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Protocol: Understanding the link between gum disease and heart attacks

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Background information

Heart attacks are one of the leading causes of death in the UK and across the world. The most common cause of heart attacks is the blockage of the arteries that supply the heart. These blockages are caused by a disease called atherosclerosis. Atherosclerosis occurs when fat (cholesterol) accumulates in the arteries, resulting in the inflammation, thickening, and hardening of the artery vessel wall. People with periodontal disease - a chronic infection of the gums - are especially likely to develop atherosclerosis.(Chistiakov D *et al*. 2016) However, many people are unaware that gum disease puts individuals at a greater risk of having a heart attack.(Munz et al. 2018, Dietrich *et al.* 2013, Bahekar e*t al*. 2007) Moreover, the precise link between atherosclerosis and periodontal disease remains a mystery. A greater understanding of the link between these diseases is essential in order to reduce the number of heart attacks.

The insulin-like growth factor(IGF) system plays an essential role in growth, metabolism and development. Dysregulation of the IGF system is seen in many diseases processes, with changes in one particular binding protein called Insulin-like Growth Factor Binding Protein-6 (IGFBP6),altered in many of these, particularly in cancers (nasopharyngeal, gastric, rhabdomyosarcoma) where levels are mainly decreased. (Bach 2016)

The wingless (Wnt) signalling protein family is essential for embryogenesis and adult tissue homeostasis. It is emerging that Wnt signalling is a driving force in many disease states including atherosclerosis. Wnt signalling can promote an inflammatory response in monocytes/macrophages (a type of white blood cell) and causes dysfunction of endothelial cells (a type of cell that lines the inner walls of arteries)-keys steps in the development of atherosclerosis.(Blankesteijn *et al.* 2015) Some studies in lung cancer cells and heart muscle cells have shown that IGFBP6 can inhibit the Wnt signalling pathway, illustrating an important interaction between the IGF and Wnt families. (Bach 2016)

Preliminary data from our laboratory studies has established that monocytes from the blood of people with periodontal disease had reduced levels of IGFBP-6 and this reduction can enhance invasion of monocytes into coronary arteries- a key stage in atherosclerosis. Furthermore, we looked at proteins that can be influenced by IGFBP-6 and saw significantly enhanced levels of Egr1 in monocytes from people with periodontal disease compared to the levels detected in healthy controls. Egr1 is a protein that can be activated as a result of infection and plays a role in the pathophysiology of atherosclerosis (Blaschke *et al.*2004).

We have also identified that Wnt proteins in patients with periodontal disease are enhanced compared to controls. A preliminary analysis demonstrated that NOTUM, an antagonist of Wnt signalling, was decreased in both the plasma and monocytes from patients with periodontal disease. These changes in Wnt signalling may promote atherosclerosis in people with periodontal disease.

The main benefit of this research will be a fuller understanding of the pathogenesis between periodontitis and atherosclerosis, and in the longer-term, the identification of new treatments and biomarkers, for better diagnosis, management, and life quality for patients. The findings will be transferrable to other inflammatory conditions, such as stroke, diabetes mellitus, and rheumatoid arthritis.

Study Aim & Objectives

Aim:

The aim of this study is to determine if the monocytes from people with periodontal disease behave differently from those taken from healthy individuals in a way that heightens the risk of atherosclerosis. We will test the hypothesis that reduced levels of IGFBP-6 and altered Wnt signalling contribute to altered monocyte/macrophage pro-atherosclerotic behaviour in patients with periodontitis. Consequently, modification of IGFBP-6 and/or Wnt signalling will retard atherosclerosis and IGFBP-6, and Wnt signalling proteins will act as suitable biomarkers of atherosclerosis disease progression.

Objectives and methods:

1. To determine the underlying mechanism of regulation in monocytes/macrophages from people with periodontal disease compared with healthy controls we will assess four key stages that are essential for the development of atherosclerosis, mainly:

    a. *Monocyte adhesion to endothelial cells:* human monocytes will be isolated using standard techniques. They will be labelled with calcein-AM for 30 minutes, and then incubated with a monolayer of cultured human coronary artery endothelial cells purchased from Promocell.
    After 30 minutes the number of adherent monocytes will be quantified by image analysis.

    b. *Invasion/Migration assays*: isolated human monocytes will be differentiated into macrophages by culture with 20ng/ml M-CSF for 7 days. Macrophages will be cultured in

Matrigel-coated transwells with 50ng/ml MCP-1 as chemoattractant in the lower chamber. After 72 hours, the number of migrated macrophages will be quantified by image analysis as described previously

c. *Phenotype of macrophages*: macrophage phenotype will be assessed by quantification of M1 (iNOS, IL-6) and M2 (arginase, IL-10) markers by Western blotting and ELISAs.

d. *Lipid content of macrophages*: Monocyte/macrophages will be incubated with 10µg/ml ox-LDL for 24 hours. Lipid content will be quantified by oil red O staining. Endocytosis will be quantified using zymosan bead. Changes in RNA will be assessed using qPCR.

The *in vitro* experiments have been designed to determine the effect of increasing or decreasing the level of IGFBP6 and Wnt signalling using various approaches outlined in Table I below.

Table I: summary of the design of in vitro experiments

Exp	Group 1	Group 2	Group 3	Experimental Question
A	Control + siRNA control	Control + BP6 siRNA	Periodontitis + siRNA control	Does **silencing BP6** in control cells mimic efforts observed in cells from periodontal patients?
B	Control + IgG control	Control + BP6 Nab	Periodontitis + IgG control	Does **neutralizing IGFBP6** in control cells mimic effects observed in cells from periodontal patients?
C	Control + vehicle	Periodontitis + vehicle	Periodontitis + BP6	Does **addition of IGFBP6** to cells from periodontal patients mimic control cells?
D	Control + vehicle	Periodontitis + vehicle	Periodontitis + ETC-159	Does **inhibition of Wnt secretion** (using ETC-159) in cells from periodontal patients mimic control cells?
E	Control + vehicle	Periodontitis + vehicle	Periodontitis + iCRT	*Does* **inhibition of Wnt/b-catenin signalling** *(using iCRT) in cells from periodontal patients mimic control cells?*
F	Control + vehicle	Control + 400ng/ml Wnt3a	Periodontitis + vehicle	*Does treatment of control cells with* **Wnt3a mimic** *cell from periodontal patients?*

2. Assessing the potential use of IGFBP6 and Wnt proteins as suitable biomarkers

a) Peripheral blood and saliva will be collected from patients with periodontal disease both pre and post treatment and age and gender matched healthy controls.

b) Saliva samples will be collected from people with severe periodontitis and age and gender matched controls.

The seven Wnt proteins and IGFBP6 identified by our previous proteomic analysis will be quantified in plasma, isolated monocytes and saliva, and the levels in patients and controls statistically compared. Standard laboratory techniques including western blot and specific enzyme-linked immunosorbent assays (Bio-Plex, Bio-Rad Laboratories Ltd. Hertfordshire, UK) will be used to assess for changes.

This will determine whether IGFBP6 and/or any of the seven Wnt pathway proteins are associated with periodontitis and could lead to future work investigating the role of these proteins in disease progression. Is it likely that other novel findings will be identified and scope to explore these will be undertaken under the current protocol.

See protocols 1-3.

Study sites

- University Hospitals Bristol and Weston NHS Foundation Trust ,Lower Maudlin Street, Bristol, BS1 2LY

  Translational Health Sciences, Research Floor, Level 7, University of Bristol, Bristol Royal Infirmary, Upper Maudlin Street, BS2 8HW (location of laboratory and storage location for samples)

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Participants and recruitment

Clinicians or a member of the dental team at the University of Bristol Dental School will approach participants who are attending the hospital for either periodontal assessment or regular dental appointments (healthy volunteers).

- Potential donors will be provided with the same participant information leaflet (see enclosed) containing information about the research. Sufficient time will be provided to participants prior to
- Written consent will be obtained from participants to allow for a basic periodontal examination, and collection of a blood sample. Every 5th person will be asked to provide a sample of saliva.

Inclusion/exclusion criteria for people with periodontitis

Inclusion:

- All people over the age of eighteen (18)
- Capacity to consent
- Attending a scheduled dental appointment at the University of Bristol Dental Hospital
- People with unstable severe periodontitis or a high susceptibility to periodontitis (as defined by the British Society of Periodontology 2019 implementation of the 2017 World Workshop Classification of periodontal and peri-implant diseases and conditions: Stage III/IV and Grade C.)
- At least 18 erupted teeth

Exclusion

- All people under the age of eighteen (18)
- Those who lack capacity to consent
- History of cardiovascular disease such as hypertension, angina, previous myocardial infarction, heart failure, aortic disease, arrhythmia, heart valve disease.
- History of autoimmune/autoinflammatory diseases such as Sjogren's syndrome, rheumatoid arthritis, inflammatory bowel disease, systemic lupus erythematosus, or systemic sclerosis
- History of endocrine disease including diabetes mellitus types 1 and 2
- Currently taking antibiotics or has had antibiotics in the preceding 6 months
- People taking statins, non-steroidal anti-inflammatory medications, immunosuppressive medications or corticosteroids.
- Smokers of tobacco (including Vaping/e-cigarettes)
- History of salivary gland disease or oral mucosa disease

Inclusion/exclusion criteria for healthy volunteers

Inclusion:

- All people over the age of eighteen (18)
- Capacity to consent
- Attending a scheduled dental appointment
- People with no evidence of periodontitis
- At least 18 erupted teeth

Exclusion

- All people under the age of eighteen (18)
- Those who lack capacity to consent
- History of cardiovascular disease such as hypertension, angina, previous myocardial infarction, heart failure, aortic disease, arrhythmia, heart valve disease
- History of autoimmune/autoinflammatory diseases such as Sjogren's syndrome, rheumatoid arthritis, inflammatory bowel disease, systemic lupus erythematosus, or systemic sclerosis
- History of endocrine disease including diabetes mellitus types 1 and 2
- Currently taking antibiotics or has had antibiotics in the preceding 6 months
- People taking statins, non-steroidal anti-inflammatory medications, immunosuppressive medications or corticosteroids.
- Smokers of tobacco (including Vaping/e-cigarettes)
- History of salivary gland disease or oral mucosa disease

Ethical Considerations and information

Blood and saliva samples will be collected from participants who are capable of informed decision making. Treating clinicians or an appropriately qualified member of their team will obtain informed consent from participants. In general terms, participants will be asked to provide a sample of blood and saliva that will be used in this study to better understand the link between periodontal disease

and atherosclerosis. Participant information leaflets and consent forms will be provided (see enclosed).

Sample Collection

Objective 1 and 2b

Upon obtaining consent, the clinical practitioner will collect two tubes of peripheral blood (approximately 20mls in total) from the forearm of the person using standard venepuncture techniques.

The clinical practitioner will ask every 5th person to provide a saliva sample. Whole saliva will be collected in polypropylene vials using the passive drool method (2mls in total). The person will undergo a basic periodontal screen to assess gum health using the previously validated 'Basic Periodontal Exam' (BPE)/ 'Periodontal Probing Depth', as well as recording if there is 'bleeding on probing'. (Dietrich *et al.* 2019). The exam and collection of samples will occur before dental treatment begins. After blood and saliva has been collected, and the periodontal screen performed, the person will have no further participation in the study.

Objective 2a

Upon obtaining consent, the clinical practitioner will collect two tubes of peripheral blood (approximately 20mls in total) from the forearm of the person using standard venepuncture techniques, and saliva will be collected (2mls in total).

The person will undergo a basic periodontal screen to assess gum health using the previously validated 'Basic Periodontal Exam' (BPE)/ 'Periodontal Probing Depth' as well as recording if there is 'bleeding on probing'. (Dietrich *et al.* 2019)

The person will then undertake their routine dental treatment. The time it takes for the successful completion of dental treatment varies, as do the number of necessary appointments, but typically it can take approximately 24 weeks. On completion of this dental treatment the person will be asked to provide one additional peripheral blood sample (approximately 20mls) and saliva (2mls in total) sample. After this second sample of blood and saliva has been collected these participants will have no further participation in the study.

For all the objectives listed above, the clinical practitioner will then anonymise all samples by allocating a code to the samples. Only the clinical practitioners will have access to the code that links the samples and people, not the researchers. Once anonymised, samples will be made available to the researchers, who will analyse the samples using established laboratory techniques as mentioned above.

Duration of participant involvement

Objectives 1 and 2b: participants will be actively involved in the study for approximately 20 minutes at their assessment appointment.

Objective 2a: from the time of their assessment appointment to the successful completion of their dental treatment, the participants will have taken part in the study for approximately 24 weeks. The total amount of time the participant will be actively involved in the study will be approximately 35 minutes, spread over the two appointments.

Sample size determination

From our previous data with similar experimental design and analyses, we have calculated the sample size (**power analysis**) using an online sample size calculator (https://clincalc.com/stats/samplesize.aspx) and $p<0.005$

Objective 1: Mechanism of regulation

Approximately 150 gum disease (severe periodontitis) participants and 150 control participants (good gum health) will be approached. From these approaches we hope to obtain samples of blood from 125 severe periodontitis and 125 control participants. The sample size is based on previously published studies and our own previous work, and shown to provide a 90% power to detect a 50% change. (Willi *et al.* 2014; Nanbara *et al.* 2012)

Objective 2: Biomarker potential

a) Approximately 20 gum disease (severe periodontitis) participants and 20 control participants (good gum health) will be approached. From these approaches we hope to obtain samples of blood and saliva from 12 severe periodontitis and 12 control participant The sample size is required to provide >90% power to detect a 30% change in protein expression.

b) Approximately 30 gum disease (severe periodontitis) participants and 30 control participants (good gum health) will be approached. From these approaches we hope to obtain saliva samples from 20 people with severe periodontitis and 20 control participants .The sample size is required to provide a 80% power to detect a 20% change. (Jasim *et al.* 2018)

Analysis

Data will be analysed by t-tests when two groups are compared or by ANOVA if more than two groups are assessed.

Withdrawal of participants

Participants will be informed that they can withdraw from the study at any stage- no reason needs to be provided. Withdrawal from the study will not affect their dental care.

Any blood and saliva donated by a participant is a gift, and any samples or data collected up until the point the participant wants to withdraw will be kept, used and included in analysis.

Safety and adverse events

There is minimum risk to taking blood from participants. All clinical study procedures will be carried out on clinics at Bristol Dental Hospital by appropriately trained staff. All cross infection controls will be implemented including those related to COVID 19. All study material is participant specific and all clinical study staff are trained in Good Clinical Practice (GCP). All procedures are performed by clinical staff that have appropriate qualifications and training.

A slight discomfort and bruising may be experienced due to withdrawal of blood. There should be no major adverse effects, pain, discomfort, distress, intrusion, inconvenience or changes to lifestyle as this is a routine blood donation of small volume. No adverse effects are associated with collecting saliva.

Any adverse events should be reported to Dr Dáire Shanahan who will review, record and if necessary, report all events to the UHBW R&I office via the University Hospitals Bristol and Weston adverse event reporting system (UHBW undertake this role on behalf of the Sponsor).

Data management

Confidentiality:

The link between the participant and their coded tissue and periodontal score will be held by the clinical care team. Initially recorded on paper, the link between participants and the code will be transferred to a password-protected University computer. Paper copies of the code will be destroyed securely using NHS confidential waste streams. The researchers will not have access to the code.

Storage and use of data after the study:

Anonymised study data will be stored on the University Research Data Storage Facility (RDSF) and analysed by the researchers. The RDSF is a secure facility that is backed up daily. Access is granted by the University Data Steward at the request of the project Chief Investigator, and will be restricted to members of the research team. After the study, with the consent of the participant, research data will be made available as "open data" on the data.bris Research Data Repository. This means the data will be publicly available and so able to be used by other researchers to support additional research in the future. All data relating to participants will be anonymised and so it will not be possible to identify participants from these data.

Consent forms (hard copies) will be kept in a locked cabinet in a locked office within the dental clinical trials unit. Only dental clinical trials team members have access to this space. They will also be scanned onto University of Bristol electronic storage space which is protected by the University's firewall and is password protected. Consent forms will be held until the blood/saliva is used up or disposed. The researchers using the blood/saliva are in a separate building and will not have access to the paper consent forms, or be able to access the electronic scanned copies.

Auditing and inspection:

The University of Bristol has an arrangement with University Hospitals Bristol and Weston NHS Foundation Trust such that they monitor studies sponsored by the University of Bristol.

Sponsorship

University of Bristol is the Research Sponsor

Insurance

The University of Bristol has arranged Public Liability insurance to cover the legal liability of the University as Research Sponsor in the eventuality of harm to a research participant arising from management of the research by the University. The University of Bristol holds Professional Negligence insurance to cover the legal liability of the University for harm to participants arising from the design of the research, where the research protocol was designed by the University. The University of Bristol's Public Liability insurance policy provides an indemnity to our employees for their potential liability for harm to participants during the conduct of the research

These policies do not in any way affect an NHS Trust's responsibility for any clinical negligence on the part of its staff (including the Trust's responsibility for University of Bristol employees acting in connection with their NHS honorary appointments).

Publication policy:

Data may be published in peer-reviewed journals, conference abstracts and used for future grant applications, but in anonymised form only. As such, it will not be possible to identify participants from these data.

Conflicts of interest:

The study personnel declare no conflicts of interest.

References:

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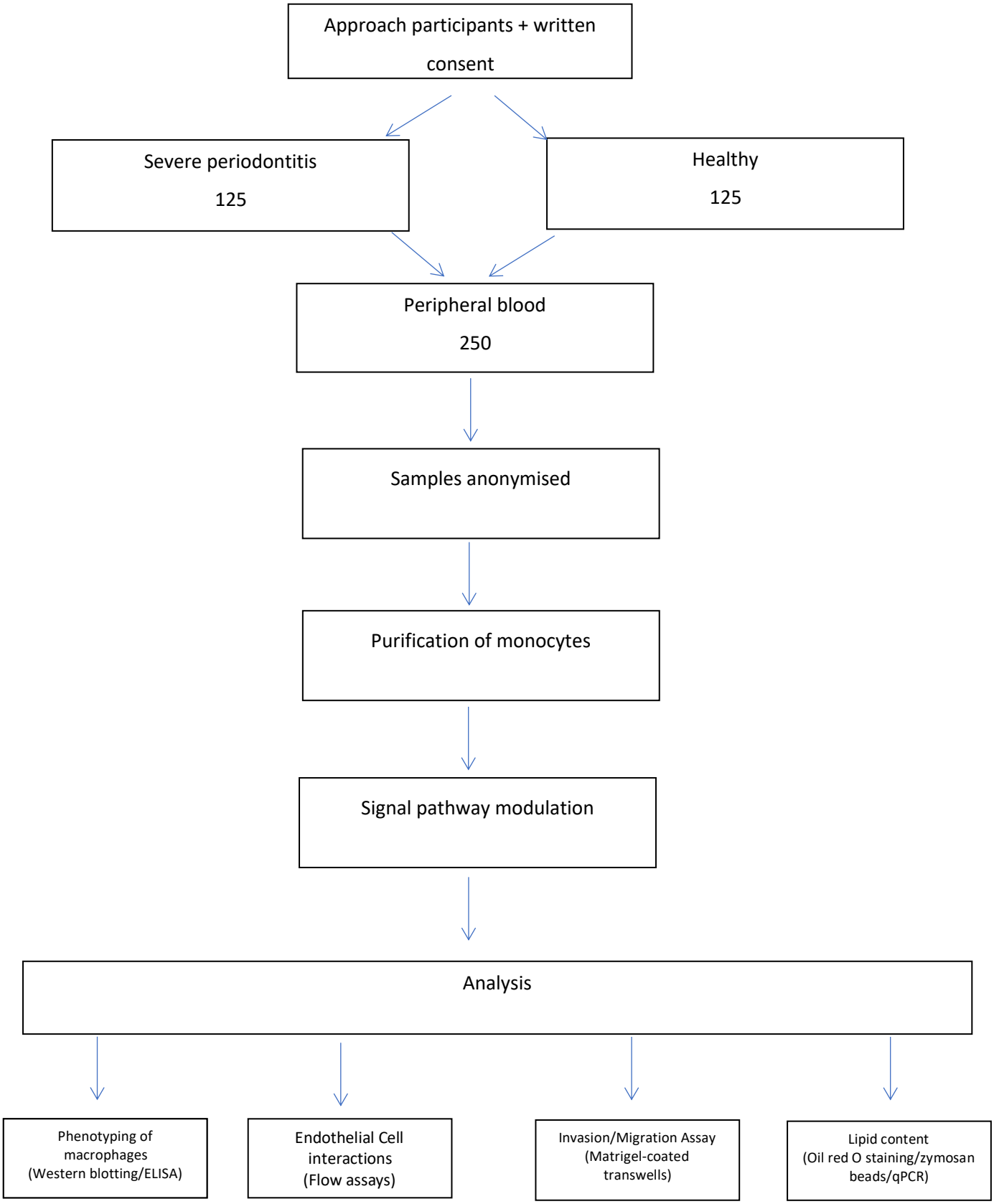
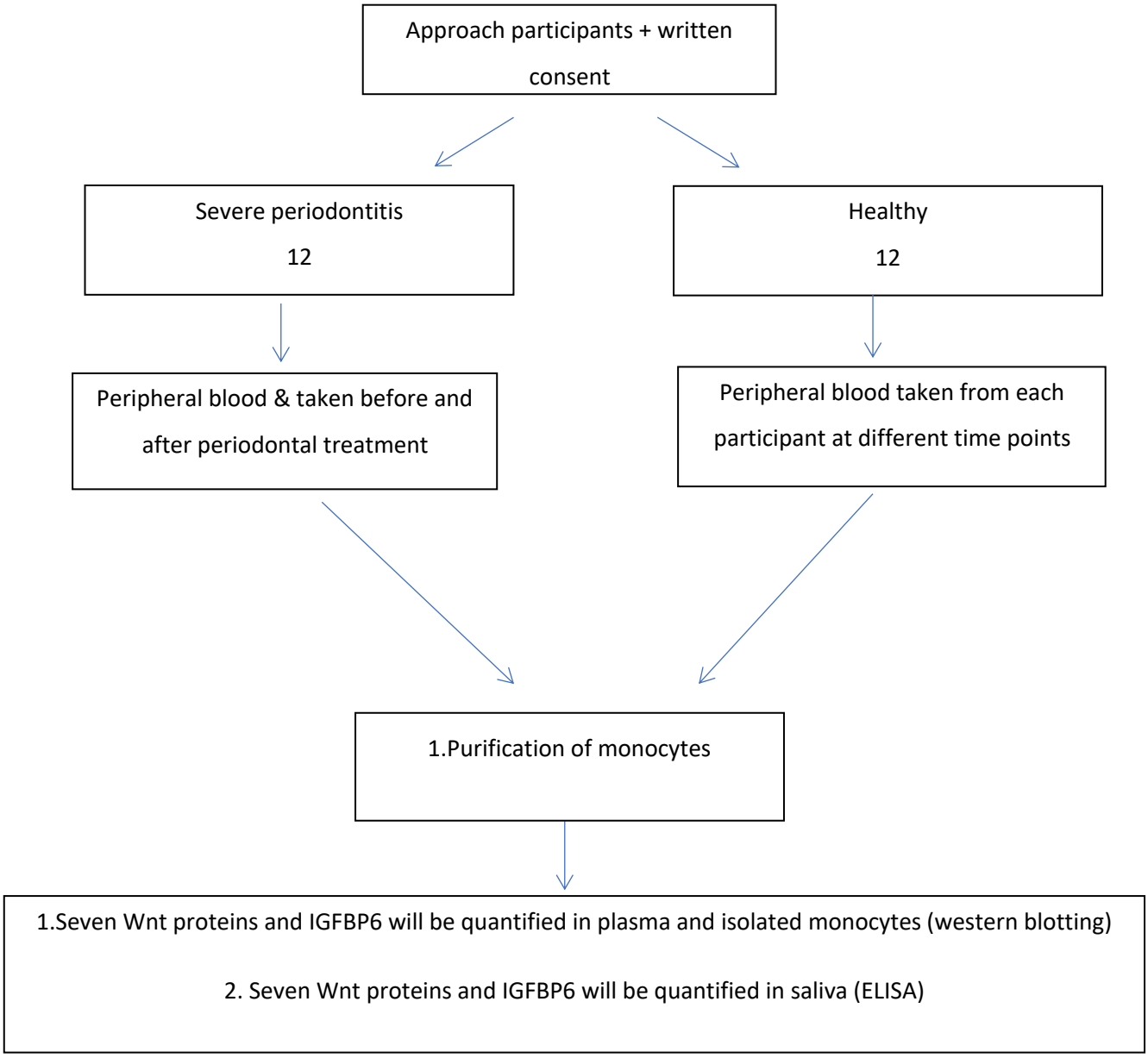
Protocol flow chart 1: Understanding the link between gum disease and heart attacks



Protocol flow chart 2: Understanding the link between gum disease and heart attacks



Protocol flow chart 3: Understanding the link between gum disease and heart attacks