besides being a prognostic marker e IF4E can also be targeted for therapeutic intervention.[8, 13, 19]

The TP53 and retinoblastoma pathways are almost universally disrupted in HNSCCs, indicating the importance of these pathways in head and neck tumorigenesis. More than 50% of HNSCC harbor TP53 gene mutations and over 50% demonstrate chromosomal loss at 17p the site where the TP53 gene resides.[1]

The eukaryotic protein synthesis initiation factor e IF4E has been found to be elevated in Carcinoma breast and HNSCC but not in benign lesions or normal mucosa. Recurrence of HNSCC was found to be more common in patients with elevated e IF4E in surgical margins. No other marker has provided evidence for being effective in detecting malignant alteration in cells. Since recurrence in HNSCC usually occurs within the first 2 years the prognostic value of e IF4E can be used in a relatively short follow up time[9].

Since both the institutions receive HNSCC patients representative of sample population the results can be validated to impact. This collaborative trial between two countries has set a precedence to build and continue the partnership for future studies, education and guide protocols in diagnosis and treatment.

## Ethics and Dissemination

All patients (or their legally authorised representative) included in this study will sign a consent form that describes this study and provides sufficient information for patients to make an informed decision about their participation**.** The written consent from every patient, at both centres will be obtained on the HREC/IRB-approved consent form, before that patient's biopsy specimen undergoes immunohistochemistry. Any protocol amendments will be communicated to investigators, HREC/IRB, participants and Australian New Zealand clinical trials registry, as deemed necessary.

Clinical and histopathological information about study participants will be accessible only to the site investigators and kept confidential by them. Identifiable data collected from electronic and hardcopy patient files by SJ will be stored securely on a password protected computer and external hard drive. De - identified data will be used for analysis and interpretation of the results.

Paraffin sections and slides will be stored in the departmental repository.

Results of the study will be submitted for publication and presented as a dissertation and at departmental meetings and conferences.

**Author Contributions:** Sheela Joseph (SJ), Rajinikanth Janakiraman (JR), Geeta Chacko (GC), Rama Jayaraj (RJ), Mahiban Thomas (MT), Meera Thomas (MeT) and Sramana Mukhopadhyay (SM) made substantial contributions to the conception or design of the study or acquisition of data, drafting the manuscript or revising it critically for important intellectual content. All the authors read and approved the final manuscript.

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**Collaborators**: *Australia* - Royal Darwin Hospital – Mahiban Thomas, Cameron Scott, James Badlani. Charles Darwin University – Rama Jayaraj, Sheela Joseph. *India* - Christian Medical College, Vellore – Rajinikanth Janakiraman, Geeta Chacko, Meera Thomas, Sramana Mukhopadhyay.

**Competing interests:** None

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**Figure Legends:**

Figure 1: Flow chart of research activity and the involvement of key personnel

Figure 2: Positive control (Glioblastoma with p53 mutation) for p53 antibody at 200X magnification showing unequivocal brown stain of the nucleus

Figure 3: Positive control (Carcinoma breast) for eIF4E antibody at 400X magnification showing unequivocal brown stain around the nucleus



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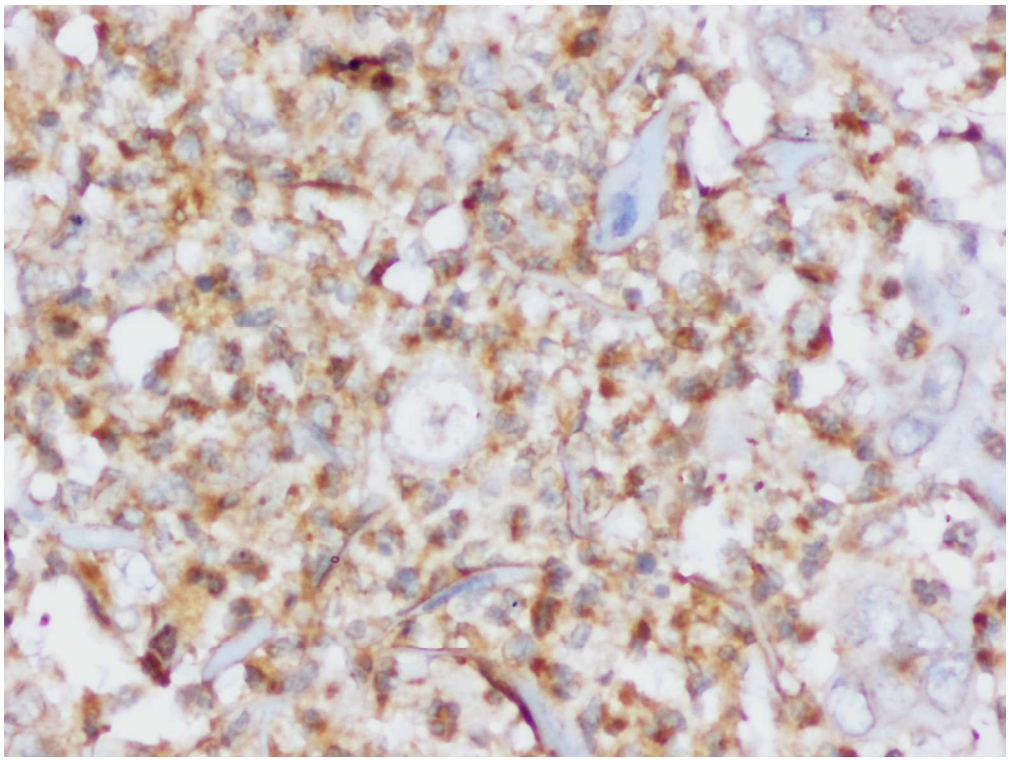

Figure 3: Positive control (Carcinoma breast) for eIF4E antibody at 400X magnification showing unequivocal brown stain around the nucleus

152x114mm (300 x 300 DPI)

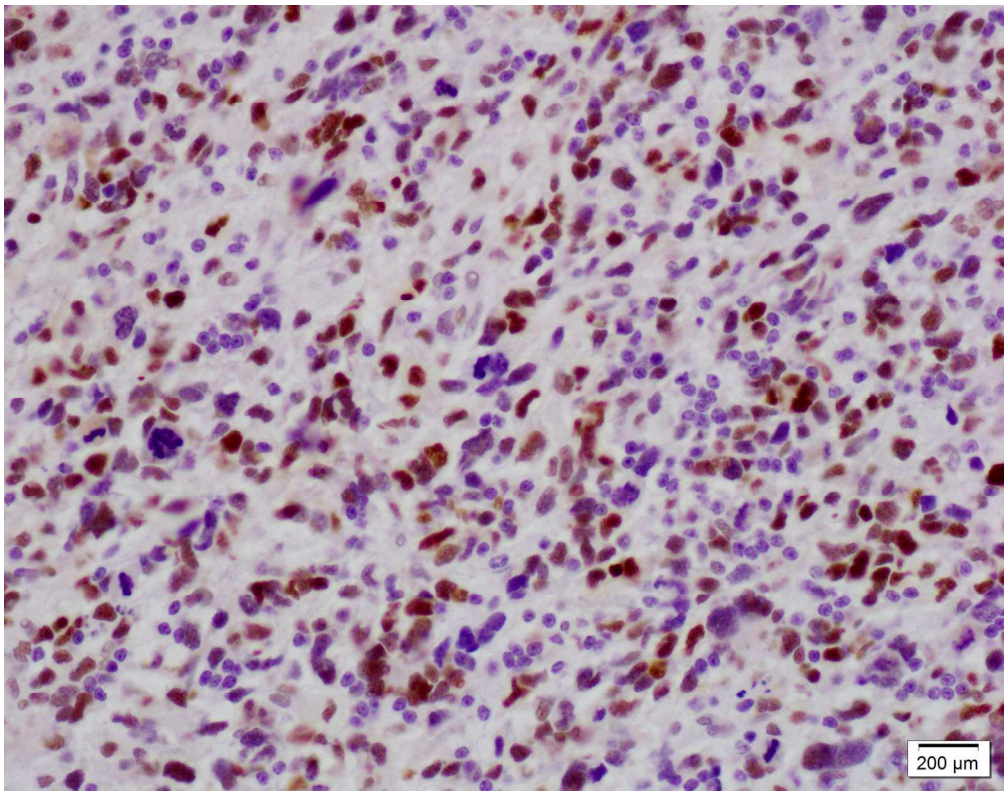


Figure 2: Positive control (Glioblastoma with p53 mutation) for p53 antibody at 200X magnification showing unequivocal brown stain of the nucleus

152x119mm (300 x 300 DPI)

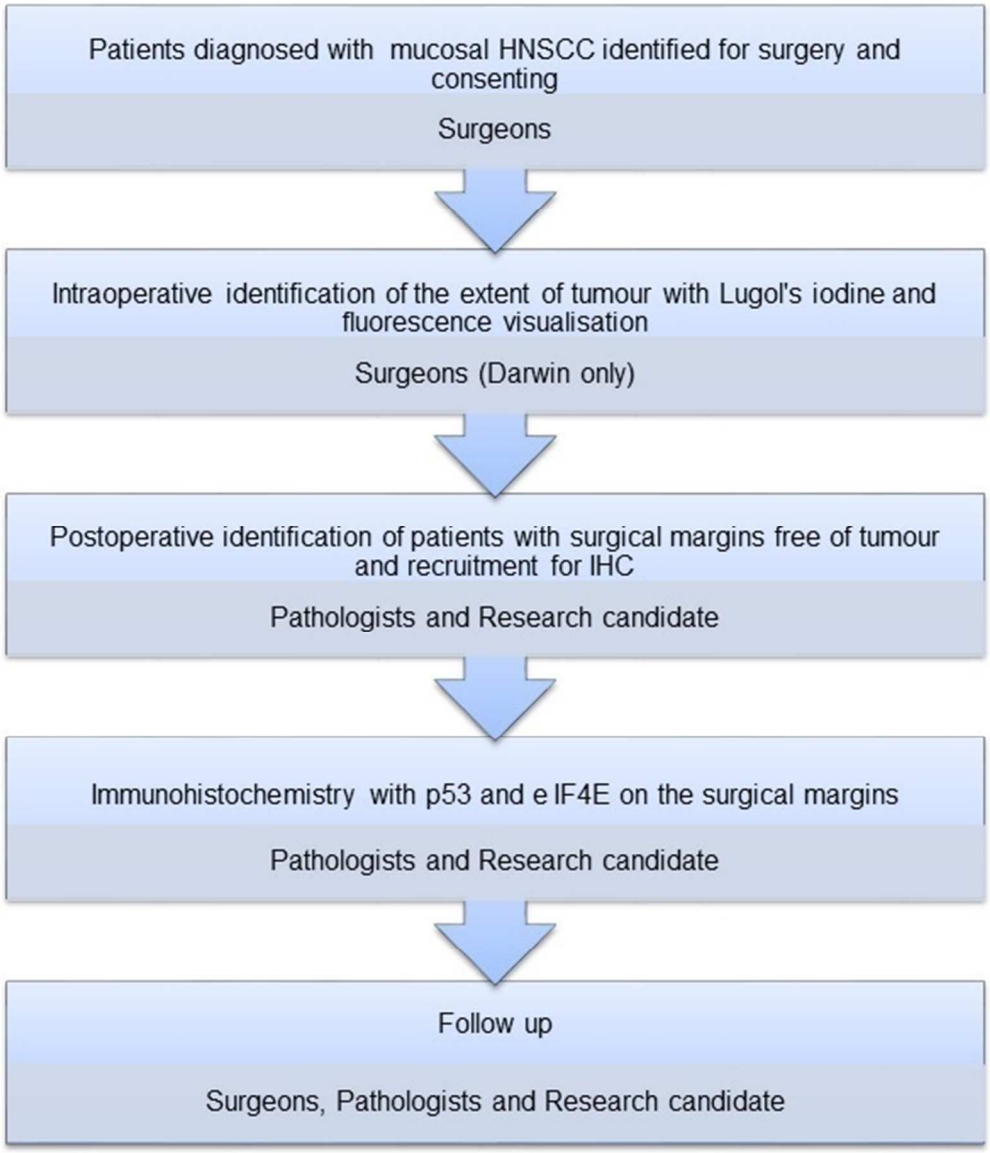


Figure 1: Flow chart of research activity and the involvement of key personnel

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SPIRIT
STANDARD PROTOCOL ITEMS: RECOMMENDATIONS FOR INTERVENTIONAL TRIALS

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

| Section/item | Item No | Description |
|---|---|---|
| **Administrative information** | | |
| Title<br>**Page 1** | 1 | Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym |
| Trial registration<br>**Page 2** | 2a | Trial identifier and registry name. If not yet registered, name of intended registry |
| | 2b | All items from the World Health Organization Trial Registration Data Set |
| Protocol version | 3 | Date and version identifier – ~~1/11/16~~ 4/11/16 |
| Funding<br>**Page 13** | 4 | Sources and types of financial, material, and other support |
| Roles and responsibilities | 5a | Names, affiliations, and roles of protocol contributors |
| | 5b | Name and contact information for the trial sponsor |
| **Page 1** | 5c | Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities |
| | 5d | Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee) |
| **Introduction** | | |
| Background and rationale<br>**Page 4 & 5** | 6a | Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention |
| | 6b | Explanation for choice of comparators |
| Objectives<br>**Page 6** | 7 | Specific objectives or hypotheses |

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| Trial design<br>**Page 6** | 8 | Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory) |
| **Methods: Participants, interventions, and outcomes** | | |
| Study setting<br>**Page 6** | 9 | Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained |
| Eligibility criteria<br>**Page 6, 7 & 8** | 10 | Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists) |
| Interventions<br>**Page 8 & 9** | 11a | Interventions for each group with sufficient detail to allow replication, including how and when they will be administered |
| | 11b | Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease) |
| | 11c | Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests) |
| | 11d | Relevant concomitant care and interventions that are permitted or prohibited during the trial |
| Outcomes<br>**Page 10** | 12 | Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended |
| Participant timeline<br>**Page 7 & 9** | 13 | Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure) |
| Sample size<br>**Page 6** | 14 | Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations |
| Recruitment<br>**Page 7** | 15 | Strategies for achieving adequate participant enrolment to reach target sample size |
| **Methods: Assignment of interventions (for controlled trials)** | | |
| Allocation: | | |

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| Sequence generation | 16a | Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions |
| Allocation concealment mechanism | 16b | Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned |
| Implementation | 16c | Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions |
| Blinding (masking) | 17a | Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how |
| | 17b | If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial |
| **Methods: Data collection, management, and analysis** | | |
| Data collection methods **Page 9** | 18a | Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol |
| | 18b | Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols |
| Data management **Page 10** | 19 | Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol |
| Statistical methods **Page 10** | 20a | Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol |
| | 20b | Methods for any additional analyses (eg, subgroup and adjusted analyses) |
| | 20c | Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation) |

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| **Methods: Monitoring** | | |
| Data monitoring | 21a | Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed |
| | 21b | Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial |
| Harms | 22 | Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct |
| Auditing | 23 | Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor |
| **Ethics and dissemination** | | |
| Research ethics approval<br>**Page 2** | 24 | Plans for seeking research ethics committee/institutional review board (REC/IRB) approval |
| Protocol amendments | 25 | Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators) |
| Consent or assent<br>**Page 7 & 8** | 26a | Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32) |
| | 26b | Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable |
| Confidentiality | 27 | How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial |
| Declaration of interests<br>**Page 13** | 28 | Financial and other competing interests for principal investigators for the overall trial and each study site |
| Access to data | 29 | Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators |
| Ancillary and post-trial care | 30 | Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation |

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| Dissemination policy | 31a | Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions |
| | 31b | Authorship eligibility guidelines and any intended use of professional writers |
| | 31c | Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code |
| **Appendices** | | |
| Informed consent materials | 32 | Model consent form and other related documentation given to participants and authorised surrogates |
| Biological specimens | 33 | Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable |

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.