PROTOCOL TITLE:

Role of the oral microbiome & mucosal immunity in COVID-19 disease: diagnostic/prognostic utility in South Asian populations

Short title: Oral Microbiome & Mucosal Immunity in COVID-19 Disease

Acronym: MIMSA

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IRAS number 295430
EDGE number 142218
ClinicalTrials.gov Identifier: NCT05212766
REC reference: 22/SC/0105

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Study Synopsis

Title	**Role of the oral microbiome & mucosal immunity in COVID-19 disease: diagnostic/prognostic utility in South Asian populations**
Protocol Short Title/Acronym	Oral Microbiome & Mucosal Immunity in COVID-19 disease (MIMSA)
Protocol Version number and Date	Version 2.0 02/05/2023
Study Phase if not mentioned in title	1
Is the study a Pilot?	No
Study Hypothesis	That differences in morbidity and mortality of South Asian populations following COVID-19 disease are related to differences in mucosal immunity and the oral microbiome.
Study Duration	18 months duration
Methodology	Cross sectional with longitudinal component • South Asian (SA) and White British (WB) populations • Blood and stimulated whole mouth fluid (SWMF) • Uninfected subjects, and COVID-19 patients in both populations. • Medical History, Oral disease questionnaire and clinical examination • Separation of SWMF into pellet for DNA extraction for microbiomics and supernatant for cell phenotype analysis, cytokines and antibodies to SARS-CoV2 antigens
Sponsor name	King's College London/GSTT
Chief Investigator	Professor Stephen Challacombe
REC number	22/SC/0105
Medical condition or disease under investigation	COVID-19 disease
Purpose of clinical trial	To collect clinical samples from defined patient groups for laboratory analysis
Primary objective	Determining whether in the mouth there are differences between the participant groups in the nature and activity of mucosal innate immunity, in immune responses to SARS-COV2 antigens, or in the oral microbiome
Secondary objective (s)	1. To determine whether differences in mucosal immunity or differences in the oral microbiome correlate with susceptibility to COVID-19

	infection, or to disease severity or indicate speed or efficacy of disease control. 2. Determining whether the presence of pre-existing oral disease is related to susceptibility to SARS-CoV2 infection, to severity of COVID-19 disease or relates to the nature of mucosal immunity and to the composition and activity of the oral microbiome
Number of Subjects/Patients	Up to 800 subjects will be recruited, comprising up to 400 SA and 400 WB, each including patients, recovered and uninfected subjects.
Trial Design	Cross sectional with longitudinal component. Samples of SWMF, and blood and a detailed mouth examination on all participants. Lymphocyte phenotypes in blood and SWMF compared, cytokines and serum and secretory antibodies to SARS-CoV2 antigens. DNA extraction from SWMF for meta-analysis of oral microbiome, and metabolome and proteome analysis
Endpoints	1. Patients recruited 2. Samples collected and processed 3. Generation of raw data
Main Inclusion Criteria	• *South Asian* and White British persons and those diagnosed with current or past symptomatic or asymptomatic SARS-CoV2 infection, or uninfected.* • *Aged 18 or over.* • *Able to understand and consent.* • *For in patient groups: Confirmed COVID-19 positivity, symptoms and symptom onset within the past 21 days;*
Statistical Methodology and Analysis	A total sample size of up to 800 participants, up to 400 of which will provide samples at three further time points. Between 30 and 50 values per parameter would be needed to demonstrate statistical differences on a cross sectional basis and 30-40 per parameter on a sequential basis. The microbiome, clinical data and immunome data will be associated using Spearman correlations. A Mann–Whitney U-test will be used to determine the median differences of continuous clinical variables. For more than two groups, the Kruskal–Wallis test with post-hoc Dunn test will be used.

*South Asian defined as those of Indian sub-continent origin.

There is no device or product used in this study

Glossary of Terms and Abbreviations

AE Adverse Event

AR	Adverse Reaction
ASR	Annual Safety Report
CA	Competent Authority
CI	Chief Investigator
CRF	Case Report Form
CRO	Contract Research Organisation
DMC	Data Monitoring Committee
EC	European Commission
GAfREC	Governance Arrangements for NHS Research Ethics Committees
GSTT	Guys & St Thomas Hospital
ICF	Informed Consent Form
ISRCTN	International Standard Randomised Controlled Trial Number
KCH	King's College Hospital
KCL	King's College London
MA	Marketing Authorisation
MS	Member State
Main REC	Main Research Ethics Committee
NHS R&D	National Health Service Research & Development
PI	Principal Investigator
QA	Quality Assurance
QC	Quality Control
Participant	An individual who takes part in a clinical trial
RCT	Randomised Controlled Trial
REC	Research Ethics Committee
SAE	Serious Adverse Event
SDV	Source Document Verification
SOP	Standard Operating Procedure
SSA	Site Specific Assessment
SWMF	Stimulated Whole Mouth Fluid
TMG	Trial Management Group
TSC	Trial Steering Committee

1. Introduction

Background

SARS-CoV2 primarily infects respiratory mucosae, yet mucosal immunity has barely been studied. There is increasing evidence of the importance of both natural immunity (innate) and of immune responses (adaptive) of mucosal surfaces of the whole of the upper aerodigestive tract and of the lower respiratory tract in protection against disease, but very little is known in relation to COVID. Minor salivary glands appear to be a major site of SARS coronavirus replication (Liu et al 2011) and in the virological phase (first week) extremely high viral loads can be found in saliva (To et al 2020) and may be higher than in the nasopharynx (Yoon et al 2020): The oral cavity is rich in ACE2 receptors (Xu et al 2020; Huang et al 2020) especially in the epithelial cells of the many hundreds of minor salivary glands (Wu et al 2020). SARS-CoV2 can also be found in gingival crevicular fluid. It is known that in viral diseases T-cells and cytokines are altered in both systemic and mucosal immunity but have not been much explored in the mucosal response of the upper aero-digestive tract.

The role of oral mucosal immunity has been well elucidated in a number of oral and systemic diseases (Feller et al., 2013; van Splunter et al., 2018; Naiff et al., 2014). The host immune response elicited by SARS-CoV2 upon dysregulation leads to high morbidity and mortality. Thus, the role of mucosal immunity in the pathogenesis of COVID-19 is of paramount importance. Although a number of host immune factors including cytokines (notably IL-6, IL-1β, IFN-γ) and T cell phenotypes (Th1, Th2, Th-17, Tregs, Tfh cells) have been implicated in a few reports to play important roles in systemic immunity to SARS (Chen and Wherry, 2020; McElvaney et al., 2020), their role in mucosal immunity remains largely unexplored.

Dysregulated immune responses have been shown to orchestrate disease pathogenesis in many viral infections including dengue and HIV (Uno and Ross, 2018; Wang et al., 2016). Decrease in Tregs, Tfh cells and germinal centre formations have been shown in lung autopsies of severe COVID-19-19 patients (Duan et al 2020). Although class switching from IgM to IgG antibodies takes about 7 days in most infections, specific anti-SARS-CoV2 IgM and IgG responses have been detected within 2-3 days (Thevarajan et al., 2020; Suhandynata et al., 2020). These findings implicate B cell responses as being differentially orchestrated by the T cells. Taken together, these data indicate that COVID-19 is a disease driven via host immune system so that understanding the immune players and the molecular pathways involved is of utmost importance for rational patient management.

Recent years have seen great increases in our understanding of the oral microbiome in health and disease and its relationship to gut, lung and nasal microbiomes. All mucosal surfaces have a normal stable microbial flora (the microbiome) which protects against invasion by pathogens. However, in COVID any role of a mucosal microbiome is completely unknown. Oral diseases, especially periodontal diseases, allow bacteria and viruses to readily enter the blood stream. It is unclear whether this plays a role in systemic manifestations of COVID. Microbial dysbiosis appears to be a factor in COVID-19 severity. Metagenomic analyses of patients infected with SARS-CoV2 have frequently reported high reads of cariogenic and periodontopathic bacteria,

endorsing the notion of a connection between the oral microbiome and COVID-19 complications (Chakraborty 2020). There is considerable evidence that periodontopathic bacteria are involved in the pathogenesis of several respiratory diseases, and are associated with chronic inflammatory systemic diseases including type 2 diabetes, hypertension, and cardiovascular disease (Patel and Sampson 2020). Advances in high-throughput sequencing have revolutionised our understanding of human-microbe interactions and have led to a substantial rise in knowledge of the human microbiome. With this increased understanding has come an awareness of the significant role that the microbiome of mucosal surfaces plays in several diseases.

Notably, recent studies support the hypothesis that the oral microbiota is of relevance in both acute and chronic lung disease, with poor oral health associated with an increased risk of respiratory disease (Mammen et al 1999). A question is, therefore, whether inflammation in the oral cavity may predispose to SARS-CoV2 infection (Sampson et al 2020). We know that improving oral health results in a reduction in respiratory infections in ICU and nursing home patients (Azarpazhooh et al 2006). Of particular relevance to the current COVID-19 pandemic, risk factors including coughing, hyperventilation and mechanical ventilation have been shown to provide a pathway for members of the oralmicrobiota to penetrate the lower respiratory tract and cause disease (Bao et al 2020). Co-infections have been significant causes of mortality in many previous viral pneumonia pandemics, including the recent 2009 swine flu outbreak (MacIntyre,et al 2018), and these phenomena appear to be repeating for the current COVID-19 pandemic. Early metatranscriptome analyses indicate the presence of elevated levels of oral commensal bacteria in the bronchoalveolar lavage of COVID-19 patients (Shen et al 2020).

There is increasing evidence of disparities in susceptibility to SARS-CoV-2 infection and subsequent morbidity and mortality amongst groups of different ethnic origin. In the UK, South Asian populations have been identified as a particularly susceptible group, with a high percentage mortality in contrast to similar populations in India, even after taking into account the effect of socio-economic level and of co-morbidities. The extent to which social, environmental and biological factors differ in the two populations and contribute to differences in outcomes from COVID-19 is not known.

Our research will compare the oral microbiome, salivary innate and specific mucosal antibody responses and oral disease among SARS-CoV2-positive patients and controls in the UK and determine their diagnostic and prognostic utility. We will compare mucosal and systemic immunity in order to reveal biomarkers for risk of disease progression so as to enable early initiation of treatment.

The study is part of a UK-India partnership and at the end of the research, data from the UK will be compared with that derived from a similar study with our collaborators in India.

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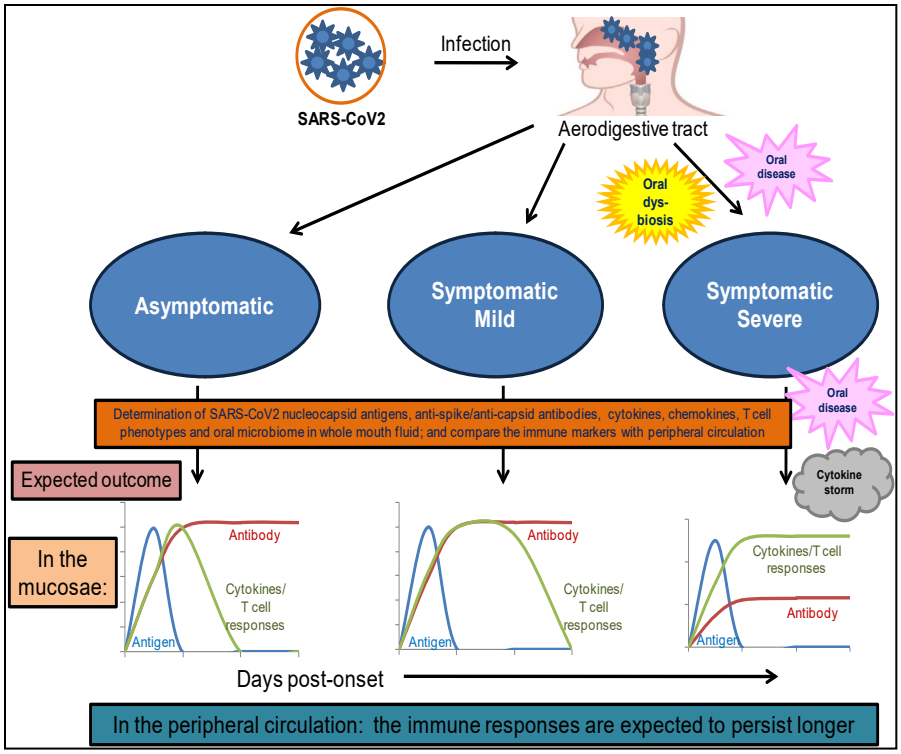

Figure: Model of possible interrelationships between innate cellular immunity, cytokines and mucosalantibody in patients with asymptomatic, mild or severe COVID-19 disease. Oral mucosal immunity influenced by host immune dysregulation, underlying oral disease and/or oral dysbiosis determines the level of disease severity in COVID-19. When an individual gets infected with SARS-CoV2, there will be an immediate peak in viral proteins due to the initial proliferation of the virus (virological phase), which lasts about 5-7 days. This antigen peak is shown as a blue line in all the three groups. When the individual's cytokines and T cell responses are modulated appropriately the virus is cleared with no clinical symptoms (asymptomatic) or with very minimal symptoms (symptomatic–mild). The cytokines and T cell responses (green line on the graphs) are short-lived in these two groups. On the other hand, when there is immune dysregulation, underlying oral disease and/or dysbiosis, the cytokines and T cell responses remain upregulated for a long period (cytokine storm) thereby causing tissue destruction (symptomatic–severe), which in turn increases the morbidity and mortality (immunological phase). The protective/virus neutralizing antibodies (red line on the graphs) potentially appear after one week and remain high in the asymptomatic and symptomatic-mild groups of patients. However, in the symptomatic-severe group, the anti-SARS-CoV2 antibodies potentially remain lower and hence might not confer any protection. The graphs depict the expected outcome in the whole mouth fluid. These markers are expected to persist even longer in the peripheral circulation. The proposed study is designed to address these key points and potentially unveil the host immune players that drive the disease pathogenesis in COVID-19.

2 Trial Objectives, Design and Statistics

2.1. Trial Objectives

- Primary Objective

The key aim of this research in South Asian populations and White British populations, is to find out whether differences in COVID-19 morbidity and mortality in similar populations in the UK might be related to mucosal immunity expressed in the mouth and to the oral microbiome (the total bacteria, fungi and viruses living in the mouth). This will be achieved by determining

whether in the mouth there are differences between the participant groups of mucosal natural immunity (innate immunity), immune responses to SARS-COV2 antigens (adaptive immunity), or the oral microbiome

- Secondary Objectives

1. To determine whether differences in mucosal immunity or differences in the oral microbiome correlate with susceptibility to COVID-19 infection, or to progression and recovery from the disease.
2. To determine whether the presence of pre-existing oral disease is related to susceptibility to SARS-CoV2 infection, to severity of COVID-19 disease or relates to the nature of mucosal immunity and to the composition and activity of the oral microbiome

- Primary End Point
    - Collection, processing and analysis of samples. Statistically significant quantitative differences in measured mucosal innate immune factors, mucosal adaptive immune factors and/or in the oral microbiome in relation to 1) a comparison between South Asian and White British populations in the UK 2) the degree of severity of COVID in South Asian and White British participants in the UK

- Secondary End point
    - Generation of raw data.
    - *a comparison between South Asian populations in the UK and in Chennai, India.

***Note:** *This is the UK part of an international research collaboration between the UK (funded by the MRC) and India (funded by the DBT) with research being carried out separately but in parallel in the two Countries and generating results which will be directly compared.*

2.2 Trial Design & Flowchart

Proposed Methodology: In this combined cross sectional and prospective longitudinal study, non-COVID infected subjects and SARS-CoV2-positive patients of South Asian origin or White British origin diagnosed to have, or who have had, asymptomatic, mild, moderate or severe disease as per WHO or the equivalent NIH criteria will be recruited. Peripheral blood (approx 17 ml divided into 5ml clotted, 2ml EDTA, 2ml fluoride, 8ml heparinised) and stimulated whole mouth fluid (SWMF) samples (approx 4ml) will be collected from all patients and from subgroups at three further time points at approximately 2, 4 and 12 weeks. (SWMF is a combination of saliva, gingival crevicular fluid, desquamated epithelial cells, some nasopharyngeal secretions, some serum transudate). In addition to medical data available for each patient, short questionnaires on oral health and diet, a clinical examination for oral health using "PSR akaBPE" – periodontal screening and recording/basic periodontal examination of periodontal health will be undertaken. The maximum periodontal pocket depth in mm on all standing teeth using a graduated periodontal probe and the presence of bleeding on probing will be recorded for each tooth. Missing teeth will be noted.
Further information on ethnicity of South Asians will be sought, eg from Pakistan, Bangladesh, North India, South India or Sri Lankan [Sinhalese, Tamil or Muslim].

Sequence of consent, examination and samples:

Each of the two population cohorts (South Asian and White British) has four sub-groups (clinically uninfected subjects, asymptomatic/mild, symptomatic moderate/severe, and recovered). After patient information sheets and consent, then oral health questionnaires (5mins), SWMF samples (5-15 mins), oral health (periodontal) examination (10 mins), and blood via venesection (5 mins). Patients will chew on a pellet of wax until approximately 4ml SWMF is recovered by drooling into sterile universal containers (between 5 and 10 minutes normally) after which the chewed wax will be wrapped in gauze and disposed with other clinical waste in adherence to infection control procedures. Blood samples may be taken before, during or after the SWMF collection. Repeat blood and stimulated SWMF will be taken at approximately 2 weeks, 4 weeks and 12 weeks after baseline for those participants willing to take part in the sequential studies. Oral examination and partial mouth recording will not be repeated unless any material change in oral condition is evident.
Uninfected subjects will be further defined as those who do not show antibodies to nuclear capsid protein antigens. The presence of such antibodies would indicate exposure to SARS-CoV2 rather than immunisation, and would be classified as asymptomatic if no history of symptoms.

Number of samples: With South Asian and White British participants, there will be up to 100 patient samples in each of the four sub-groups (uninfected subjects, asymptomatic/mild and moderate/severe disease, and recovered.). All will be assayed for immunological parameters, and the first 50 in each group for microbiome analysis, with the remainder stored for future analysis. The first 50 participants in each group will be invited to provide sequential samples.

Times of samples: There will be repeat samples of SWMF and blood at approximately 2, 4 and 12 weeks. Those SWMF at 4 weeks will be analysed for the microbiome in addition to the immunology (see table 1).

Choice of time points: Mucosal immunological responses have a different and often shorter time frames than systemic responses. The 2 week post infection or post vaccination time point as well as the 4 week time point is to determine whether a response has been generated, and indicate possible kinetics of the mucosal response in comparison with systemic (serum) responses. The 12-week sampling point was selected as optimal to indicate longevity of any mucosal response, and any differential between a possible protective response of longer duration from a response of shorter duration.

Handling of samples: After collection, samples for GSTT haematology (Viapath) and blood glucose (if results not already available) will be sent to GSTT haematology. Study pseudoanonymised samples will be transported to the Centre for Host-Microbiome Interactions (CHMI) laboratories in Guy's Tower by the clinical research dentist. SWMF will be transported on ice. All samples will be subject to current infection control measures and containers pre-labelled. In the lab, SWMF samples will be centrifuged and immune factors including cytokines and anti-SARS-CoV2 antibodies assayed in the supernatant, whilst cellular factors determined in reconstituted pellet. DNA extraction for the microbiome studies will be performed on the saliva pellet. Peripheral blood T cell phenotypes will be assayed in the supernatant from plasma.
Recruitment: Patients identified by the clinical team will be recruited by a clinical research assistant and consented. Uninfected subjects and ambulant patients will be recruited within Guys

and St Thomas' Hospital sites, from KCL sites and for General Medical Practice PIC (participant identification centre) sites. Symptomatic patients will be recruited from within outpatients or on the wards. From each subject, basic medical data will be collected and an oral health questionnaire applied, followed by SWMF, an oral examination and blood samples as above.

Timing of research: We have recognised that the success of vaccination in the UK has reduced the potential patient pool, although the Omicron strain has produced a surge in cases. Samples will be taken over 15 months and may include a greater proportion of ambulant patients than originally planned, including those identified as asymptomatic carriers from routine testing, and those to be vaccinated. Although samples will be batch analysed, we do not anticipate that robust data can be determined until towards the end of the collection period, and then compared with the data derived from the study in Chennai. Findings will be disseminated as widely as possible in academic journals, academic meetings and in appropriate form in public media and a study website.

2.3 Trial Flowchart

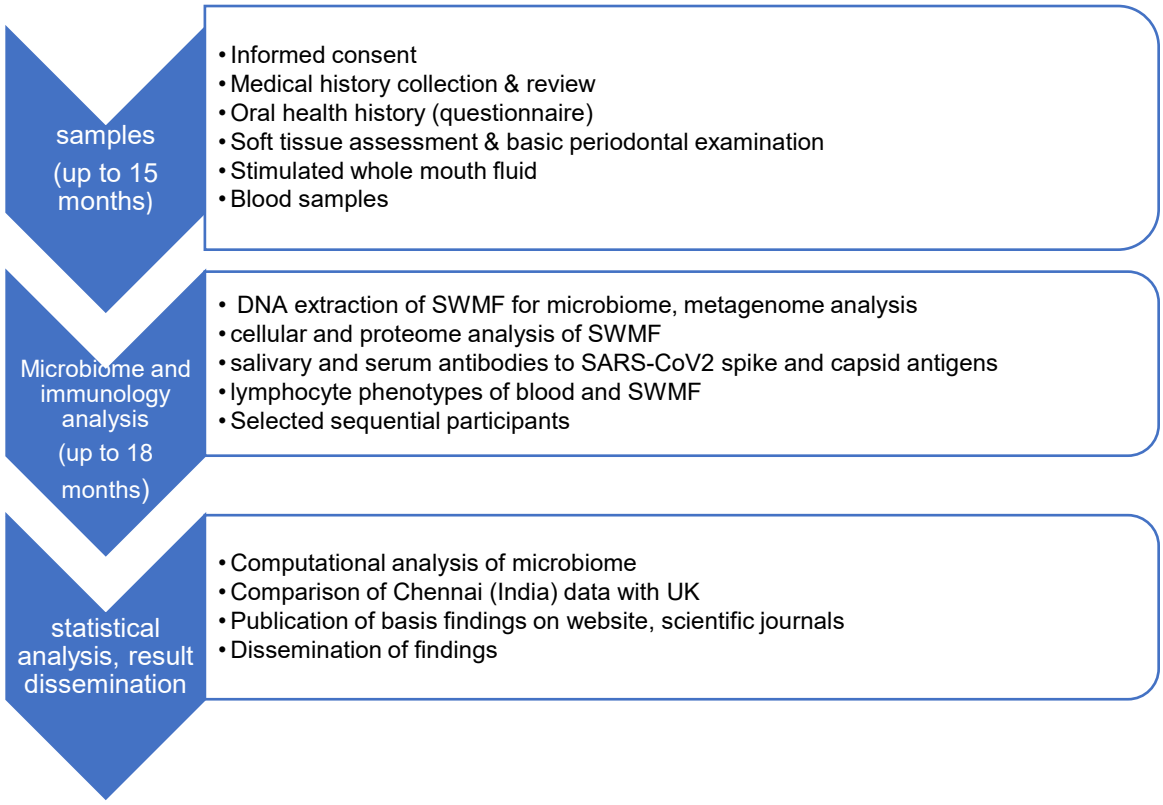

Table 1 Summary of Groups, Recruitment numbers, screening and sample collection

Patient Categories and timelines

		Baseline Im+Mb	10-14d	4 weeks	12 weeks*
South Asian	SA1 Uninfected	100	-	Im+Mb*	
	SA2 Asymptomatic/mild	100	Im	Im+Mb	Im
	SA3 Moderate/severe	100	Im	Im+Mb	Im
	SA4 Recovered	100		Im+Mb	Im+Mb
White British	WB1 Uninfected	100	-	Im+Mb*	
	WB2 Asymptomatic/mild	100	Im	Im+Mb	Im
	WB3 Moderate/Severe	100	Im	Im+Mb	Im
	WB4 Recovered	100		Im+Mb	Im+Mb
TOTAL	Participants	800	200	400	300
	Immunology samples	800	200	400	300
	Microbiome samples	800	-	400	100
	Blood samples	800	-	400	300

Samples for immunology studies (blood and stimulated whole mouth fluid- (SWMF) and microbiome (SWMF) are taken from all participants at baseline. Sequential samples of SWMF for immunology will be taken at approximately 2, 4 and 12 weeks in the first 50 of the COVID positive groups. Sequential samples for microbiome analysis will be performed on these samples at 4 weeks, and at 12 weeks in the recovered groups. The aim (following statistical advice) is to sample a minimum of 50 participants per group in the cross sectional part of the study, and a minimum of 30 per group in the sequential part.
* Post vaccination sample for subset of 50 two weeks after first and second vaccination.

2.4 Trial Statistics

Statistical advice indicates that with expected variation in both immunological and microbiome parameters, between 30 and 50 values per parameter would be optimal to demonstrate statistical differences on a cross sectional basis (more than 21 for immunological samples, more than 30 for microbiome samples) and that 30-40 per parameter would be optimal on a sequential basis. We

have therefore aimed for a minimum of 50 and a maximum of 100 participants for the cross sectional comparison, and between 30 and 50 participants on a longitudinal basis. Groups of 100 for the cross-sectional part of the study and 50 for the sequential also allow for the unexpected withdrawal of participants and leave sufficient numbers of samples for statistical analysis.

The microbiome, clinical data and immunome data will be associated using Spearman correlations. A Mann–Whitney U-test will be used to determine the median differences of continuous clinical variables. For more than two groups, the Kruskal–Wallis test with post-hoc Dunn test will be used. The association between the prototype prevalence, immunome and clinical data will be modelled with single (univariate) or multiple (multivariate) dependent variables (clinical & immunome metadata features) and will be performed using multinomial or binomial logistic regression.

Standard pipelines and statistical methods already in use within the group will be used for oral metagenome data. The data will be initially quality controlled and trimmed. Human contaminant sequences will be removed from reads by mapping against a human reference genome using Bowtie2. Scaffolds will be assembled from filtered reads using SPAdes. SAMtools will be applied to generate statistics for each filtered scaffold. The scaffolds will be merged to one scaffold file and bacterial genes from the file will be identified using Prodigal. The genes will be clustered and redundant genes will be removed using CD-HIT. Next all the unmapped reads will be mapped to the list of assembled genes and using METEOR software suite the gene counts will be generated for each sample and according to the depth of the sample sequence data, the count table will be downsized. Then the updated count table will be normalized to the gene length and converted to a frequency matrix, which we call the Gene Abundance Matrix.

The gene richness analysis will be done based on the updated count matrix to obtain the diversity of the samples at the gene level. A Wilcoxon test follow by Benjamini-Hochberg multiple testing correction will be used to obtain the significant changes between the cohorts. Next, we will map each sample to our already generated 1,942 oral Metagenomic Species Pangenomes (MSP), as an updated format for the metagenome species. The relative abundance of an MSP will be calculated using the mean abundance of its 100 ‘marker’ genes. To contrast the species abundance in different cohorts, we will perform Benjamini-Hochberg multiple testing correction of a Wilcoxon test. Functional analysis will also be performed using the PFAM and KEGG databases. Antimicrobial resistance analysis will be performed by searching reads using the CARD (Comprehensive Antimicrobial Resistance Database) database. In addition, “ortotyping” will be performed using the microbial information on the genus-level abundance with a Hellinger transformation with a Dirichlet multinomial mixtures (DMM) approach to cluster individual samples to distinct groups based on their microbiome composition, and a Fisher’s exact test will be used to identify the prevalent ortotype.

3. Sample Size, Selection and Withdrawal of Subjects

The success of the national vaccination programme has necessitated casting the recruitment net more widely than originally anticipated. Sample size is discussed in 2.4

Uninfected (vaccinated or unvaccinated) will be identified by advertising within KCL and GSTT sites, and at PIC sites, via social media and by recruitment at vaccination centres at GSTT or

testing centres at KCL/GSTT. Patients admitted to wards for non-COVID reasons and found to be SARS-CoV2 negative will be invited to participate. Uninfected (vaccinated) will be identified by recruitment at vaccination centres and by advertising within KCL and GSTT and PIC sites. Uninfected participants will be further defined as those who do not show antibodies to nuclear capsid protein antigens. The presence of such antibodies would indicate exposure to SARS-CoV2 rather than immunisation, and would be classified as asymptomatic if no history of symptoms.

All will be given the Patient Information Leaflet (PIL) about the study and access to the study website (https://www.kcl.ac.uk/research/mimsa)

Asymptomatic/mild subjects will be recruited from those identified as positive for SARS-CoV2 carriage in routine testing at KCL or GSTT. In both cases local staff will inform them about the trial and give out a patient information sheet. A medical history will be taken from those who consent to take part in the research

Symptomatic moderate/severe patients will be recruited within Guys and St Thomas Hospital, mainly in conjunction with the GSTT COVID infection teams. Patients will be recruited from those admitted or planned to be admitted to GSTT with a recent onset (⩽21 days) of symptoms of COVID-19 and a confirmed positive nasal/nasopharyngeal PCR test for SARS-CoV-2. They will be identified and approached by the clinical staff caring for them on the wards who will discuss the trial in outline and offer the PIL. Should the patient wish to consider participation after reading the PIL, the clinical research dentist (summoned by clinical ward staff) will obtain signatures of consent.

Recovered participants will be patients who have had COVID and fulfil the inclusion criteria. They will have either been discharged from GSTT or be attending either the post-COVID or ICU follow-up outpatient clinics and will be sent letters of invitation to participate. They will also be recruited from participating GP PIC sites, KCL sites and GSTT sites

Number of subjects	Subject selection
Group 1. SA1, BW1 **Uninfected Subjects** **(n=100 each)**	Uninfected subjects, both South Asian and White British will be recruited by advertisement within KCL or social media, from non-COVID in-patients or outpatients or attending A&E at GSTT and at SARS-CoV2 antigen testing centres and vaccination centres and at PIC sites. They will be furnished with the participant information leaflet and invited to take part in the study. After obtaining informed consent, oral examination, oral & general health questionnaires and samples of blood and stimulated whole mouth fluid (SWMF) (see below) will be taken either on the wards (inpatients), A&E or at the department of Oral Medicine at Guys Hospital
Group SA1v, BW1v (n=50)	In a subset of uninfected subjects from those attending for vaccination or volunteers, blood and SWMF will be taken 2 weeks after the first vaccination and 2 weeks after the second vaccination for immunology and microbiome analysis.

Group 2. SA2, BW2. Asymptomatic/mild n=100 each	**Asymptomatic/mild SARS-CoV2 positive group** Volunteers or subjects attending SARS-CoV2 testing centres who are found to be antigen positive or In-patients who have positive Covid tests and designated as asymptomatic/mild but admitted for non-covid reasons or those attending A&E with COVID but designated as mild/asymptomatic will be furnished with the participant information leaflet and invited to take part in the study After obtaining informed consent, oral examination, oral health & medical history questionnaires and blood and stimulated whole mouth fluid (SWMF) (see below) will be taken at the department of Oral Medicine at Guys Hospital or on the wards or in A&E at GSTT and KCH. Groups of 100 South Asian and White British participants will be recruited.
Groups 3 SA3, BW3 n=100 each	**Moderate/Severe Symptomatic patients** Patients either seen at GSTT or admitted as in-patients to GSTT or KCH and found to be SARS-CoV2 positive by RT-PCR in nasopharyngeal samples and designated as moderate/severe on the NIH/NIMR COVID severity scale will be furnished with the participant information leaflet and invited to take part in the study and will be enrolled in the study after obtaining written informed consent. After obtaining informed consent, oral examination, oral health & medical history questionnaires and blood and stimulated whole mouth fluid (SWMF) (see below) will be taken at the department of Oral Medicine at Guys Hospital or on the wards or in A&E. Groups of 100 South Asian and White British participants will be recruited.
Groups 4. SA4, BW4 n=100 each	**'Recovered' patients** These will be patients who have had COVID. They may be invited directly to participate by advertising at sites specified above or be attending either the post-COVID or ICU follow-up outpatient clinics. Patients may cover the COVID severity range and include those with a severe/moderate COVID history. Those attending the post-COVID clinic may include those referred in by primary care including those with long-COVID. Covid positive patients discharged from GSTT and fulfilling the inclusion criteria will be sent letters of invitation to participate. This data is available to the COVID clinical research team and participants will be identified with the Clinical Research Dentist.

3.1 Inclusion Criteria

Inclusion criteria	• South Asian and White British persons and those diagnosed with symptomatic or asymptomatic COVID-19 infection, or recovered from COVID infection or previously uninfected. • Aged 18 or over. Able to understand and consent. • For inpatient groups: Confirmed COVID-19 positivity, symptoms and symptom onset within the past 21 days; Be recently (within 7 days) hospitalised with COVID-19 disease; Have COVID-19 disease proven by PCR testing for SARS-CoV-2 within the last 21 days; • Uninfected subjects: no history of COVID-19; not vaccinated; (negative for anti-SARS-CoV2 nucleoprotein antibodies at lab)

	• Recovered groups: Have had COVID-19 disease proven by PCR testing for SARS-CoV-2. • Those willing to participate on a single occasion but unwilling to participate with longitudinal samples will not be excluded. • Smoking, obesity, diabetes, heart disease, antibiotics or treatment related to COVID is not excluded.

3.2 Exclusion Criteria

Exclusion criteria	• Those patients unwilling to participate, those unable to understand sufficiently to give informed consent and those unable to participate due to the severity of COVID-19 disease. • Those not classified as of either South Asian or White British heritage. • Patients with malignancy, who are pregnant, on long term immune suppression, or unable to give informed consent due to inability to communicate in, understand or read English • Those who are not willing to have an oral examination, or donate blood or saliva samples. • Those who cannot chew / drool to provide a SWMF sample due to severe/critical conditions. • Participation in other current research that is designed to, or is expected to alter the immune response. • Diabetes not excluded but screening for diabetes will be performed: glucose will be assessed in blood/serum sample (150ul).

3.3 Criteria for Premature Withdrawal

- If subjects withdraw consent
- Patients no longer wanting to continue with study

Participants have the right to withdraw from the study at any time for any reason.

4. Study procedures

4.1 Informed Consent Procedures

Participant Information Sheets will be given at the first point of contact regarding the study. For GSTT patients and volunteer participants in PIC sites this will normally be the clinical team. For volunteer participants responding to invitations to participate, this will normally be the clinical research dentist. Potential participants will be given a participant information sheet, and the research will be clearly explained verbally.
The participants will be given a minimum of 30 minutes to provide consent after discussion of the study. If any participants require a longer period of time to make a decision, this will be arranged.

If in the sequential part of the study a participant decides not to continue, samples and data that has already been collected will be included unless the participant specifically tell us not to.

4.2 Screening Procedures

Within GSTT, there is a dedicated COVID research team including nurses and consultants in infection, consultants in intensive care who have indicated willingness to help identify suitable patients meeting the inclusion criteria. Voluntary participation in the research project will be emphasized. Clinical care will continue as normal if the participant decides to leave the research at any time. Verbal and written consent will be obtained at the second visit by a member of the research team. Volunteers will be recruited from staff at GSTT and KCL through advertisement and Participant Identification Centers will be opened to advertise the study locally.

4.3 Randomisation Procedures

Samples will not be randomized.

4.4 Schedule of Treatment for each visit

Please see section 2.2 trial design. There is no treatment involved in this study.

Patients having been identified by the clinical treatment team as being suitable for recruitment to the study, will be asked whether they would like to participate in the study and given a participant information sheet (PIS) by the clinical team providing their care. The patient will be given at least 30 minutes to read and decide whether they would like to proceed to attend the pre-sampling visit. Patients who do not wish to participate will receive standard clinical care as normal. Volunteers also will be provided the PIS and have at least 30 mins to read through before providing informed consent.

After the patient information sheet has been given and consent obtained, the medical and oral health questionnaires will be asked, followed by the collection of SWMF (5-15 mins). Patients will chew on a pellet of wax until approximately 4ml SWMF is recovered by drooling into sterile universal containers (normally 5-10 minutes).The chewed wax will be wrapped in gauze and disposed with other clinical waste in adherence to infection control procedures. The oral health examination (10 minutes) will take place. Blood samples total 15ml (approximately 5 minutes) may be taken before, during or after the SWMF collection. Ward visits and follow up visits of patients previously seen at GSTT will be recorded in patients' hospital notes using EPR. For volunteer ambulant participants not previously patients of GSTT, appointments will be made at Guys Dental Hospital and patients pre-registered. Visits will be recorded on EPR and SALUD. Each participant in the three infected sub-groups (mild, moderate, recovered) once enrolled in the study, and willing to participate in the sequential part, would have a total participation time of approximately 85 minutes and four visits over 12 weeks. Uninfected subjects would be either a single visit of 40 mins or 60 minutes with a follow-up visit at four weeks (but not including travelling time)

Oral Health Clinical Assessment

We wish to record the severity and extent of active inflammation of the periodontium: a situation where, cumulatively around the mouth, very large areas of eroded or ulcerated gingival sulcular and pocket epithelium may be present. The standard in the field is "PSR/BPE" – periodontal screening and recording/basic periodontal examination. We will record the deepest pocket on every standing tooth as an accurate cumulative measure of inflamed surface area using disposable, appropriately marked, WHO probes. PSR simplifies this to a single code per sextant.

4.5 Follow up visits

Participants will be reviewed as per schedule above (2.3) and repeat samples of SWMF and blood taken. The oral health questionnaire and the whole mouth examination will not be repeated, unless there are major changes in the clinical condition. Participants will be given a window of +_ 5 days for scheduling appointments.

4.6 End of Study Definition

For subjects in cross sectional study– when collection and analysis of samples has been completed.
For subjects in the sequential study, following two or three further visits and sample collection. When target numbers of SWMF and blood samples have been processed and analysed.

5. Laboratories *(if Applicable)*

5.1 Central/Local Laboratories

Blood samples designated for routine blood tests and glucose will be sent to the hospital blood pathology lab for processing and analysis according to routine processes.
The remaining blood samples will be processed and prepared in the Centre for Host Microbiome laboratory on Floor 17, Tower Wing, Guy's Hospital. Microbial DNA extraction will be performed in these laboratories. Extracted microbial DNA identified by study number only will be sequenced by specialised commercial companies or academic institutions in the UK or non UK.

The salivary microbiome will be analysed on 50-100 patients per group after microbial DNA extraction with standardised protocol, using shotgun metagenomic sequencing on an Illumina NovoSeq generating > 20 million reads/sample. Sequencing will be performed by a commercial provider. Analysis of the sequencing data will be performed at KCL under the supervision of CHMI academics as described in section 2.4. The salivary metabolome will be determined using NMR. The longitudinal immune response and shifts in the oral microbiota will be correlated with COVID-19 severity as well as both COVID-19 and non-COVID-19 oral disease. We will identify significant differences in oral microbiota & immune responses during transition from viral to immunological to post-COVID-19 phases, identifying specific host factors as therapeutic targets.

Salivary secretory IgA antibody assay to study mucosal responses to antigens associated with COVID-19.

SARS is a closely related virus to SARS-CoV2. Several crystal structureshave been published that describe how different antigens recognize the SARS spike protein receptor binding domain. These highlighted antigenic regions in the SARS spike protein. Structures are also now available for the homologous SARS-CoV2 spike protein receptor binding domain. The amino acid sequence of the spike protein from SARS & SARS-CoV2 were compared. Major differences were identified and 10 peptides constructed. Three of these have been selected and will be assayed by ELISA along with Capsid antigens obtained commercially. The group has extensive experience with ELISA techniques, and has performed preliminary experiments to verify their use in SWMF. (This kit has been developed and validated in King's College London, and methodology shared with VHS, Chennai for sample testing.)

Blood and SWMF lymphocyte phenotyping

Blood and SWMF pellet T cell phenotyping will be performed by flow cytometry (BD FACSLyric) using monoclonal antibodies (BD Biosciences) to T cell markers. Pan T cells – CD3; Helper Tcells – CD4; CD4 T cell activation marker – CD25; Cytotoxic T cells – CD8; CD8 T cell activationmarker – CD38; NK cells – CD56 and HLA-DR; Th17 and Th22 cells – CCR4, CCR10; Tfh cells - ICOS; Tregs – Foxp3; T cell exhaustion marker – PD-1; effector and memory T cells – CD45RA and CD45RO/CCR7; mucosal homing marker – CD69; airway homing marker – integrin β1 and β7. Tcell phenotypes will be reported as percentages of the total T cells. Cells will be stained with the defined cocktail of antibodies for 15 minutes at room temperature in the dark; fixed and permeabilised; and then stained with intracellular antibodies (as appropriate) for 15 minutes; washed and analysed by flow cytometry.
For SWMF analysis, the samples will be gently centrifuged and a portion of the pellet resuspended in buffered saline, and incubated with the fluoresceinated antibodies before passing through an analyser.

Cytokine profiles will be analysed and quantified using the CBA kits (BD Biosciences) as per the manufacturer's instructions. Cytokines include IL-2, IL-4, IL-6, TNF-α, IL-1β, IFN-γ, IL-17, IL-22, IL-23, IL-8, IL-12p70, TGF-β, IP-10, MIG, MCP-1, CCL-20. CBAs will be analysed by flow cytometry (BD FACSLyric). Total IgG will be detected in the serum/plasma and SWMF supernatant samples by ELISA as per the manufacturer's instructions.

All of the techniques have been established, used and validated in the CHMI laboratories.

5.2 Sample Collection/Labelling/Logging

Whole mouth saliva will be collected into a universal tube. All samples will be pseudo-anonymised and code break sheet will be located in CHMI offices in a secure password protected database in Microsoft Excel or Access on an encrypted PC.

5.3 Sample Analysis Procedures

See 5.1

5.4 Sample Storage Procedures (if applicable)

All samples will be stored in an HTA licensed -80°C freezer until ready for testing. DNA extracted from samples will be stored in an HTA licensed -80°C freezer until ready for sequencing. (Designated Individual Dr Cheryl Gillett , HTA License number 12521, Person Designate: Professor Gordon Proctor)

5.5 Data Recording/Reporting

The data obtained during this study will comply with community standards for research data. King's College London aspires to ISO 27001 standards for data security. All hard copy data and link codes will be kept in a GSTT locked and secure filing cabinet in a locked room based in the PI's office Department of Periodontology, Faculty of Dentistry, Oral and Craniofacial Sciences in Guys Dental Hospital. Additional back up of link codes will be stored on secure GSTT storage. All pseudoanonymised electronic data will be held on KCL firewalled computers, subject to KCL standard audits In addition, data will be stored at KCL high performance computers with weekly backup program. Data will be recorded by a dedicated technician who will be HTA trained.

Data analyses:

The immunological markers will be compared between the healthy controls, asymptomatic and symptomatic COVID-19 patients using Mean, Median and non-parametric tests like Anova and Mann-Whitney rank sum tests. The immunological markers in the blood and SWMF will be compared and analysed at various time points of the disease progression. The key immunological parameters will be compared with the oral microbiome data to identify key biomarkers that may be indicative of the pathogenesis of the disease. These parameters will also be compared between the South Asian and the British-Caucasian population recruited in the UK. At the conclusion of the study, all the above analyses of the immunological and oral microbiome parameters will be compared among the age-matched and disease-matched participants in the UK and a collaborative, parallel study performed by collaborators in Chennai, India.

5.6 Sample Transfer

The SWMF, and blood/serum will not be transferred to any party not identified in this protocol and are not to be processed and/or transferred other than in accordance with the patients' consent.

6. Assessment of Safety

We do not anticipate the study to raise significant issues, other than the inconvenience of having to attend additional appointments for sequential samples. There is no interference with clinical care. There may be possible slight discomfort from taking of blood sample, or rarely from oral examination. No adverse effects anticipated from whole mouth fluid collection.
Repeat visits if not coincidental with medical visits will cause some inconvenience and potential travel expense.

6.1 Specification, Timing and Recording of Safety Parameters

Adverse events will be recorded following reports at any point during study visits. The chief investigator will report to the co-sponsors any SAE within 24 hours (see appendix) and report to the MREC within 15 days of learning of the event

6.2 Procedures for Recording and Reporting Adverse Events

In this study no products or devices will be used. Any abnormal blood tests will be recorded in the participant file and reported to the participant's GP or hospital care team if an inpatient. Any SAE apparently unrelated to the study but occurring during the study period will be recorded by the chief investigator and reported to the lead sponsor within 24 hours of awareness of the event.

6.3 Reporting Responsibilities

King's College London, as the lead sponsor, will be responsible for reporting any adverse events, and the Chief Investigator for recording any adverse events.

6.5 Ethics Reporting

Reports of related and unexpected SAEs will be submitted to the Main REC within 15 days of the chief investigator becoming aware of the event, using the NRES template. The form will be completed in typescript and signed by the chief investigator. The Coordinator of the main REC will acknowledge receipt of safety reports within 30 days. A copy of the SAE notification and acknowledgement receipt should be sent to the R&D Directorate at GSTT.

8. Ethics & Regulatory Approvals

This protocol and related documents have been reviewed and approved by the OXFORD Research Ethics Committee (REC). The Chief Investigator will submit a final report at conclusion of the study within the timelines defined in the Regulations

9. Data Handling

Dr David Moyes will act as custodian for the study data. The following guidelines will be strictly adhered to:

- Patient data will be pseudo-anonymised (unique code + initials).
- All data will be stored on a password protected computer.
- The study will be run to EU General Data Protection Regulations, 2018 and the UK Data Protection Act, 2018

10. Data Management

The data obtained during this study will comply with community standards for research data. King's College London aspires to ISO 27001 standards for data security. However, all psudeoananymised electronic data will be held on KCL firewalled computers, subject to KCL standard audits. All hard copy data and link codes will be kept in a locked and secure filing cabinet in a locked room based in the PI's office (Professor Ide). Department of Periodontology, Faculty of Dentistry, Oral and Craniofacial Sciences in Guys Dental Hospital. Additional back up of link codes will be stored on secure GSTT storage. In addition, study data will be stored at KCL high performance computers with weekly backup program.
The Principal Investigator will maintain study documentation containing clinical data for all participants entered into the study in a secured, locked and alarmed room, ensuring the confidentiality of the information collected. Securing records includes placing written forms in locked file cabinets and/or sealed and labelled storage boxes in a locked room and alarmed room. Access will be denied to all persons with the exception of the Principal Investigator and his designees.

Case Report Form

The CRF will record all information below-

Basic clinical parameters and safety recording
Patient demographics including ethnicity
Relevant Medical history including current medication
Relevant Dental History
Mouth Examination (this will include any previous dental habits)
"PSR/BPE" – periodontal screening and recording/basic periodontal examination.

Adverse Events,
The CRF will also have a withdrawal from study form which will record participants reason for withdrawal when/if known.

Patient Metadata and tissue collection form
Sample ID
Date of collection
Time of collection
Initials of clinician undertaking

Patient Metadata
Age of patient
Sex M/F
Oral Hygiene Habits
Does Patient use toothpaste yes/ no
Patient regularly sees dentist yes/no

History of previous periodontitis yes /no
If yes to give further details
If patient is a smoker (standard cigarettes) yes/ no
Patient smokes e-cigarettes yes/ no

Sample collection
SWMF: stimulated whole mouth fluid sample yes/no
Volume of SWMF collected (to be detailed at laboratory)
Blood samples yes/no

The CRF will be updated at all 3 study visits.
The CRF will be completed by the primary investigator at all study visits as well as any named staff working on the trial. All participants will be added to an enrolment log.

Record Retention and Archiving
Source documents containing or relating to clinical data will be kept for five (5) years after termination of the study. The Principal Investigator will maintain study documentation for all participants entered into the study in a secured, locked and alarmed room, ensuring the confidentiality of the information collected. Securing records includes placing written forms in locked file cabinets and/or sealed and labelled storage boxes in a locked room and alarmed room. Access will be denied to all persons with the exception of the Principal Investigator and his designees. Pseudo-anonymised data derived from the study will be kept for 10 years and then archived by Kings College London.

Compliance
The CI will ensure that the trial is conducted in compliance with the principles of the Declaration of Helsinki (1996), and in accordance with all applicable regulatory requirements including but not limited to the Research Governance Framework, Trust and Research Office policies and procedures and any subsequent amendments.

11. Finance and Publication Policy

The intention is for findings from the study to be published in appropriate peer-reviewed scientific journals. Summary findings will be placed on the study website **https://www.kcl.ac.uk/research/mimsa**

12. Insurance / Indemnity

The lead sponsor, King's College London, will take primary responsibility for ensuring that the design of the study meets appropriate standards and that arrangements are in place to ensure appropriate conduct and reporting. King's College London also provides cover under its No Fault Compensation Insurance, which provides for payment of damages or compensation in respect of any claim made by a research participant for bodily injury arising out of participation in a healthy volunteer study (with certain restrictions). The co-sponsor, Guy's & St Thomas' Foundation NHS Trust, take responsibility for arranging the initiation and management of this research, and will take responsibility for ensuring that appropriate standards, conduct and reporting are adhered to regarding its facilities and staff involved with the project.

13. Confidentiality

All information will be link anonymised for analysis of all laboratory samples.
All personal identifiable data will be kept secure on encrypted GSTT computers and in manual files which are kept in a locked and alarmed room, which is only accessed by CHMI research team (including research principal investigators, Research nurses, Clinical Research Dentists)
Laboratory samples (serum, SWMF and microbial DNA) and research data generated from them will be identified by study numbers only. All data handling will comply with university research guidelines and with the DPA.
Participants will be identified by their assigned study identification number on all case report forms and other documents.
Documents that identify the participant by name (e.g., the signed informed consent form and medical history form) will not be submitted to the sponsor, but will be maintained in strict confidence by the principal investigator, except to the extent necessary to allow auditing by the appropriate national or local authorities or co-sponsor staff personnel.

Appendix 1 – Information with regards to Safety Reporting in Non-CTIMP Research

	Who	When	How	To Whom
SAE	Chief Investigator	-Report to Sponsor within 24 hours of learning of the event -Report to the MREC within 15 days of learning of the event	SAE Report form for Non-CTIMPs, available from NRES website.	Sponsor and MREC
Urgent Safety Measures	Chief Investigator	Contact the Sponsor and MREC Immediately Within 3 days	By phone Substantial amendment form giving notice in writing setting out the reasons for the urgent safety measures and the plan for future action.	Main REC and Sponsor Main REC with a copy also sent to the sponsor. The MREC will acknowledge this within 30 days of receipt.
Progress Reports	Chief Investigator	Annually (starting 12 months after the date of favourable opinion)	Annual Progress Report Form (non-CTIMPs) available from the NRES website	Main REC
Declaration of the conclusion or early termination of the study	Chief Investigator	Within 90 days (conclusion) Within 15 days (early termination) *The end of study should be defined in the protocol*	End of Study Declaration form available from the NRES website	Main REC with a copy to be sent to the sponsor
Summary of final Report	Chief Investigator	Within one year of conclusion of the Research	No Standard Format However, the following Information should be included:- Where the study has met its objectives, the main findings and arrangements for publication or dissemination including feedback to participants	Main REC with a copy to be sent to the sponsor