UNIVERSITY OF BIRMINGHAM

Effect of Density Gradient Material upon *Ex-Vivo* Neutrophil Behaviour

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Abstract

Neutrophils play a critical role in innate immunity and orchestrate and partake in key aspects of acquired immunity. They are routinely isolated from peripheral venous blood to study their behaviour. Isolation requires an efficient, aseptic and reproducible method to obtain non-activated and viable cells, and *ex vivo* handling should not influence neutrophil behaviour, in particular induction of inappropriate activation. Neutrophil activation is characterised by the production of reactive oxygen species (ROS), which relies on nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and increased oxygen uptake in the respiratory burst. The generation of NADPH oxidase and the consequent ROS production is indispensable for neutrophils to implement their defensive mechanisms; phagocytosis and neutrophil extracellular trap (NET) formation. There is currently no comparative data on the most appropriate isolation method to study neutrophils. Therefore, this study investigated the use of Percoll (GE Lifesciences) and Histopaque (1077 and 1119, Sigma-Aldrich) density gradient materials for isolating neutrophils upon ROS production, NET generation, cell viability and metabolic activity. Venous blood from 10 healthy volunteers was collected and neutrophils prepared on Percoll and Histopaque density gradients. ROS and NET production were analysed in unstimulated cells, interferon-α primed cells and cells stimulated with *Fusobacterium nucleatum* (Toll-like receptors) or opsonised *Staphylococcus aureus* (FcγRII). Percoll isolation of neutrophils provided higher cell yields. Histopaque isolated cells demonstrated higher levels of baseline unstimulated ROS production and consistent with that, higher metabolic activity, implying higher baseline activation, than Percoll prepared cells. Subsequent ROS generation and NET formation in unstimulated and stimulated cells with *F.nucleatum* and opsonised *S.aureus*, were lower for

Histopaque. Moreover, metabolic activity was lower at the point of NET release (180m) for Histopaque. In conclusion, the current density gradient method used by the Periodontal Research Group in Birmingham, (Percoll, with NH_4Cl lysis), proved to provide a higher yield, a more sensitive response to toll-like receptor (*F.nuc)* and FcγR (opsonised *S.a*) stimulation, and also expressed increased sensitivity to NET production. Percoll will remain the gradient material of choice, as opposed to Histopaque, for all future studies involving ROS and/or NET production.

Contents

List of Figures and Tables

Abbreviations

AFU	Arbitrary fluorescent units
AMP	Antimicrobial peptide
ATP	Adenosine-5'-triphosphate
BSA	Bovine serum albumin
CGD	Chronic granulomatous disease
GCF	Gingival crevicular fluid
EDTA	Ethylenediaminetetraacetic acid
FCγR	FC gamma receptor
gPBS	PBS supplemented with glucose and cations
IFNα	Interferon alpha
Ig	Immunoglobulin
IL	Interleukin
LPS	Lipopolysaccharide
MNase	Micrococcal nuclease
MPO	Myeloperoxidase
NADPH	Nicotinamide adenine dinucleotide phosphate
NCTC	National collection of type cultures
NET	Neutrophil extracellular trap
OD	Optical density
PBS	Phosphate buffered saline
PMA	Phorbol 12-myristate 13-acetate
PMNL	Polymorphonuclear leukocyte
PVP	Polyvinylpyrrolidone
RCF	Relative centrifugal force
RLU	Relative light units
ROS	Reactive oxygen species
RPM	Revolutions per minute
SEM	Standard error of the mean
TEM	Transmission electron microscopy
TLR	Toll-like receptor

Chapter 1: Introduction

1.1 Introduction to neutrophils

Neutrophilic polymorphonuclear leukocytes (neutrophils/PMNLs) play a pivotal role in the innate immune system, where they are the most abundant leukocyte and typically the first immune cell to be recruited to sites of infection (Hayashi *et al.,* 2003). Following the first reports of neutrophils in the late nineteenth century by Paul Ehrlich (1880), later observations by Metchnikoff (1893) revealed these puzzling cells do not actually promote infection, but instead play a critical role in host defence. Neutrophils are terminally differentiated effector cells of myeloid lineage, which play a role in cytokine/chemokine production (Garlet *et al.,* 2003), antigen presentation (Beauvillain *et al*., 2007), pathogen transport to lymph nodes (Maletto *et al*., 2006) and T-cell responses (Tacchini-Cottier *et al.,* 2000).

1.2 Neutrophil activation and function

Subsequent to neutrophil production in the bone marrow, neutrophils enter the circulatory system, where they survive for approximately 5.4 days (Pillay *et al.,* 2010). During this period neutrophils may encounter stimulated endothelial cells of the vessel walls, as a result of both host and bacterial derived inflammatory signals, such as interleukin 17 and lipopolysaccharide respectively. If this encounter does occur, neutrophils initiate their endothelium migration into the tissue, which relies on many molecules to achieve the characteristic “tethering, rolling and firm adhesion”. Once in the tissue, the recognition of pathogen-associated patterns by molecules such as Toll-like receptors (TLR), further enhance neutrophil activation, enabling the cells to elicit the multiple antimicrobial mechanisms at their disposal; principally degranulation, phagocytosis and NETosis (Amulic *et al.,* 2012). Neutrophil granules

contain antimicrobial peptides and signalling molecules, which are released upon activation into either the phagosome or extracellularly via secretory vesicles. Dependent on the content and also the time at which produced during haematopoeisis, granules are subdivided into azurophilic (primary), specific (secondary) and gelatinase (tertiary); however there is significant overlap between the granule cargo (Faurschou & Borregaard 2003). Phagocytosis is the leading mechanism employed by neutrophils to overcome pathogens and is the process by which neutrophils internalise the pathogen into a phagosome. Degranulation products and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase fusion to the phagosome provide a lethal environment, enabling the removal of the majority of microbes (Warren *et al.,* 2003). Despite the long held belief that neutrophils fulfil their role via just two antimicrobial mechanisms; NADPH-oxidase generation of reactive oxygen species (ROS) and granular antimicrobial peptides (AMPs) release, in 2004 a novel process was discovered that involves both the former. Brinkmann *et al* (2004) described a process by which neutrophils extrude their nuclear chromatin as a complex mix of DNA, histones and AMPs, known as neutrophil extracellular traps (NETs), believed to immobilise and kill bacteria extracellularly. The formation of these web-like structures is believed to facilitate the action of neutrophils in the containment of pathogenic microbes, by anchoring the microorganisms within the "trap" and thus preventing the spread of infection (figure 1). According to Fuchs *et al.,* (2007), NET formation is dependent upon NADPH oxidase, which is required for the generation of ROS and facilitates NETosis, a novel form of "programmed cell death". This is also supported by chronic granulomatous disorder (CGD) patients, in whom a mutation in the NADPH oxidase results in impaired ROS generation and an inability to produce NETs.

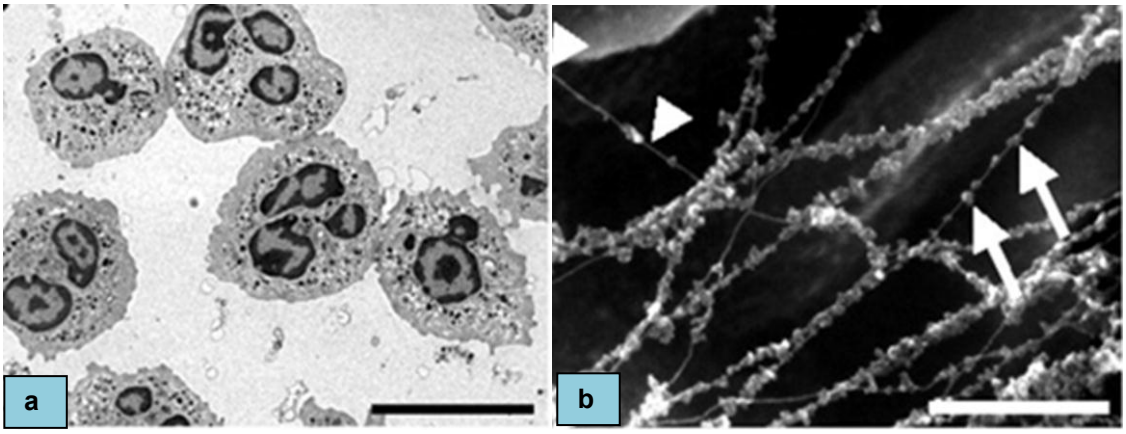

Figure 1: Naïve neutrophils (a) and NETs (b).
(a) Transmission electron microscopy (TEM) of naïve neutrophils. (b) NETs generated by either phorbol myristate acetate (PMA), interleukin-8 (IL-8) or lipopolysaccharide (LPS) activated neutrophils. Arrow heads show smooth fibres and arrows show globular domains. Scale bar represents 10μm (a) and 500μm (b). (Image taken from Brinkmann *et al.,* 2004).

1.3 Periodontitis

Neutrophils are implicated in periodontitis pathophysiology, which is characterised by periodontal pocket formation, gingival bleeding and alveolar bone destruction (figure 2). Despite bacterial pathogens initiating this chronic inflammation, disease progression appears to result from dysregulation of the host immune system, a failure to eliminate causative pathogens and to resolve the inflammatory lesion. The exact contributions of direct bacterial damage and indirect damage from the host response are not entirely clear, but it is believed that tissue destruction relies primarily on the host (Birkedal-Hansen 1993), with an estimate that bacteria account for approximately 20% of disease prevalence (Grossi *et al.,* 1994). Consistent with other infections, neutrophils are the first and most abundant cell type to respond to periodontopathogenic bacteria (Haffajee *et al.,* 1995). The role of neutrophils in periodontitis becomes evident when considering individuals with abnormal neutrophil function, for example in Chediak-Higashi syndrome, Papillon-Lefevre syndrome and

abnormalities in chemotaxis and diapedesis (Dixon *et al.,* 2000). These defects can increase susceptibility to disease, as individuals are unable to phagocytose or kill bacteria efficiently and are therefore more likely to suffer severe periodontitis. In spite of this, additional evidence suggests that neutrophils may only provide initial protection, whilst at later stages of periodontitis they may actually cause detrimental effects. In support of this, some periodontal bacteria are able to bypass neutrophils, for example *Aggregatibacter actinomycetemcomitans* is believed to have the capacity to not only decrease neutrophil activation and bacterial internalisation, but remarkably to remain viable following neutrophil ingestion (Permpanich *et al.,* 2005). Regardless of neutrophils being rendered ineffective, they continue to accumulate within the gingival and periodontal tissues, resulting in a non-resolving inflammatory state characterised by the release of ROS, serine proteases and proteolytic agents (Eick *et al.,* 2000).

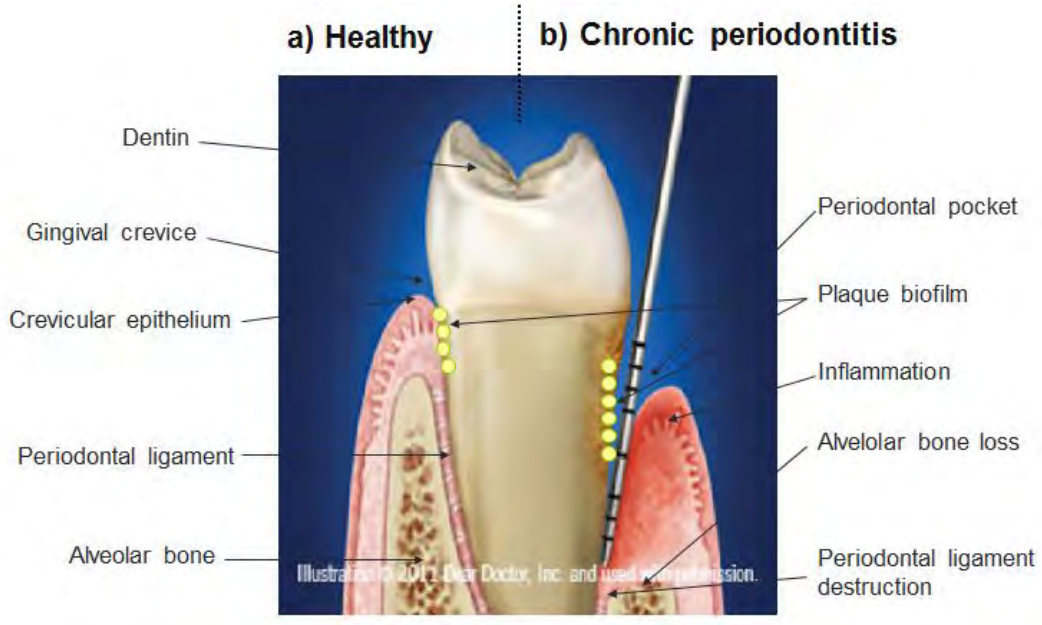

Figure 2: Schematic comparing a healthy state (a) to chronic periodontitis (b). Compared with the healthy state (a), chronic periodontitis (b) is characterised by the formation of a periodontal pocket, gingiva inflammation, alveolar bone loss and periodontal ligament destruction; if untreated this can result in tooth loss (Image adapted from the American Academy of Cosmetic Dentistry [online]).

1.4 Neutrophil isolation

To characterise neutrophils in periodontitis, as well as other inflammatory diseases, cells are often isolated from the peripheral blood of volunteers and investigated *ex vivo*. Indeed there are neutrophil-like cells, such as the HL60 human promyelocytic cell line, which can be used to circumvent problems of interpersonal variation between neutrophil donors (Martin *et al.,* 1990). Nevertheless, these immortalised cell lines do not fully reflect primary neutrophils in behaviour or in their intricate biological functions. In addition, despite 99% of murine genes having an equivalent human counterpart, murine disease models also do not fully mimic the human situation. Exemplifying this is the difference in whole blood constituents, while human neutrophils represent 50-70% of circulating PMNs, mouse neutrophils make up only 10-25% (Doeing *et al.,* 2003).

Neutrophils are considered relatively fragile cells with an *ex vivo* lifespan estimated to be less than 8 hours (Payne *et al.,* 1994), thus isolation from human blood requires an efficient, aseptic and reproducible method to obtain non-activated and viable cells. A further consideration is the effect of the isolation and purification method on neutrophil behaviour; in particular neutrophils may become activated as a result of *ex vivo* manipulation. Neutrophil activation is characterised by the production of ROS, which relies on NADPH oxidase and increased oxygen uptake in a process known as the respiratory burst. The generation of NADPH oxidase and consequent ROS production is essential for neutrophils to fulfill their role in immunity, namely phagocytosis and NET formation (Hampton *et al.,* 1998). Therefore research investigating neutrophil function needs to consider if *ex vivo* purification techniques influence neutrophil activation status. Methods of neutrophil isolation from whole

blood can differ between research groups, creating potential phenotypic differences in neutrophils even if retrieved from the same volunteer. Inconsistencies in neutrophil isolation may result in differences in neutrophil activation, which consequently may lead to results in *ex vivo* studies being misinterpreted or even being incomparable between studies.

1.5 Neutrophil isolation density gradient materials

There are currently several materials which can be employed to produce density gradients to isolate and purify neutrophils from whole blood; two of which are Percoll™ (GE Healthcare) and Histopaque™ (Sigma). The density gradient material aims to provide an appropriate separation range to isolate neutrophils at a high purity from other blood cells, whilst at the same time, not stimulating or activating the cells. Percoll is comprised of colloidal silica and coated with non-dialyzable polyvinylpyrrolidone (PVP) to avoid toxicity to cells, and is used in conjunction with sodium chloride and water to produce a gradient medium. Centrifugation of two discontinuous Percoll gradients (1.079 and 1.098 g/ml) causes the PVP coated silica particles to sediment, which generates isometric gradients and facilitates neutrophil separation (GE Healthcare). Alternatively, Histopaque is a polysucrose and sodium diatrizoate solution, which is commercially available at prepared densities of 1.077 and 1.119 g/ml. Similarly to Percoll, the centrifugation of both densities beneath whole blood allows for the isolation of a neutrophil band at the gradient interface. The retrieval of neutrophils with different density gradient materials may impact differently upon those cells, which may influence subsequent experimental results.

Indeed Grisham *et al.,* (1985) investigated the effects of four methods on neutrophil purification and function, and revealed differences in neutrophil yield and purity dependent upon the procedure used. Notably the use of two discontinous Histopaque gradients, 1077 and 1119, produced a pure neutrophil supernatant, but with a relatively low yield (table 1, method B). This particular method is based on studies by English & Anderson (1974) and whist these findings are extremely relevant, this protocol has been modified with time, for example the addition of a distilled water lysis step. It was also reported that neutrophil isolation with two Percoll gradients (60% and 80%) (Based on Thomas *et al.,* 1983) led to a moderate yield but a lower purity than Histopaque isolated neutrophils (table 1, method C). Interestingly, the authors did not observe any variability in neutrophil function as a result of the isolation method, in relation to corresponding ROS production, chemotaxis and degranulation. Thus, choice of isolation method may depend upon the nature of subsequent experiments. For example it may be desirable to have a high purity if performing a ROS assay, as erythrocyte contamination may generate scavenging effects due to erythrocyte-derived antioxidants and metabolites (Thomas *et al.,* 1985).

Table 1: Comparison of multiple parameters from neutrophils obtained by 4 methods. (A) Histopaque 1077, (B) Histopaque 1077 and 1119, (C) 80% and 60% percoll and (D) centrifugation. Parameters include the purity (%), yield (%), superoxide production (nmol/min), myeloperoxidase (MPO) release (%) and hypochlorous acid (HOCl) production (nmol/60 min). Data expressed as mean± SEM, n=4. (Grisham *et al.,* 1985).

Method	Purity (%)	Yield (%)	Superoxide production (nmol/min per 10^7 cells)	MPO release (% control)	HOCl production (nmol/60 min per $4x10^6$ cells)
A	91 ± 1	42 ± 12	70 ± 22	572 ± 233	99 ± 15
B	96 ± 0.4	22 ± 3	66 ± 15	508 ± 42	129 ± 11
C	90 ± 4	42 ± 1	63 ± 21	500 ± 36	135 ± 13
D	67 ± 3	60 ± 5	52 ± 11	530 ± 116	124 ± 24

More recent research by Rebecchi *et al.,* (2000) investigated the release of myeloperoxidase (MPO) from neutrophils isolated with Ficoll-Hypaque (1.077g/ml), a gradient material very similar to Histopaque. Based on previous contradictory findings, this paper postulated that whilst Percoll may not disrupt MPO, Ficoll-Hypaque neutrophil isolation may interfere with MPO release. Notably this study did not carry out a direct comparison between Percoll and Hypaque, but demonstrated an increased respiratory burst in neutrophils isolated by Ficoll-Hypaque, relative to spontaneous sedimentation and whole blood assays. This was further characterised by luminol chemiluminescence, from which the authors concluded that Ficoll-Hypaque isolation may interfere with ROS production and increased MPO release (figure 3). This data indicates that neutrophils are inappropriately activated as a result of the density gradient material and this may account for the confounding data in the literature (Pember & Kinkade 1983; Miyasaki *et al.,* 1991).

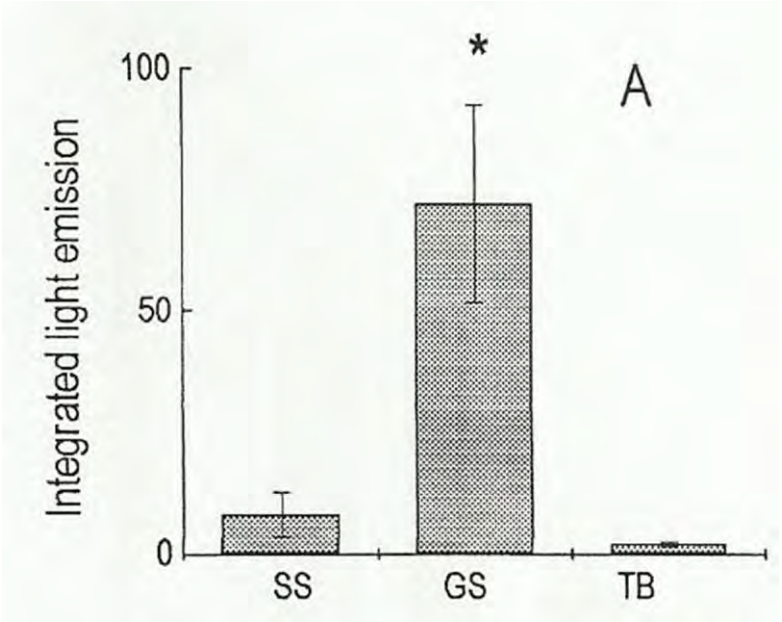

Figure 3: Luminol chemiluminescence of neutrophils isolated by 3 methods. (SS) spontaneous sedimentation, (SG) Ficoll-Hypaque density gradient and (TB) total blood. Light emission at 104 minutes, *$p < 0.02$ difference relative to TB, data expressed as mean ± SEM (Rebecchi *et al.,* 2000)

1.6 Aims

Previous reports have claimed that the neutrophil isolation procedure can affect neutrophil viability, contamination and activation state. However these few studies have not fully characterised the reported variability and also the extent to which these differences influence neutrophil activation. Based on the discrepancy in the literature and also disagreement between research groups, the present studies aimed to compare Histopaque 1077 and 1119 density gradient material with Percoll 1.079 and 1.098, the method currently used by the Periodontal Research Group in Birmingham. Outcome measures included neutrophil ROS production, to indicate whether cells are being inappropriately activated *ex vivo* and also whether activation with primers and stimuli is altered as a result of the separation method. NET visualisation and quantification were also investigated to determine whether the isolation procedure influenced NET formation. Finally, cell metabolic activity was determined at time points relevant to both the ROS and NET assays for both neutrophil isolation methods.

Chapter 2: Materials & Methods

2.1 Volunteers

Blood was collected in 6ml lithium heparin anticoagulant vacutainers (Greiner, Bio-One) from 10 volunteers, all of whom were males aged between 20-30 years, non-smokers, periodontally healthy and not currently taking anti-inflammatory or anti-microbial drugs. Volunteers were recruited from Birmingham Dental School and Hospital and samples were collected under ethical approval number 10/H1208/48 and donors gave their informed consent. Blood from the same individual was used for both Percoll and Histopaque neutrophil isolation in parallel and under identical laboratory conditions.

2.2.1 Percoll neutrophil isolation protocol

Percoll densities were prepared according to the equation below, which calculates the sedimentation of a sphere in a centrifugal field. (Provided by GE Healthcare)

Table 2: Percoll density gradient material equation and medium components

$$v = \frac{d^2 (\rho_p - \rho_l)}{18\eta} \times g$$

where v = sedimentation rate
d = diameter of the particle (hydrodynamically equivalent sphere)
ρ_p = particle density
ρ_l = liquid density
η = viscosity of the medium
g = centrifugal force

Density	1.079	1.098
Percoll	19.708 ml	24.823 ml
Water	11.792 ml	6.677 ml
NaCl	3.5 ml	3.5 ml

8ml of 1.079 density Percoll were carefully layered over 1.098 in a 25ml tube (Fisher Scientific) and 6ml of blood were layered over the gradients using a Pasteur pipette. This was centrifuged (Hettich Universal 320R) for 8min at 150 relative centrifugal force (rcf), followed by 10min at 1200rcf. Plasma, monocytes, lymphocytes and density gradient material were aspirated off and discarded, allowing for the transfer of the neutrophil layer to 30ml lysis buffer (2.2.2) in a 50ml centrifuge tube (Fisher Scientific), which was then topped up to 50ml with lysis buffer. Following a gentle inversion, the centrifuge tube was incubated for 15min at room temperature to lyse any remaining erythrocytes. Next, the tube was centrifuged for 6m at 500rcf to pellet the neutrophils. The supernatant was aspirated off, whilst the pellet was re-suspended in 2ml of lysis buffer and incubated for 5min at room temperature, prior to centrifugation for 6min at 500rcf. The supernatant was again aspirated off and the neutrophil pellet washed in 2ml of PBS (2.2.3), followed by re-centrifugation (6min at 500rcf). The supernatant was discarded and the cells again re-suspended in 2ml of PBS (2.2.3) and counted with a haemocytometer (Naubauer, Reichart) and a light microscope (Leitz Laborlux s). Cell viability was confirmed with trypan blue exclusion in a 1:1 dilution.

2.2.2 Lysis Buffer (1 litre):

8.3g NH_4Cl (Sigma A9434), 1g $KHCO_3$ (Sigma P9144), 0.04g Na_2 EDTA $2H_2O$ (Sigma E5134) and 2.5g bovine serum albumin (BSA; Sigma A4530) were added to 1 litre of sterile, endotoxin-free water (Versol) and stored at 4°C.

2.2.3 Phosphate Buffered Saline (1 litre):

7.75g NaCl (Sigma S9625), 0.2g KH_2PO_4 (Sigma P5379) and 1.5g K_2HPO_4 (Sigma P8281) were added to 1 litre of sterile, endotoxin-free water (Versol) and stored at 4°C.

2.2.4 PBS supplemented with glucose and cations (gPBS, 1 litre):

1.8g glucose (Sigma), 0.15g $CaCl_2$ (BDH 10070), 1.5mls $MgCl_2$ (1M; BDH 22093) were added to 1 litre previously made PBS and stored at 4°C. This was made up in order and each component mixed before adding the next.

2.3 Histopaque neutrophil isolation protocol

3ml of H1077 density Histopaque were carefully layered over 3ml H1119 in a 15ml tube (Fisher Scientific). 6ml of blood were layered over the gradients using a Pasteur pipette, which was then centrifuged (IEC Centra CL3R) for 50min at 800rcf. Plasma, monocytes, lymphocytes and density gradient material were aspirated off and discarded, allowing for the retrieval of the neutrophil layer from the H1077 and H1119 interface (figure 4). Neutrophils were transferred to 5ml of Dulbecco's PBS (Sigma D8537) in a fresh 15ml centrifuge tube and centrifuged for 6min at 400rcf, followed by manual aspiration of the supernatant. The neutrophil pellet was re-suspended in 10ml Dulbecco's PBS, prior to re-centrifugation for 6min at 400rcf. The supernatant was again aspirated off and the cells re-suspended in 1ml of Dulbecco's PBS prior to erythrocyte lysis, where 5ml distilled ice cold water were added, followed by the immediate addition of 2ml 4x PBS (OXOID) after 30sec. This was then centrifuged for 8min at 450rcf and finally re-suspended in 2ml Dulbecco's PBS and counted with a haemocytometer (Naubauer, Reichart) and a light

microscope (Leitz Laborlux s). Cell viability was confirmed with trypan blue exclusion in a 1:1 dilution.

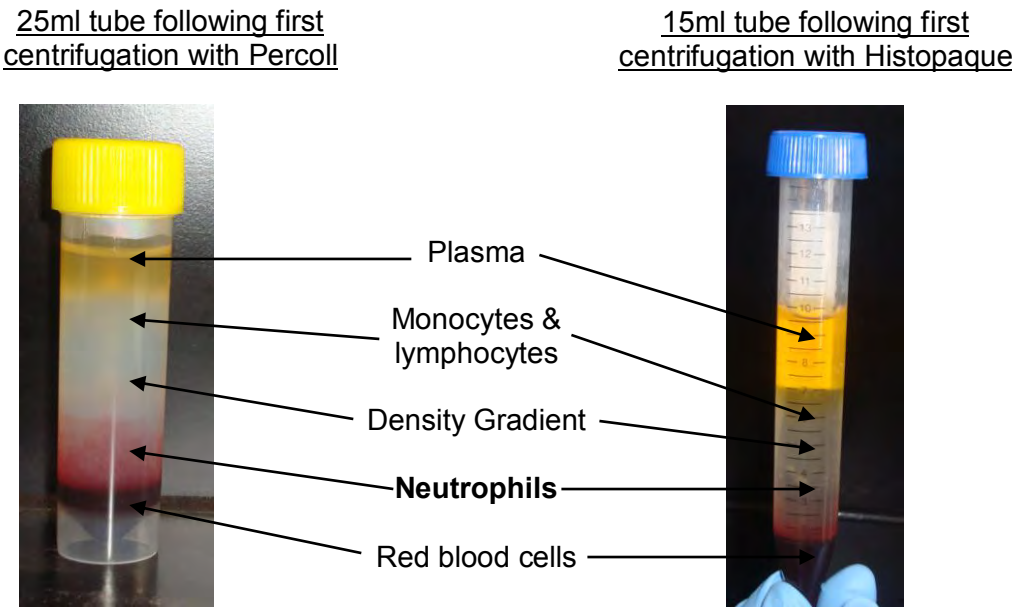

Figure 4: Comparison of blood separation after centrifugation with Percoll and Histopaque. Centrifugation of blood over 2 discontinuous density gradients causes particles of the same density to separate, enabling the isolation of neutrophils from whole blood.

Both Percoll and Histopaque isolated neutrophils were immediately re-suspended at 10^5 cells per well in either gPBS (2.2.4) or RPMI (L0500 Biosera supplemented with 0.5% glutamine) for the ROS and NETs assays respectively.

2.4.1 Chemiluminescence protocol

A 96-well white microwell plate (Microlite2; Dynex Technologies Ltd., Worthing, UK) was blocked with 200µl 1% BSA (2.4.2) at 4°C overnight. The plate was washed with PBS (2.2.3) (BioTek Plate washer ELx50), followed by the addition of 10^5 Percoll or Histopaque-isolated cells in 100µl gPBS (2.2.4), 35µl additional gPBS

(2.2.4) and 30µl luminol (2.4.3). The plate was placed in the luminometer (EG&G Berthold LB96V, 37°C, WinGlow) and after a 30min baseline period; selected wells were primed with 10µl of IFNα (2.4.4) and read for a further 30min before the addition of 25µl of stimulus; *F. nuc* (2.4.6) or opsonised *S.a* (2.4.5). Wells were also selected to act as a control, whereby cells received blocking buffer (2.4.2) as a primer and gPBS (2.2.4) as a stimulant to mimic pipetting disruptions. Subsequent to stimulation, the total volume per well was 200µl, which was read for a further 120min. All readings were expressed as relative light units (RLUs) and read at 340-650 nanometres (nm). Table 3.

2.4.2 Blocking Buffer (1 litre):

1% BSA in PBS was produced by adding 10g BSA (Sigma A4503) to 1 litre PBS, which was aliquoted and stored at -20°C prior to use.

2.4.3 Luminol

Luminol measures intracellular and extracellular ROS production, predominantly in the form of HOCl (Dahlgren & Stendahl 1983). 30mM stock solution was made by dissolving 0.5g luminol (Sigma A8511) in 94.05ml 1mM NaOH and stored at 4°C. The working solution was produced by diluting 1ml stock with 9ml PBS and the pH adjusted to 7.3.

2.4.4 Priming: Interferon alpha (IFNα)

Periodontitis patients are reported to have elevated levels of IFNα in their peripheral blood, which may "prime" neutrophils for an exaggerated response when exposed to a second stimulus (Wright *et al.,* 2008, Dias *et al.,* 2011). IFNα was used as a

priming agent in both the ROS and the NETs assays: $2.56x10^6$ units/ml IFNα (PBL) stock was used at a working concentration of 8µg/ml and aliquots defrosted when needed (10µl added to selected wells).

2.4.5 Bacterial Stimulant: Opsonised *Staphylococcus aureus* (*S.a*)

S.a is a gram-positive coccus, which is present on the skin, mucous membranes and the oral cavity, although not considered a primary periodontal pathogen, *S.a* associates with NET stimulation (Brinkmann *et al.,* 2004). Neutrophil activation by opsonised *S.a* occurs via neutrophil FC-gamma receptor (FCγR) binding to the Fc end of bacterially-bound immunoglobulin G (IgG) molecules. *S.a* (National collection of type cultures [NCTC] 6571 Fisher Scientific) was cultured from a bead, by inoculating manitol salt agar plates (OXOID). Following an aerobic overnight incubation (5% CO_2, 37°C), bacterial colonies were transferred to 500ml sterile tryptone soya broth (OXOID) in a glass universal, using a flame sterilised loop. The inoculated broth was incubated overnight (5% CO_2, 37°C), after which the culture contained stationary phase bacteria. The culture was divided into 10 x 50ml centrifuge tubes (Fisher Scientific), each of which was centrifuged for 10min at 700rcf to pellet the bacteria (IEC Centra CL3R). The supernatant was removed and the pellet re-suspended in 20ml sterile PBS (2.2.3) by vortex mixing. Suspensions were pooled and re-centrifuged twice more producing 2 x 50ml tubes containing bacterial pellets. Upon the third centrifugation, the pellet was re-suspended in 20ml formal buffered saline for 1hour (h) at room temperature. To remove the fixative, the bacteria were subjected to washing by centrifugation and re-suspension in 20ml sterile PBS (2.2.3) in triplicate. Bacterial growth was determined by measuring the optical density of the suspension with a spectrophotometer and counting visible

colonies, which was estimated to be $2.8x10^{12}$ and then diluted in sterile PBS (2.2.3) to yield a suspension of $2.8x10^{7}$. The bacterial pellets were finally re-suspended in 5ml sterile PBS in 2 x 50ml tubes (Fisher Scientific) and frozen at -80ºC. To opsonise the bacteria, 1 x 50ml tube was defrosted and heat treated for 20m at 80ºC, followed by the addition of Vigam (33µl per 10^{9} cells), a fluid containing active human IgG. (Bio Products Laboratory). This was incubated overnight at room temperature on a shaker (Stuart Scientific SI20H). Opsonised bacteria were washed twice by centrifugation (10min at 700rcf) and re-suspension in PBS (2.2.3), divided into 1ml aliquots and stored at -80ºC.

2.4.6 Bacterial Stimulant: *Fusobacterium nucleatum sp Polymorphum (F. nuc*)

F. nuc is a gram negative bacterium implicated in dental plaque biofilm formation and in periodontitis pathogenesis, due to its ability to co-aggregate with other bacteria and its quorum sensing properties (Darveau *et al.,* 1997). *F. nuc* activates neutrophils via Toll like receptors (TLR), in particular TLR2 is reported to recognise cell wall components of the organism (Kikkert *et al.,* 2007). *F. nuc* (American Type Culture Collection [ATCC] 10953) was kindly provided by Dr Mike Milward and 1ml aliquots stored at -20ºC were defrosted when needed.

Table 3: ROS plate design. Percoll or Histopaque cells were subjected to priming (IFNα) and stimulation (*F.nuc* or opsonised *S.a*), subsequent to the measurement of ROS.

		Control				*F. nuc*				Opsonised *S.a*			
	Density gradient material	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	**Percoll (Control)**	100µl cells in gPBS 30µl luminol 35µl gPBS 10µl blocking buffer 25µl gPBS				100µl cells in gPBS 30µl luminol 35µl gPBS 10µl blocking buffer 25µl *F. nuc*				100µl cells in gPBS 30µl luminol 35µl gPBS 10µl blocking buffer 25µl opsonised *S.a*			
B	**Percoll (IFNα)**	100µl cells in gPBS 30µl luminol 35µl gPBS 10µl IFNα 25µl gPBS				100µl cells in gPBS 30µl luminol 35µl gPBS 10µl IFNα 25µl *F. nuc*				100µl cells in gPBS 30µl luminol 35µl gPBS 10µl IFNα 25µl opsonised *S.a*			
C	**Histopaque (Control)**	100µl cells in gPBS 30µl luminol 35µl gPBS 10µl blocking buffer 25µl gPBS				100µl cells in gPBS 30µl luminol 35µl gPBS 10µl blocking buffer 25µl *F. nuc*				100µl cells in gPBS 30µl luminol 35µl gPBS 10µl blocking buffer 25µl opsonised *S.a*			
D	**Histopaque (IFNα)**	100µl cells in gPBS 30µl luminol 35µl gPBS 10µl IFNα 25µl gPBS				100µl cells in gPBS 30µl luminol 35µl gPBS 10µl IFNα 25µl *F. nuc*				100µl cells in gPBS 30µl luminol 35µl gPBS 10µl IFNα 25µl opsonised *S.a*			

2.5 NET Quantification

Following treatment with Micrococcal nuclease (MNase; LS004797 Worthington Biochemical Corporation), to digest extracellular DNA, and centrifugation, NETs were fluorometrically quantified with Sytox green (Invitrogen S7020). A 96-well plate was blocked by adding 200µl syringe-filtered 1% BSA (2.4.2) and stored at 4°C overnight. The supernatant was removed from the plate using a vacuum pump, followed by the addition of 10^5 Percoll or Histopaque isolated cells in 100µl RPMI (L0500 Biosera supplemented with 0.5% glutamine) and 35µl additional RPMI. After a 30min baseline incubation period (37°C, 5% CO_2), selected wells were primed with 10µl of IFNα (2.4.4) and incubated for a further 30min (37°C, 5% CO_2) before the

addition of 25µl stimuli; *F. nuc* (2.4.6) or opsonised *S.a* (2.4.5). Wells were also selected to act as controls, whereby cells received blocking buffer (2.4.2) as a primer and RPMI medium (L0500 Biosera supplemented with 0.5% glutamine) as a stimulant. Subsequent to stimulation, the plate was covered and incubated for 2h (37°C, 5% CO_2). Post-incubation, 15µl of 14.3units/ml MNase were diluted in PBS (2.2.3), added to each well and incubated at room temperature for 10min. The contents of each well were mixed and centrifuged (10min at 1800rcf, Hettich Universal 320R). Next, 150µl of the supernatant were transferred to the wells of a fresh plate, with the addition of 15µl of 10µM Sytox green (Invitrogen S7020) diluted 1/500 in RPMI medium, to quantify any free DNA within the supernatant. Fluorescence was read in arbitrary fluorescent units (AFU) by a fluorometer (Twinkle LB970, Berthold Technologies; excitation 485nm and emission 525nm) 3 times at 37°C. Table 4.

Table 4: MNase plate design. Percoll or Histopaque cells were subjected to priming (IFNα) and stimulation (*F.nuc* or opsonised *S.a*), subsequent to the quantification of NETs.

		Control				***F. nuc***				**Opsonised *S.a***			
	Density gradient material	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	**Percoll (Control)**	100µl cells in RPMI 35µl RPMI 10µl blocking buffer 25µl RPMI				100µl cells in RPMI 35µl RPMI 10µl blocking buffer 25µl *F. nuc*				100µl cells in RPMI 35µl RPMI 10µl blocking buffer 25µl opsonised *S.a*			
B	**Percoll (IFNα)**	100µl cells in RPMI 35µl RPMI 10µl IFNα 25µl RPMI				100µl cells in RPMI 35µl RPMI 10µl IFNα 25µl *F. nuc*				100µl cells in RPMI 35µl RPMI 10µl IFNα 25µl opsonised *S.a*			
C	**Histopaque (Control)**	100µl cells in RPMI 35µl RPMI 10µl blocking buffer 25µl RPMI				100µl cells in RPMI 35µl RPMI 10µl blocking buffer 25µl *F. nuc*				100µl cells in RPMI 35µl RPMI 10µl blocking buffer 25µl opsonised *S.a*			
D	**Histopaque (IFNα)**	100µl cells in RPMI 35µl RPMI 10µl IFNα				100µl cells in RPMI 35µl RPMI 10µl IFNα				100µl cells in RPMI 35µl RPMI 10µl IFNα			

		25µl RPMI	25µl *F. nuc*	25µl opsonised *S.a*

2.6 NET visualisation

A 24-well plate was blocked with 500µl syringe-filtered 1% BSA (2.4.2) and stored at 4°C overnight. The supernatant was removed from the plate with a vacuum pump, followed by the addition of 10^5 Percoll or Histopaque isolated cells in 200µl RPMI (L0500 Biosera plus 0.5% glutamine) and 35µl additional RPMI. After a 30min baseline incubation period (37°C, 5% CO_2), selected wells were primed with 25µl of IFNα (2.4.4) and incubated for a further 30min (37°C, 5% CO_2) before the addition of 75µl stimuli; *F. nuc* (2.4.6) or opsonised *S.a* (2.4.5). Wells were selected to act as a control, whereby cells received blocking buffer (2.4.2) as a primer and RPMI medium (L0500 Biosera plus 0.5% glutamine) as a stimulant. Subsequent to stimulation, the plate was covered and incubated for 2h (37°C, 5% CO_2). Post-incubation, 25µl of 10µM Sytox green (Invitrogen S7020) diluted 1/500 in RPMI was added to each well. NETs were visualised with an epi-fluorescent microscope (Nikon Eclipse TE300) with fluorescent filter excitation 472nm and emission 520nm (BrightLine® GFP-3035B, Semrock) at x20 magnification. Images were captured on a digital camera (Nikon Coolpix 990). Table 5.

Table 5: NET visualisation plate design. Percoll or Histopaque cells were subjected to priming (IFNα) and stimulation (*F.nuc* or opsonised *S.a*), subsequent to the visualisation of NETs.

		Control		*F. nuc*		Opsonised *S.a*	
	Density gradient material	**1**	**2**	**3**	**4**	**5**	**6**
A	**Percoll (Control)**	200µl cells in RPMI 25µl blocking buffer 75µl RPMI		200µl cells in RPMI 25µl blocking buffer 75µl *F. nuc*		200µl cells in RPMI 25µl blocking buffer 75µl opsonised *S.a*	
B	**Percoll (IFNα)**	200µl cells in RPMI 25µl IFNα 75µl RPMI		200µl cells in RPMI 25µl IFNα 75µl *F. nuc*		200µl cells in RPMI 25µl IFNα 75µl opsonised *S.a*	
C	**Histopaque (Control)**	200µl cells in RPMI 25µl blocking buffer 75µl RPMI		200µl cells in RPMI 25µl blocking buffer 75µl *F. nuc*		200µl cells in RPMI 25µl blocking buffer 75µl opsonised *S.a*	
D	**Histopaque (IFNα)**	200µl cells in RPMI 25µl IFNα 75µl RPMI		200µl cells in RPMI 25µl IFNα 75µl *F. nuc*		200µl cells in RPMI 25µl IFNα 75µl opsonised *S.a*	

2.7 Metabolic Activity Assay

Incubation of neutrophils with CellTiter-Glo Luminescent substrate quantifies metabolic activity by quantifying ATP (adenosine-5'-triophospate) and is also used to estimate viable cell number. A-96 well microplate was blocked with 200µl 1% BSA (2.4.2) at 4°C overnight. The plate was washed with PBS (2.2.3), followed by the addition of 80µl 10^5 Percoll and Histopaque isolated cells in gPBS (2.2.4) to represent ROS cells and RPMI (L0500 Biosera plus 0.5% glutamine) for NET quantification and visualisation cells. The plate was placed in the luminomter and CellTiter-Glo Luminescent substrate (Promega) was added to both Percoll and Histopaque isolated gPBS cells at the start, 30, 60 and 90min. To mimic the longer incubation period of the NET assays, CellTitre-Glo was added to Percoll and Histopaque RPMI suspended cells initially, and at 30, 60 and 180min.

Chapter 3: Results

All statistical analyses were performed using the InStat3 statistical software package (Graphpad Instat 3). Statistical tests employed for the purpose of this study were at a significance of 0.05, parametric and paired when appropriate.

3.1.1 Percoll and Histopaque neutrophil yield

Following neutrophil isolation from whole blood, cells were counted to enable use of the appropriate number of re-suspended cells for subsequent ROS and NET assays, and also to provide relative yields following Percoll and Histopaque purification. Across 10 paired isolations, with the exception of one experiment the neutrophil yield retrieved from the same volume of blood was higher for Percoll ($p=0.0038$, paired t-test, figures 5&6 n=10).

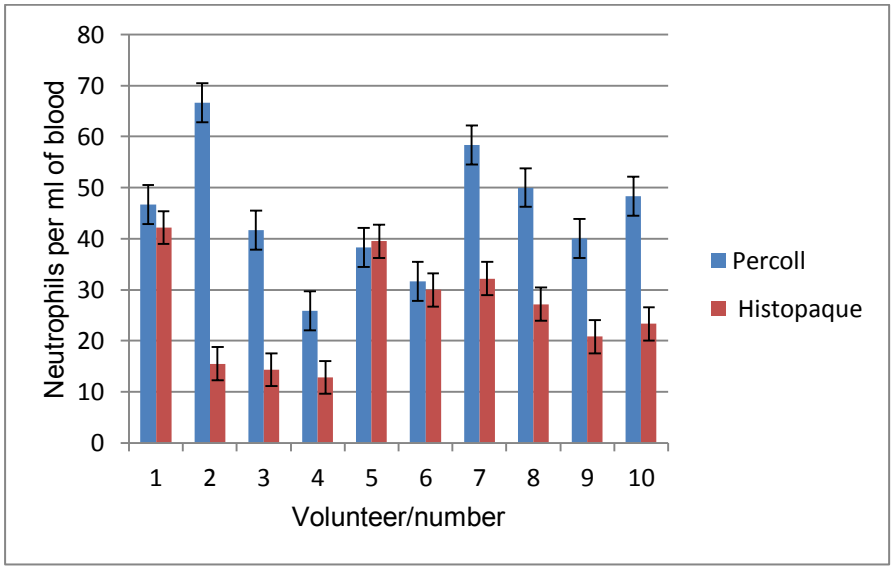

Figure 5: Number of neutrophils isolated from 1 mL of blood with percoll or histopaque. Error bars indicate standard error of the mean (±SEM), n=10.

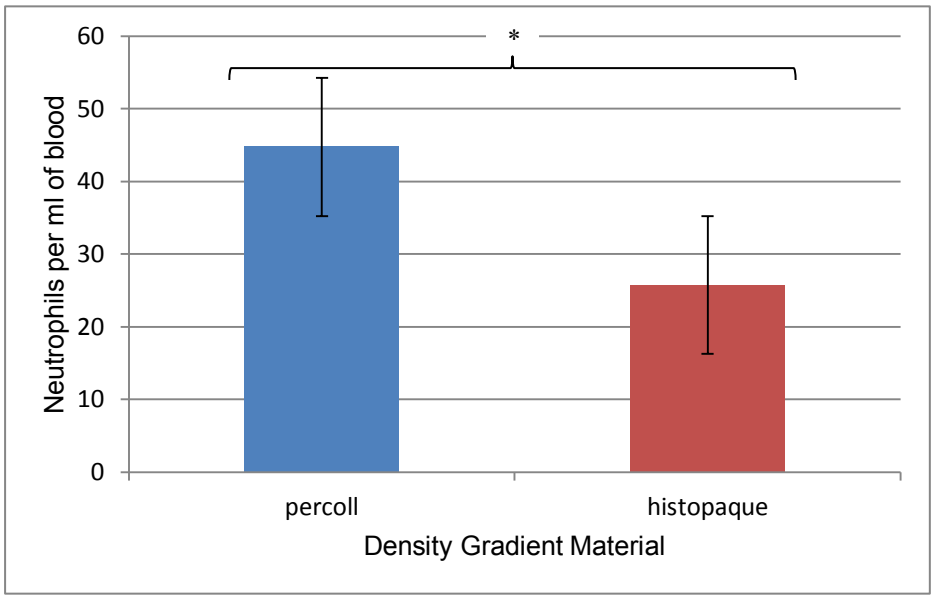

Figure 6: Average number of neutrophils isolated from 1 millilitre of blood with Percoll or Histopaque. (Data expressed as mean ± SEM, n=10, *p=0.0038 [paired t-test]).

3.1.2 Percoll and Histopaque control cells

Subsequent to isolation, both Percoll and Histopaque separated neutrophils were assessed for ROS generation. Cells were left to settle for 30min in the luminomter prior to any treatment (Baseline; time point A). Following this baseline period, selected wells were primed with IFNα and light emission was read for a further 30min (time point B). Finally, selected wells were stimulated with either *F. nuc* or opsonised *S.a*, which was read for 1h (giving the final time point; C). Wells were also selected to act as a control, whereby cells received blocking buffer as a primer and gPBS as a stimulant. These control wells allowed for further comparisons between Percoll and Histopaque, as these untreated neutrophils should in theory be indistinguishable. Histopaque isolated neutrophils demonstrated higher ROS production by untreated cells at baseline and subsequent time points. However these observed differences did not reach statistical significance (p=0.4, 0.2 and 0.3 for time points A, B and C respectively [paired t-tests], figure 7, n=10 in

quadruplicates). Notably, ROS production by both Histopaque and Percoll unstimulated neutrophils decreased over time.

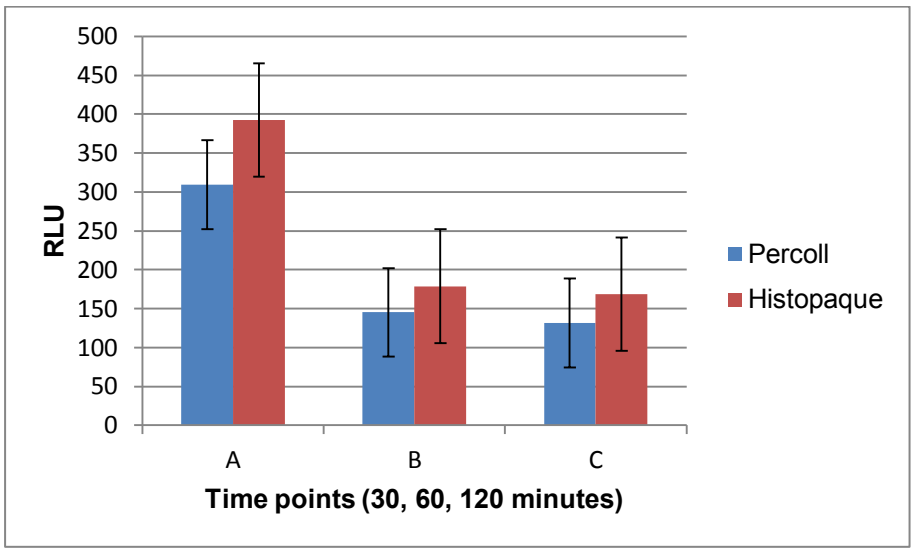

Figure 7: ROS production by unprimed and unstimulated Percoll and Histopaque isolated neutrophils at different time points. P=0.4, 0.2, 0.3 for A (30min), B (60min), C (120min) respectively (paired t-test). (Data expressed as mean ± SEM, n=10, in quadruplicates).

3.1.3 The effect of IFNα-priming on Percoll and Histopaque cells

IFNα (8μg/ml) has been reported to prime neutrophils and result in an exaggerated response when exposed to a second stimulus. IFNα can also activate neutrophils directly, dependent upon the concentrations employed (Dias *et al* 2010). The addition of IFNα to percoll neutrophils led to increased ROS production after 30min ($p=0.04$), and this increase was maintained for a further 60min, but was no longer significant ($p=0.2$). Interestingly, Histopaque neutrophils did not appear to respond to the cytokine initially, but did so after 90min exposure, however this was not significant ($p=0.4$) (figure 8, n=10 in quadruplicates).

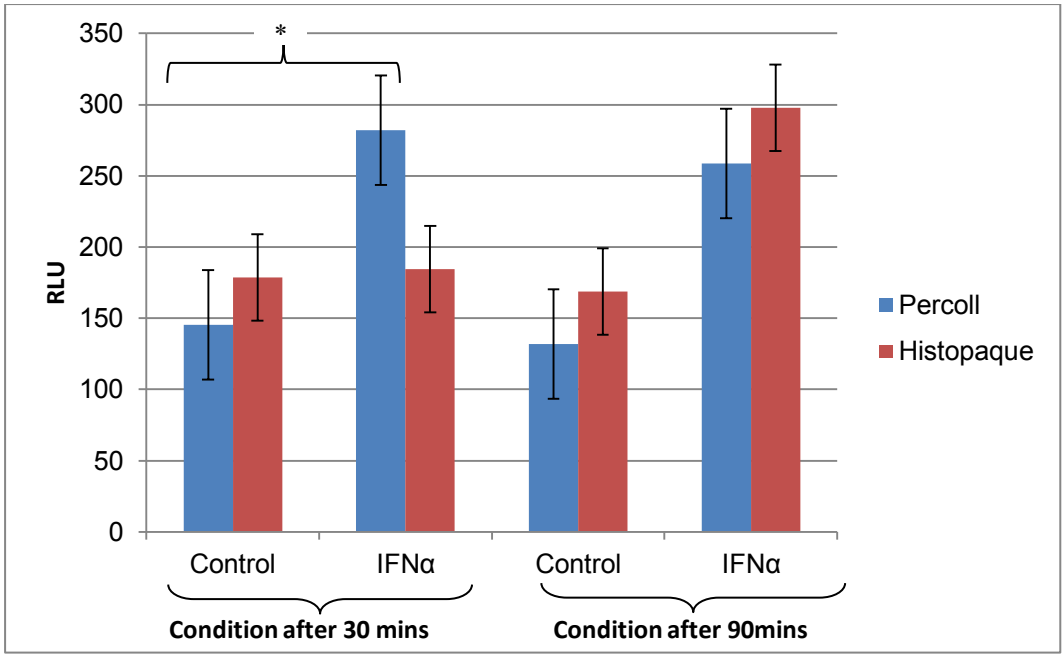

Figure 8: Control and IFNα-mediated ROS production (no 2nd stimulus) by Percoll and Histopaque isolated neutrophils 30 and 90min following priming. Percoll isolated cells showed increased ROS 30m after IFNα *p=0.04 (paired t-test). (data expressed as mean ± SEM, n=10, in quadruplicates).

3.1.4 The effect of *F. nuc* and opsonised *S.a.*-stimulation on IFNα-primed Percoll and Histopaque isolated neutrophils

ROS generation by IFNα-exposed neutrophils prior to *F. nuc* or Opsinised *S.a* was marginally higher, relative to those who were only subjected to *F. nuc* or Opsonised *S.a.* However this difference was not statistically significant. *F. nuc* stimulated neutrophils provoked a smaller response in comparison with opsonised *S.a* stimulated cells, for both percoll and histopaque neutrophils (Percoll cells p =0.0002, Histopaque cells p=0.0007 [paired t-test]). Additionally, opsonised *S.a* stimulated Percoll-isolated cells produced significantly more ROS compared to Histopaque-isolated neutrophils (no IFNα: p=0.04, IFNα pre-treatment: p=0.03 [paired t-test]). However there was no significant difference between *F. nuc* stimulated Histopaque

and Percoll isolated neutrophils (no IFNα: p=0.4 *F. nuc,* IFNα pre-treatment: p=0.95 [paired t-test] figure 9, n=10 in quadruplicates).

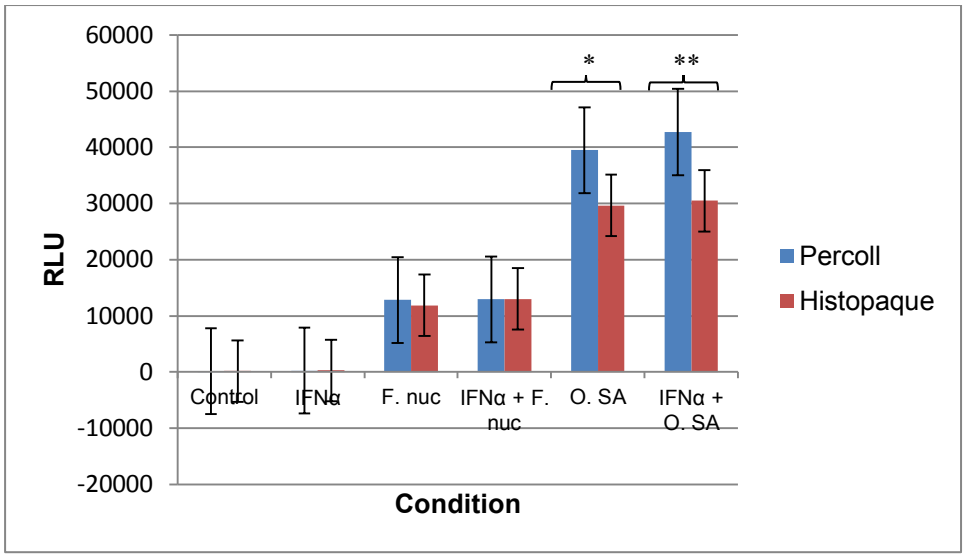

Figure 9: ROS production by Percoll or Histopaque isolated neutrophils following priming and stimulation. Opsonised *S.a* stimulated Percoll-isolated cells produced significantly more ROS compared to Histopaque-isolated neutrophils *p=0.04 (paired t-test). RLU = relative light units. (Data expressed as mean ± SEM, n=10, in quadruplicates).

3.2 ROS cell metabolic activity at relevant time points for the ROS assay

To investigate the metabolic activity of neutrophils at time points relevant to the ROS assay, ATP production of cells re-suspended in gPBS was measured at regular intervals over a 90min period. ATP generation was initially higher in Histopaque-isolated neutrophils compared with Percoll (p=0.005, paired t-test). However over the 90min period, ATP production by Histopaque-isolated neutrophils decreased by 64%. The metabolic activity of Percoll neutrophils was lower at the start of the ROS assay and despite also decreasing with time, the reduction in ATP activity was not as great as Histopaque neutrophils. However this difference between Percoll and Histopaque-isolated cell activity at 90m did not reach statistical significance (p=0.2, see figure 10, n=1 in quadruplicates).

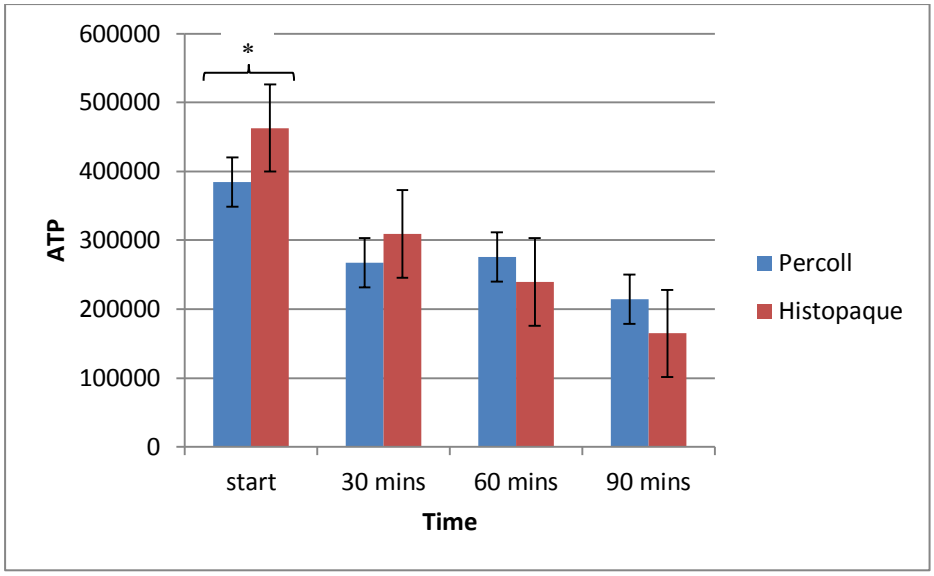

Figure 10: Metabolic activity of Percoll and Histopaque-isolated cells. Adenosine-5'-triphosphate (ATP) generation was initially higher in Histopaque-isolated neutrophils compared with Percoll *p=0.005 (paired t-test). RLU = relative light units. (Data expressed as mean ± SEM, n=1, in quadruplicates)

3.3.1 NET quantification

NET production appeared to be considerably higher in each treatment condition for Percoll isolated neutrophils, relative to Histopaque. This was most marked in neutrophils subjected to *F. nuc* (p=0.004) and IFNα + *F. nuc* (p=0.0005) (paired t-tests) treatment. Interestingly, whilst IFNα appeared to have priming effects on Percoll isolated neutrophils, producing an exaggerated response to the secondary stimuli, Histopaque isolated neutrophils did not respond to the cytokine treatment (table 6, n=10 in quadruplicates).

Table 6: NET production by Percoll and Histopaque isolated neutrophils (mean of n=10, in quadruplicates) Results represent Arbitrary fluorescent units (AFU).

	Control	IFNα	*F. nucleatum*	IFNα + *F. nucleatum*	Opsonised *S.a*	IFNα + Opsonised *S.a*
Percoll	1.30E+05	1.42E+05	3.00E+05	3.25E+05	2.40E+05	2.53E+05
Histopaque	8.59E+04	9.39E+04	1.75E+05	1.68E+05	1.72E+05	1.70E+05

3.3.2 NET visualisation

No NETs were observed in unstimulated (control) neutrophils, with the only visible fluorescence likely to be DNA within the nuclei of dead/dying cells, as this would account for the circular morphology of the fluorescent signal. Neutrophils stimulated with *F. nuc* produced high numbers of large NETs, with very few dead cells being apparent. Paradoxically, opsonised *S.a* stimulated neutrophils were found to produce very few NETs and a relatively large amount of dead cells. This is partly supported by the lower numbers of NETs when stimulated with opsonised *S.a* compared with those stimulated with *F. nuc* in the quantification assay. No differences in NET visualisation were observed as a result of the density gradient material employed (figure 11, representative images n=3).

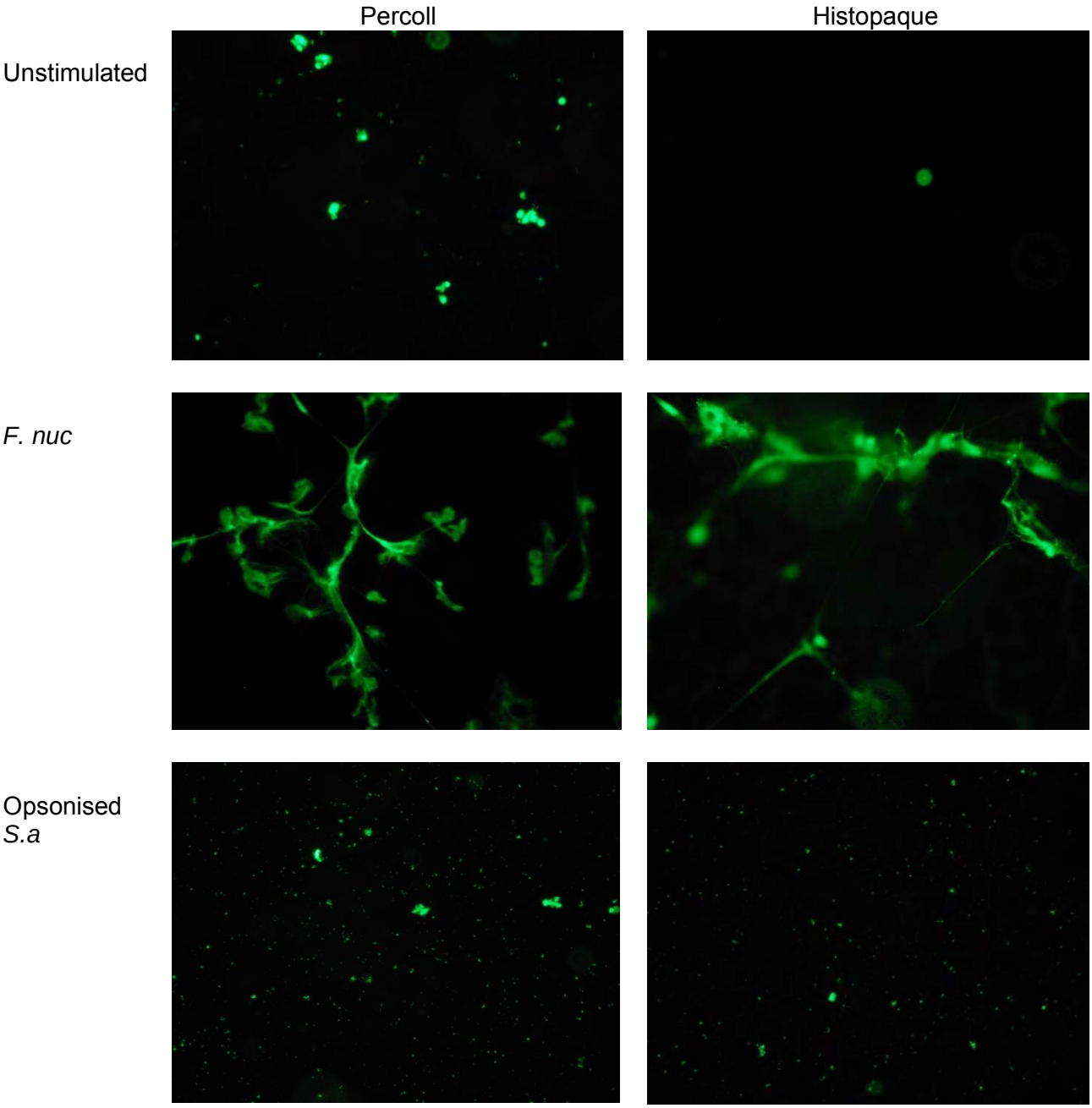

Figure 11: NET visualisation of control, *F. nuc* or opsonised *S.a* stimulated Percoll and Histopaque isolated neutrophils. Representative images n=3, visualised with an epi-fluorescent microscope at an excitation of 472nm and emission of 520nm at x20 magnification. Images were captured on a digital camera (Nikon Coolpix 990).

3.4 NET cell metabolic activity at relevant time points for the NET assay

ATP production of cells re-suspended in RPMI was measured at regular intervals over a 180min period. ATP generation was initially higher in Histopaque neutrophils compared with Percoll (p=0.03, paired t-test), consistent with the ROS data. However, over the 180min used in the NET assay, ATP production by Histopaque neutrophils decreased by approximately 94% (figure 12, n=1 in quadruplicates). The metabolic activity of Percoll neutrophils was lower at the start of the observation period and despite decreasing with time also, the reduction in cell activity was not to the same extent as Histopaque neutrophils. However this difference in cells at 180min was not significant (p=0.5, paired t-test).

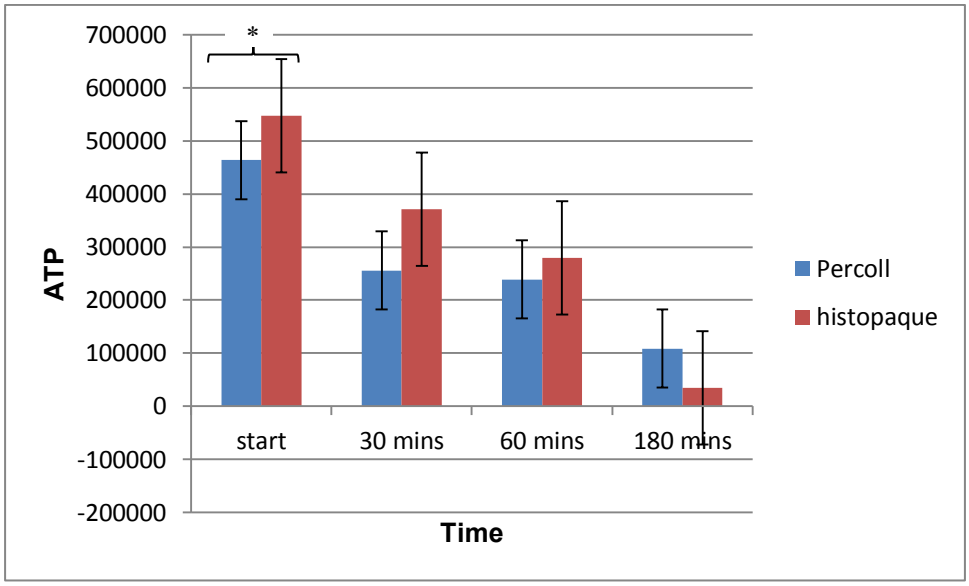

Figure 12: Metabolic activity of Percoll and Histopaque isolated cells. Adenosine-5'-triphosphate (ATP) generation initially higher in Histopaque-isolated neutrophils compared with percoll *p=0.03 (paired t-test). AFU = arbitrary fluorescent units. (data expressed as mean ± SEM) n=1, in quadruplicates).

Chapter 4: Discussion

The aim of this project was to compare two methods of neutrophil isolation from peripheral blood with respect to ROS and NET production under various conditions of priming and stimulation (or no treatment). The rationale for the study was the inconsistent and conflicting reports in the literature on the effects of different density gradient materials on neutrophil behaviour. Parameters investigated included cell yield, viability (trypan blue exclusion), metabolic status (Cell Titre Glo assay), ROS release (Luminol chemiluminescence) and NET formation (MNase assay and microscopy). Overall, results revealed that there are indeed dissimilarities between the chosen density gradient materials in neutrophil yield, baseline activation status and subsequent ROS and NET responses.

The numbers of neutrophils obtained from 12ml of blood from the same individual were significantly higher when Percoll was used for purification, with Histopaque isolation producing 48% fewer neutrophils. This is consistent with the findings of Grisham (1985), who reported that the use of two discontinuous Percoll gradients also produced a cell yield of approximately 50% higher than two different Histopaque gradients. Despite the fact that this difference in yield is corrected in subsequent ROS and NET assays, with each well containing 10^5 cells, neutrophil yield is an important variable as blood collection from one individual is usually required for several assays and may influence assay logistics. Neutrophils have traditionally been regarded as having a short life span, but recent *in vivo* analyses have demonstrated longevity within the blood stream of 5.4 days (Pillay *et al.,* 2010). Nevertheless, they are terminally differentiated cells, meaning they cannot be passaged in tissue culture without undergoing apoptosis (Gottlieb *et al.,* 1995).

Considering the problems already discussed surrounding HL60 cells and mouse models, neutrophils are somewhat difficult cells to study. *Ex vivo* research with the use of density gradients represents a method for studying neutrophils that is most reflective of their *in vivo* state and thus the number of viable cells attainable from a limited amount of venous blood governs the ability to conduct later experiments. Subsequent trypan blue counts revealed 98% viability of both Percoll and Histopaque purified neutrophils.

It was important to determine whether the isolation method had activated the cells to any degree, since this may adversely affect subsequent studies. Therefore, neutrophils were placed in the luminometer immediately after isolation to study baseline ROS production over a 30min period, as a measure of their activation status. Although data did not reach statistical significance, there was a general trend towards higher ROS production in neutrophils isolated with Histopaque. Variability of Histopaque data was also high, indicating large inter-individual variability and that higher volunteer numbers would likely result in statistical significance being achieved. At this point no cells had been intentionally subjected to priming or stimulation and the choice of density gradient material represented the only variable between these cells. The data suggest that Histopaque isolation activates the neutrophils and provokes ROS release, which are necessary for the cell to fulfill its role in immunity (Hampton *et al.,* 1998). ROS accumulation in neutrophils has been suggested to initiate apoptosis, as a result of ligand-independent death receptor signaling. Neutrophils co-cultured with free radical scavengers, *N*-acetylcysteine or desferrioxamine, were shown to exhibit decreased death signaling, for example ceramide generation and CD95 expression; which inhibited apoptosis. These

findings infer that upon exposure to a threshold level of ROS, as a result of activation, neutrophils will increase death receptor signal expression; leading to apoptosis (Scheel-Toellner *et al.,* 2004).

In order to verify the baseline activity status of neutrophils following Percoll and Histopaque purification, metabolic activity was determined by CellTiter-Glo Luminescent detection of ATP at 30min intervals over 90min. ATP functions as a coenzyme in many cells and has been implicated in neutrophil activation, where it is believed to act as a substrate in protein kinase C signaling (Lu & Grinstein 1990) and also plays an essential role in calcium ion uptake (Klempner 1985). Consistent with the hypothesis that Histopaque activates neutrophils during purification, ATP quantification revealed that Histopaque cells were metabolically active initially, however less ATP was produced over time, indicating either that internal feedback mechanisms were switching neutrophils to a less active state, or that potentially apoptosis was indeed being induced (Scheel-Toellner *et al* 2004). By contrast, Percoll-purified neutrophils appeared less metabolically active at baseline, but activity levels reduced to a lesser degree with time. Neutrophil apoptosis was not assessed in these studies due to time constraints, but would be interesting to determine as a future piece of work. Scheel-Toellner *et al.,* (2004) did not explore the temporality of ROS-induction and apoptosis, and given that the cells are in the luminometer for a total of only 2h, further investigation is warranted to determine whether apoptosis accounts for the reduced response in Histopaque neutrophils to subsequent stimulation.

Selected Percoll and Histopaque isolated cells were also subjected to priming with IFNα, a cytokine released by innate immune cells (e.g. Langerhans/dendritic cells) in response to bacterial stimulation, and shown to both prime and stimulate peripheral blood neutrophils (Wright *et al* 2008; Dias *et al* 2011) present in the biofilm in periodontitis. Neutrophil ROS production following IFNα treatment was recorded following 30 and 90min, which revealed that whilst Percoll neutrophils responded to the cytokine promptly, Histopaque neutrophils displayed a delayed activation until 90min. Interestingly, this delayed response to IFNα by Histopaque neutrophils was higher than the Percoll response, implying that Histopaque-derived neutrophils may be more lethargic in response to activation. Wright *et al.,* (2008) demonstrated that periodontitis patients have elevated levels of IFNα in their peripheral blood and display a type-1 interferon-gene expression profile. The same group subsequently showed that dependent on the concentration, IFN-α was able to both "prime" neutrophils for an exaggerated response if exposed to a second stimulus, as well as to directly stimulate neutrophils, dependent on the concentration (Dias *et al* 2011). The reference priming values (0.025, 0.25, and 2.5 units) are significantly lower than the concentration of IFNα used in this particular study (2500 units) for ROS and NET priming. It is likely that the IFNα was stimulating the neutrophils rather than priming for an exaggerated response when exposed to bacterial stimuli. Therefore the IFN data produced in this project will not be used to act as a comparison for Percoll and Histopaque isolation and further work is needed to look at priming with IFN at the correct concentration.

Percoll and Histopaque-purified neutrophils were also compared in terms of their ability to produce NETs, both quantitatively (MNase assay) and qualitatively

(fluorescence microscopy). Neutrophils were stimulated with *F. nuc* and opsonised *S.a* to induce NET production, according to previous methods (Palmer 2012 *et al* and Brinkmann *et al* 2004, respectively). Both Percoll and Histopaque-derived neutrophils produced NETs, however upon quantification, Percoll neutrophils were found to produce consistently more, in particular when stimulated with *F. nuc.* This suggests that Percoll neutrophils are more sensitive to NET production following bacterial stimulation. A further consideration is the results of the metabolic activity assay at NETosis-relevant time points. Similar to the ROS assay, Percoll and Histopaque neutrophils were incubated with the CellTiter-Glo substrate, but cells were suspended in RPMI and read over 180min (rather than 90min). Histopaque neutrophils were found to have increased metabolic activity initially, as indicated by ATP levels, but activity decreased over time. Notably, the MNase and visualisation assays are conducted at 180min, at which point the metabolic activity of Histopaque neutrophils is significantly lower than that of Percoll-isolated cells; which may account for the decreased NET production in the latter. NET qualitative visualisation did not demonstrate any variability between Percoll and Histopaque neutrophils, however *F. nuc* stimulated cells produced numerous large NETs, relative to opsonised *S.a*-stimulated cells. This coincided with the lower quantities of opsonised *S.a*-induced NET release in the MNase assay. Although not pursued this study, considering that *F. nuc* is a periodontal pathogen this raises questions in relation to whether NET production in periodontitis is protective or destructive.

A further consideration is that neutrophils isolated with Percoll were subjected to multiple centrifugation and wash steps, whereas isolation with Histopaque required less handling and transfers between tubes; suggesting Histopaque neutrophils

should be less activated. Both gradient protocols take approximately 90min, thus with regard to neutrophils' relatively short life span, neither gradient is superior in terms of time. One modification to the traditional Histopaque protocol was the addition of a 30sec erythrocyte lysis step (with distilled water), as initial experiments demonstrated erythrocyte contamination. This is distinct from Percoll-isolated neutrophils, which are subjected to an ammonium chloride (NH_4Cl) lysis step. Although it has been suggested that distilled water-induced erythrocyte lysis causes less neutrophil activation, the ROS results in this particular study indicate this may not be the case under these particular assay conditions, as baseline ROS in Histopaque neutrophils was higher than for Percoll-derived cells. This is also supported by Vuorte *et al.,* (2001), who reported that neither hypotonic shock with distilled water or NH_4Cl lysis influenced neutrophil integrity or activation. Of relevance however, is recent research suggesting a 15min NH_4Cl lysis can provoke neutrophil activation and the introduction of a 2sec NH_4Cl lysis step, followed by immediate addition of 1x Hank's balances salt solution may reduce neutrophil disruption (Maqbool *et al.,* 2011).

Chapter 5: Conclusion

This project has described a series of investigations into the use of two density gradient materials for isolating neutrophils from peripheral whole blood, for subsequent *ex vivo* investigation of NET biology. Percoll is currently the gradient material used by the Periodontal Research Group at Birmingham, as well as many other laboratories, but Histopaque is used by many other research groups. Currently, the impact of the gradient material and associated purification method upon NET production and detection following *ex vivo* neutrophil priming/stimulation is unknown. This project is also one of very few studies investigating the two methods in relation to ROS-release, but previous research has suggested that the isolation method may produce differences in neutrophil yield, viability and most interestingly activation (Grisham *et al.,* 1985, Rebecchi *et al.,* 2000).

The results revealed that Histopaque isolated fewer neutrophils compared to Percoll within the same blood samples from the same healthy volunteers. Following isolation, Histopaque-prepared neutrophils produced higher baseline (unstimulated) ROS, suggesting that Histopaque neutrophils may be activated more than their Percoll-derived counterparts. Analysis of metabolic activity supported this thesis and these observations may account for why Histopaque neutrophils did not respond to stimulation to the same extent as Percoll-derived cells. Whether the underpinning mechanism relates to ROS production inducing apoptosis (Scheel-Toellner *et al.,* 2004), or feedback mechanisms involving cytosolic/membrane-bound NADPH-oxidase sub-unit assembly/dis-assembly remains to be determined. As previously mentioned, further work is needed to analyse the effect of density gradient material

upon priming with IFNα, as the concentration employed was not representative of *in vivo* priming (Dias *et al* 2011).

The NET assays demonstrated that Histopaque neutrophils produced lower quantities of NETs relative to Percoll-isolated neutrophils, both in unstimulated and stimulated conditions. This infers that Percoll may offer increased sensitivity for NET production and quantitation by MNase assay. Histopaque-derived neutrophils alternatively appeared less sensitive to NET production, which may be due to the gradient medium activating low level ROS and thus desensitising cells to a second stimulus, or alternatively inducing apoptosis over longer time periods of time. The Cell Titre Glo assay revealed extremely low numbers of Histopaque neutrophils when NETs were quantified, but studies of caspase-3 activation at these time points are required to explore this further.

Overall, taking into account neutrophil yield, baseline ROS production, cell viability and NET production, Percoll appears to be the gradient material of choice for ROS and NET assays. There is scope to research whether the isolation technique employed influences other neutrophil function assays, such as chemotaxis or phagocytosis, as it may be that different gradients are appropriate for different neutrophil properties. Finally, with respect to different isolation protocols being used by different research groups, it may be that alterations in centrifugation protocols or lysis steps further influence subsequent *ex vivo* neutrophil behaviour. There are also other inconsistencies in neutrophil isolation protocols which may influence experimental results and thus warrant further research, such as the anti-coagulant employed during venepuncture.

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Figure 2: Image taken from American Academy of Cosmetic Dentistry [online] Available at <http://www.aacd.com/index.php?module=cms&page=578> [Accessed 14/05/12]

UNIVERSITY OF BIRMINGHAM

Effect of Neutrophil Extracellular Traps upon the Growth and Survival of Periodontopathogenic Bacteria

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This project is submitted in partial fulfilment of the requirements for the award of the MRes

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Abstract

Neutrophils were recently found to utilise an additional mechanism in the containment of pathogenic microbes, whereby neutrophils extrude their nuclear chromatin as a complex mix of DNA, histones and antimicrobial peptides (AMPs) in a process termed neutrophil extracellular trap (NET) formation. Periodontitis is a chronic inflammatory disease which is initiated by bacterial pathogens, however the subsequent tissue destruction also relies on the interactions with the host immune response. The role of NETs in the host response to periodontopathogenic bacteria and the extent to which NETs influence bacterial growth and survival is as yet undetermined. Therefore this study investigated the effect of NETs, produced by hypochlorous (HOCl)-stimulated neutrophils, co-incubated with live bacteria (*S. intermedius, S. oralis* and *F. nucleatum*) upon bacterial growth and survival, based on optical density (turbidity) measurements. Preliminary experiments investigated *ex vivo* neutrophil viability, NET storage, bacterial growth and incubation conditions, with the aim of establishing an assay representative of the *in vivo* bacteria-NET encounter. NET-mediated killing/immobilisation of bacteria was assigned by comparing bacteria incubated with the neutrophil incubation medium RPMI, to the supernatant of unstimulated neutrophils (no NETs present) and to bacteria incubated with the supernatant of HOCl-stimulated neutrophils (NETs present). Turbidity measurements indicated that, under the assay conditions applied, NETs did not impede bacterial growth following a 24h bacteria-NET incubation. It is difficult to ascertain whether this uninhibited growth is a result of NETs not being capable of immobilising and killing these bacterial species, or currently whether the assay applied was not sensitive enough. However this preliminary work provides a robust foundation for future study and the co-incubation assay.

Contents

List of figures

Abbreviations

AFU	Arbitrary fluorescent units
AMP	Antimicrobial peptide
BSA	Bovine serum albumin
DNase	Deoxyribosenuclease
GAS	Group A Streptococcus
GCF	Gingival crevicular fluid
EDTA	Ethylenediaminetetraacetic acid
HOCl	Hypochlorous acid
MIC	Minimum inhibitory concentration
MNase	Micrococcal nuclease
MOI	Multiplicity of infection
NADPH	Nicotinamide adenine dinucleotide phosphate
NET	Neutrophil extracellular trap
OD	Optical density
PAD4	Peptidylarginine deiminase 4
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pen-strep	Penicillin streptomycin
PMNL	Polymorphonuclear leukocyte (neutrophilic)
RCF	Relative centrifugal force
ROS	Reactive oxygen species
SEM	Standard error of the mean
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy

Chapter 1: Introduction

1.1 Introduction to Periodontitis

Periodontitis is a chronic inflammatory disease, in which the bacterial initiation of an inflammatory lesion becomes dysregulated and causes periodontal pocket formation, gingival bleeding and alveolar bone destruction, which if left untreated can eventually lead to tooth loss. Periodontitis is the leading cause of tooth loss in the western world (Papapanou 1999) and disease prevalence is estimated to be between 20 and 50% worldwide (Albandar & Rams 2002). Interestingly, recent reports have suggested an association between periodontitis and systemic diseases, such as cardiovascular disease, which has fueled further interest in periodontitis (Sanz *et al.,* 2010). Periodontitis is preceded by gingivitis, which is a reversible inflammation of the gingiva, resulting from the accumulation of a bacterial biofilm at and below the gingival margin. The progression to periodontitis arises when inflammation is not limited to the gingiva, but encompasses the deeper periodontal tissues also, i.e. the periodontal ligament, root cementum and alveolar bone. However, the transition from gingivitis to periodontitis does not always occur and can be the result of environmental change, oral hygiene or a genetic predisposition; interestingly according to Loe *et al.*, (1986) 10% of the population is completely resistant to the disease. Periodontitis can be categorised as chronic or aggressive, dependent upon the rate of disease progression and the clinical manifestations (Armitage & Cullinan 2010). Whilst both disease states rely on the presence of a bacterial biofilm and an altered host response, aggressive periodontitis onset occurs at a younger age and progresses at a much faster rate; with periodontal attachment loss estimated to be three to four times faster than that of chronic periodontitis (Baer 1971).

1.2 The role of bacteria in periodontitis

Bacteria initiate and aid periodontitis progression, however tissue destruction also relies on the interactions with the host. There are estimated to be approximately 1200 predominant oral phylotypes, based on the analysis of 16S rRNA gene sequences from 400 periodontitis patients (Paster & Dewhirst 2000). Cluster and ordination analysis by Socransky *et al.,* (1998) allowed for the identification of bacterial complexes (figure 1, Socransky *et al.,* 1998), which were classified by their relationship to the clinical manifestations of periodontitis.

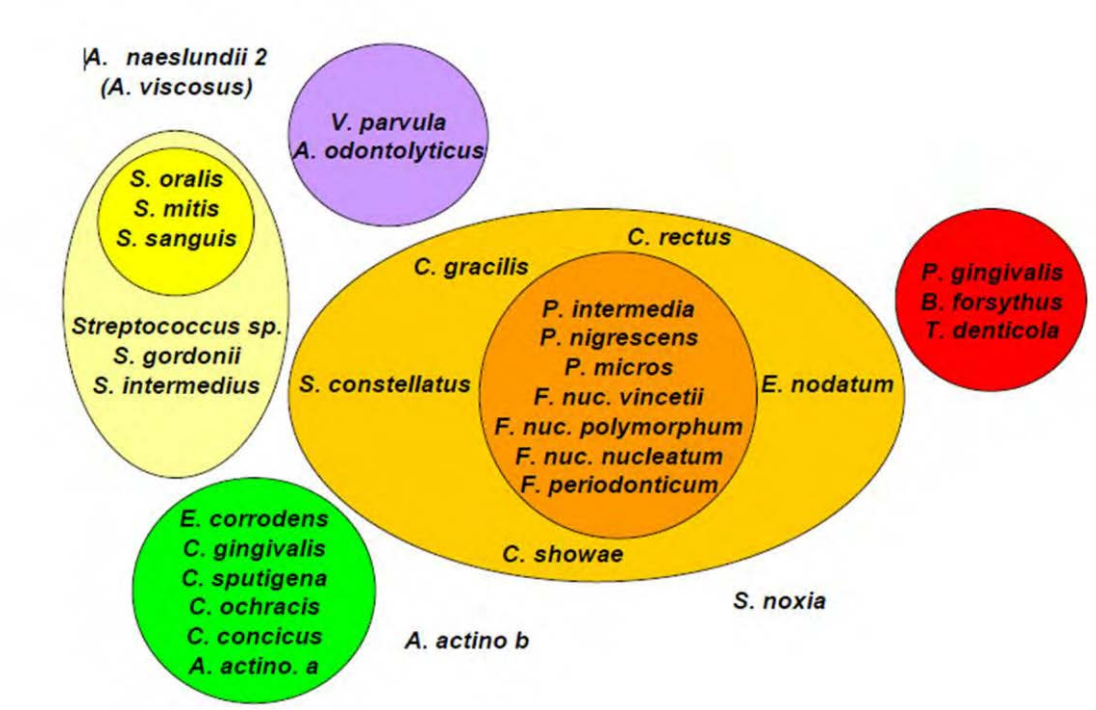

Figure 1: Diagrammatic representation summarising cluster and ordination analysis of plaque samples (periodontally healthy controls: n=25, attachment loss: n=160). The results identified 5 major complexes of bacteria in which the species interacted closely with each other. Bacteria in the red complex showed the strongest association with the clinical parameters of periodontitis, such as pocket depth and bleeding on probing. The red complex was found to be closely related to the orange complex, which could be due to the necessary colonisation of orange complex bacteria prior to the red. However the yellow and green complexes, although interactions amongst the group members, were not shown to closely associate with members of the red and orange complexes. The purple complex bacteria were strongly associated with each other, but similarly did not interact with members of other complexes. Bacteria not in a defined group did not strongly associate with each other or any of the 5 complexes. (Derived from Socransky *et al.,* 1998).

There are two main viewpoints relating to the role of the plaque microflora in periodontitis, one of which is the “specific plaque hypothesis”, which proposes that only a select few of the diverse plaque bacteria are implicated in disease development. This theory would allow for targeted treatment against selected elevated bacteria (Loesche 1979), which may be a realistic proposal in certain cases of aggressive periodontitis, where *Aggregatibacter actinomycetemcomitans* is believed to play a pivotal role (Slots 1999). A consistent flaw in this theory is that the suggested causative bacteria can also be present in healthy individuals (Socransky & Haffajee 2000). Furthermore, it is has been proposed that the bacteria isolated from diseased individuals may be opportunistic species rather than causative agents. These concerns provide support for the second theory, the “non-specific plaque hypothesis”, which suggests a threshold volume of a combination of microbes is accountable for the subsequent diseased state (Theilade 1986). Alternatively, a more recent model, which encompasses both hypotheses, is the “ecological hypothesis” (figure 2, Marsh & Divine 2011). This suggests that an increase in subgingival bacterial species triggers an inflammatory response, which in turn may initiate a shift away from microbial homeostasis and towards the growth of proteolytic and anaerobic bacterial species (Marsh & Devine 2011). These developments and the consequent expansion of gram negative anaerobes enable the progression of periodontitis.

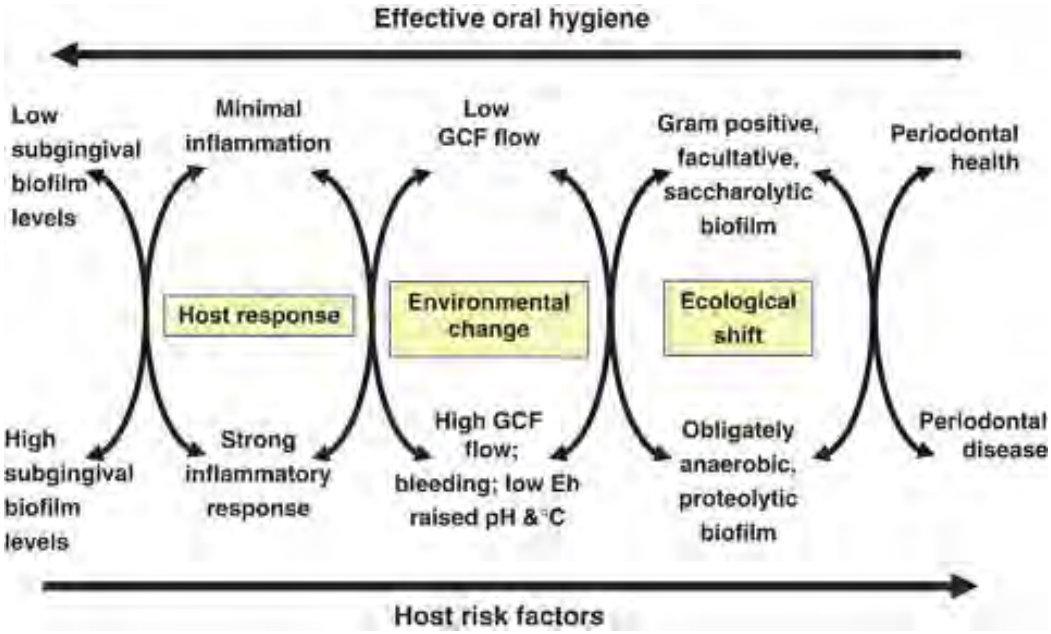

Figure 2: Ecological Plaque Hypothesis and periodontitis. This model proposes a dynamic relationship between the host oral environment and the subgingival bacteria. An increase in the number of bacteria surrounding the gingival tissue evokes a host inflammatory response, which subsequently causes a shift in the local environment (such as an increase in pH and temperature) which favours proteolytic bacteria growth. Expansion of proteolytic microbiota will drive the inflammatory response, promoting further growth of proteolytic and anaerobic bacterial species, as they are better adapted to the altered environment. This shift in environment and the subsequent bacteria expansion can pre-dispose the site to periodontitis. GCF=gingival crevicular fluid, Eh=redox potential. (Marsh & Devine 2011).

The oral environment is maintained at ~36ºC and at pH ~7, which provides favourable conditions for microorganisms to thrive and form bacterial biofilms. In order for microbial growth to prosper, bacteria must be adapted to the heterogeneous environments provided by teeth, resulting in the colonization of different habitats by certain bacteria. Factors such as competition from commensal bacteria, oxygen levels and environmental exposure all contribute to the success of microbial growth, which govern where specific bacteria can prosper (Feng & Weinberg 2006). Colonization is characterised by the formation of a biofilm, which describes the process of multiple bacterial species adhering to the tooth surface, creating a diverse bacterial community. This multi-species biofilm is not thought to

be opportunistic, but rather a sequence of events that enables the ordered adherence of each species to maximise survival (O'Toole *et al.,* 2000). Following mechanical brushing, a protein-rich substance known as the acquired pellicle is deposited onto clean teeth, which is believed to initiate the biofilm cascade by aiding the adherence of early colonisers (Clark *et al.,* 1989). Streptococci species represent 60-90% of the initial colonisers (Nyvad & Kilian 1987), which following their binding to complementary receptors in the acquired pellicle, utilise the receptor as a nutrient to aid bacterial growth (Li *et al.,* 2000). Early colonisers allow for the development of a bacterial biofilm, as secondary colonisers bind to the bacteria already adhered to the tooth surface via coadhesion and coaggregation. *Fusobacterium nucleatum* (*F. nucleatum*) is a bacterial species that is believed to play a unique role in biofilm formation, it is abundant in healthy individuals and even more so at periodontally diseased sites, however it is not generally classified as an early or late coloniser but plays a role in facilitating the binding between other bacteria. Early colonisers would not usually coaggregate with many of the late colonisers and *F. nucleatum* is believed to bridge this gap, as the majority of bacteria will interact with *F. nucleatum* (figure 3; Kolenbrander *et al.,* 2002).

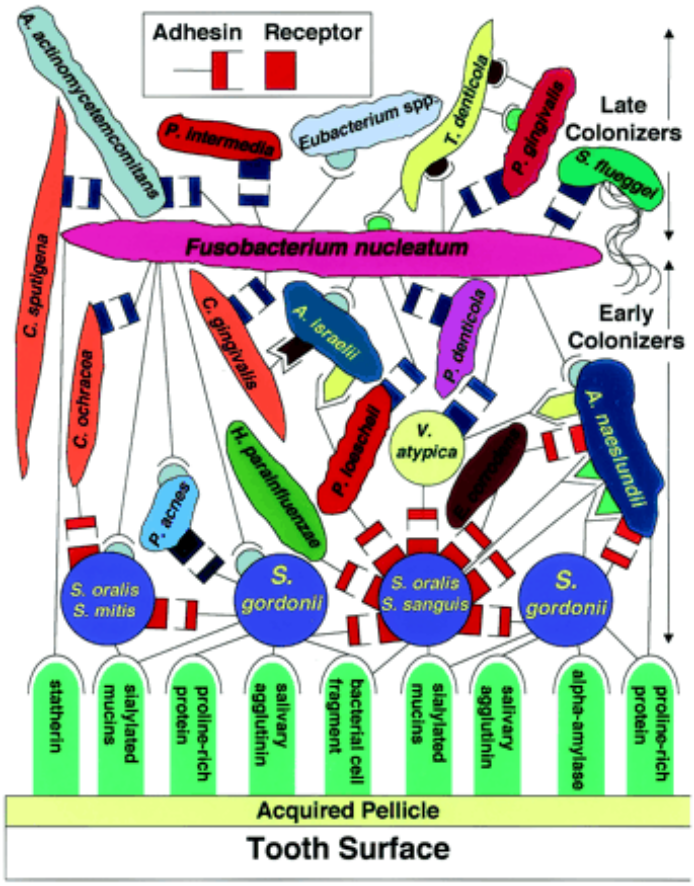

Figure 3: Model representation of complex oral bacteria colonisation. Starting at the bottom, the acquired pellicle, which contains receptors (green) is deposited onto the tooth surface. Early colonisers (blue) bind to their complementary receptors (black circular lines), followed by the adherence of secondary colonisers to the first bacteria. Sequential binding enables coaggregation between different species, which bridges the gaps between bacteria. *F. nucleatum* (pink) borders early and late colonisers, which allows for interaction between the two stages, as many late colonisers would not interact directly with early colonisers. Coagregation is represented by complementary shapes, for example the top left depicts adhesion (red and white, with stem) and it's receptor (red, no stem) (Kolenbrander *et al.,* 2002).

1.3 Neutrophil activation and function

Neutrophilic polymorphonuclear leukocytes (PMNLs) are the most abundant leukocytes and critical effector cells in the innate immune response. Subsequent to their maturation in the bone marrow, fully and terminally differentiated neutrophils are released into the blood stream, where they have recently been shown to survive for approximately 5.4 days within the human circulation (Pillay *et al.,* 2010). During this

period neutrophils undergo apoptosis and are cleared by macrophages, however if an infection arises neutrophils are typically the first immune cell to be recruited to the diseased site (Amulic *et al.,* 2012). Until recently neutrophils were thought to employ only two antimicrobial mechanisms; namely phagocytosis and degranulation (Tauber 2003, Tan & Dee 2009), however a new antimicrobial mechanism was discovered, which described the ability of activated neutrophils to extrude their nuclear chromatin as a complex mix of DNA, histones and antimicrobial peptides (AMPs) (Brinkmann 2004). This process is believed to have the purpose of immobilising and killing bacteria extracellularly and provides a further mechanism of neutrophil-mediated pathogen killing, known as neutrophil extracellular traps (NETs) (figure 4, Brinkmann and Zychlinsky 2007). NET formation is dependent upon nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which is required for the generation of reactive oxygen species (ROS) and facilitates NETosis, a novel form of "programmed cell death" distinct from apoptosis and necrosis (Fuchs *et al.,* 2007).

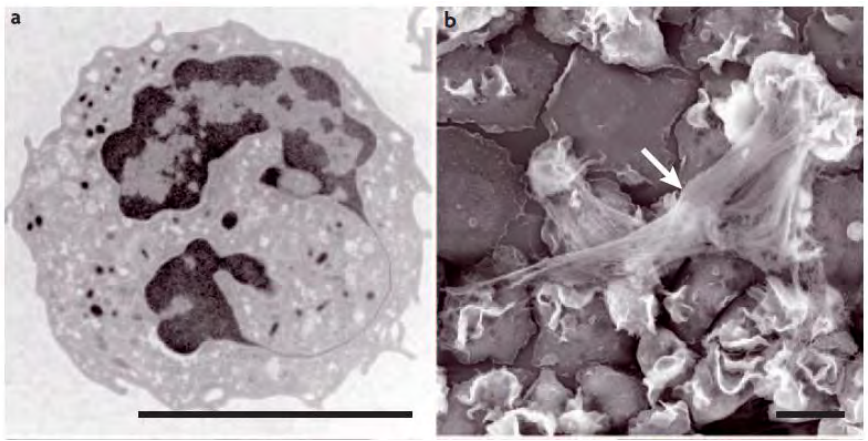

Figure 4: (a) Transmission electron micrograph (TEM) of an unstimulated human neutrophil and (b) scanning electron micrograph (SEM) of neutrophils stimulated for NET production. White arrow identifies NETs, scale bar represents 5μm (a) and 10μm (b). (Brinkmann and Zychlinsky 2007).

1.4 The role of NETs in periodontitis

The initiation and progression of periodontitis relies on the microorganisms of the subgingival crevice, which similar to other infections, evokes an immune response. The host releases an increased amount of gingival crevicular fluid from the gingival tissues that contains neutrophils and these represents the first line of defence (Goodson 2003). Neutrophil defence was initially believed to be entirely by phagocytosis (Kinane *et al.,* 2003), however considering that neutrophil-bacterial interactions also take place outside of the gingival tissue, extracellular defence mechanisms may be utilised. Indeed, Vitkov *et al.,* (2009) discovered that NETs were abundant on the gingival pocket epithelium and proposed that during bacteria's attempt to migrate and adhere to the gingival surface, they encounter NETs which shield the epithelium and prevent adhesion (figure 5, Vitkov *et al.,* 2009). Notably, NETs were also identified in the purulent crevicular exudate, suggesting NETs are combined with immobilised bacteria and removed via the exudate outflow. Interestingly the exudate bacterial counts were relatively inconsistent, which led to the theory that if phagocytosis is overwhelmed, for example due to large quantities of bacteria attempting to colonise, NETs can compensate for the increased demand on the immune system. Subsequently, Vitkov *et al.,* (2010) demonstrated that NETs are also in abundance in the gingival crevicular fluid (GCF), which they identified by the presence of citrullinated proteins. Histone citrullination by peptidylarginine deiminase 4 (PAD4), an enzyme which converts arginine and methylarginine residues to citrulline, is essential for chromatin decondensation and extracellular NET formation (Wang *et al.,* 2009). Further observations, such as the lack of neutrophils containing engulfed bacteria and the large quantities of NETs in the GCF suggest NETosis is the predominant defence mechanism employed by neutrophils in the crevicular fluid at periodontally diseased sites (Vitkov *et al.,* 2010).

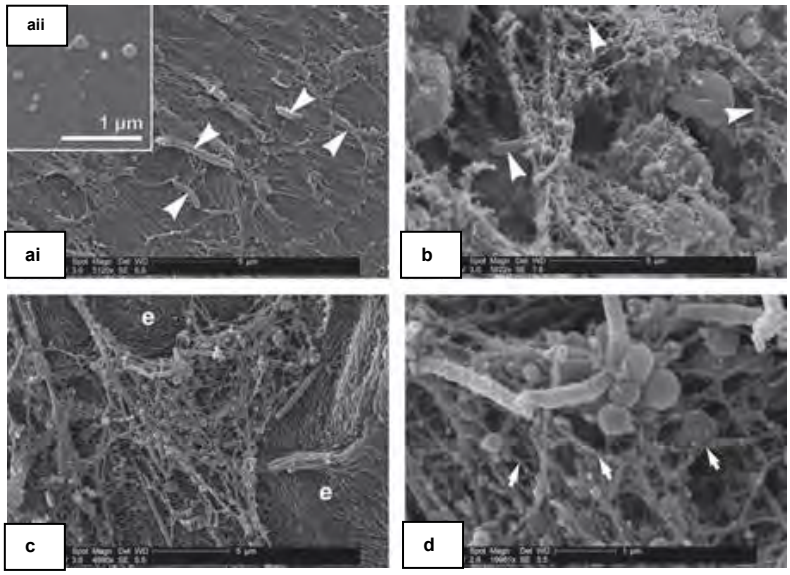

Figure 5: SEM of NETs entrapping bacteria in the crevicular exudate and pocket epithelium. (ai) Crevicular exudate containing bacteria (white arrowheads), whilst (aii), which has received DNase treatment (degrades NETs) shows no entrapped bacteria. (b) Crevicular exudate with several large NETs immobilising bacteria (white arrowheads). (c) Pocket epithelium showing entrapped bacteria, "e" represents the epithelium surface. (d) Enlarged version of c, showing NET fibers (white arrows). Scale bar represents 5µm (a1, b, c), 1µm (a2, d). Vitkov *et al.,* 2009.

1.5 The relationship between DNase and NETs

DNA represents the major constituent of NETs, and this serves as a backbone to accommodate the histones and proteases that are embedded within the NET-DNA. This was established by Brinkmann *et al.,* (2004) by observations that deoxyribosenuclease (DNase) treatment led to NET destruction. This has important implications regarding recent claims that certain bacteria produce extracellular DNase, with the believed purpose of evading NET entrapment and thus enhancing disease virulence. Buchanan *et al.,* (2006) investigated the production of DNase (Sda1) by Group A Streptococcus (GAS), which is associated with infections such as necrotizing soft tissue infections, and discovered that DNase was indeed capable of degrading NETs. Further research in necrotizing infection mouse models revealed that Sda1 mutant DNase is correlated with attenuated disease virulence, as a result

of uninhibited NET mediated defence, compared with wild-type Sda1 which degraded NETs. Paradoxically, a recent periodontitis study examined saliva samples with real-time polymerase chain reaction (PCR) and discovered *Streptococcus mutans* (*S. mutans*) presence was significantly higher in periodontally healthy individuals compared with periodontally diseased saliva samples (Iwano *et al.,* 2010). *S. mutans* is an early coloniser and has been shown to release DNase in bacterial pellet gel based assays (Palmer *et al.,* 2012); it would be interesting to speculate that degradation of NETs by *S. mutans* occurs at periodontally healthy sites, whereas if NETs are not inhibited periodontitis may progress. Alternatively, Iwano's findings (2010) that *S. mutans* is not present at periodontally diseased sites may be due to competition between the commensal bacteria and other periodontopathogenic bacteria, such as *Porphyromonas gingivalis* (*P. gingivalis*). Interestingly, it was recently discovered that *P. gingivalis*, *T. forsythensis* and *A. actinomycetemcomitans,* the three most pathogenic periodontal bacteria, were not found to produce DNase; suggesting NETs may not be aiding the host response (Palmer *et al.,* 2012). Currently it is unclear as to whether the release and accumulation of large numbers of NETs are a help or a hindrance in the host response to periodontal bacterial species.

1.6 Aims

The extent to which NETs are involved in periodontitis is not yet fully understood and specifically their interaction with periodontopathogenic bacteria and whether they are protective or destructive at periodontal tissues. Furthermore, the release of DNase by selected periodontal bacteria with the purpose of evading NET entrapment has not been fully investigated. This project aimed to investigate the association between NETs and oral species implicated in periodontitis, in particular whether NETs exert antimicrobial activity on periodontal bacteria. To address this aim, periodontal bacteria were cultured and their growth patterns and minimum inhibitory concentration (MIC) determined. NETs were produced by hypochlorous acid (HOCl)-stimulated neutrophils (Palmer *et al.,* 2012) and there antimicrobial activity assayed by optical density (turbidity) following 24h incubation with the bacteria. Preliminary work also aimed to establish the optimal conditions for bacterial growth, NET quantification and incubation conditions. Additionally, this project also considered neutrophil survival and NET storage for future experimentation.

Chapter 2: Materials and Methods

2.1 Volunteers

Blood was collected in 6ml lithium heparin anticoagulant vacutainers (Greiner, Bio-One) from males volunteers aged between 20-30 years, non-smokers, periodontally healthy and not currently taking anti-inflammatory or anti-microbial drugs. Volunteers were recruited from Birmingham Dental School and Hospital and samples were collected under ethical approval number 10/H1208/48 and donors gave their informed consent.

2.2.1 Percoll neutrophil isolation protocol

Neutrophils were isolated using Percoll at densities 1.079 and 1.098 g/ml. 8ml of 1.079 density Percoll were carefully layered over 1.098 in a 25ml tube (Fisher Scientific) and 6ml of blood were layered over the gradients using a Pasteur pipette. This was centrifuged (Hettich Universal 320R) for 8min at 150 relative centrifugal force (rcf), followed by 10min at 1200rcf. The neutrophil layer was aspirated off and added to 30ml lysis buffer (2.2.2) in a 50ml centrifuge tube (Fisher Scientific), which was then topped up to 50ml with lysis buffer. Following a gentle inversion, the centrifuge tube was incubated for 15min at room temperature to lyse any unwanted remaining erythrocytes. Next, the tube was centrifuged for 6min at 500rcf to pellet the neutrophils. The supernatant was aspirated off, whilst the pellet was re-suspended in 2ml of lysis buffer and incubated for 5min at room temperature, prior to centrifugation for 6min at 500rcf. The supernatant was again aspirated off and the neutrophil pellet washed in 2ml of PBS (2.2.3), followed by re-centrifugation (6min at 500rcf). The supernatant was discarded and the cells again re-suspended in 2ml of PBS (2.2.3) and counted with a haemocytometer (Naubauer, Reichart) and a light

microscope (Leitz Laborlux s). Cell viability was confirmed by trypan blue exclusion in a 1:1 dilution.

2.2.2 Lysis Buffer (1 litre):

8.3g NH_4Cl (A9434 Sigma), 1g $KHCO_3$ (Sigma P9144), 0.04g Na_2 EDTA $2H_2O$ (E5134 Sigma) and 2.5g bovine serum albumin (BSA; Sigma A4530) were added to 1 litre of sterile, endotoxin-free water (Versol) and stored at 4°C.

2.2.3 Phosphate Buffered Saline (1 litre):

7.75g NaCl (Sigma S9625), 0.2g KH_2PO_4 (Sigma P5379) and 1.5g K_2HPO_4 (Sigma P8281) were added to 1 litre of sterile, endotoxin-free water (Versol) and stored at 4°C.

2.3.1 NET Quantification protocol

A 96-well plate was blocked by adding 200µl syringe-filtered 1% BSA (2.3.2) and stored at 4°C overnight. The fluid was removed from the plate using a vacuum pump, followed by the addition of 10^5 neutrophils in 100µl RPMI (L0500 Biosera supplemented with 0.5% glutamine) and 75µl additional RPMI. After a 30min baseline incubation period (37°C, 5% CO_2), selected wells were stimulated with 25µl of 0.75mM HOCl (Palmer *et al.,* 2012) and incubated for 3h (37°C, 5% CO_2). Post-incubation, 15µl of 14.3units/ml MNase were diluted in PBS (2.2.3), added to each well and incubated at room temperature for 10min, prior to centrifugation (Hettich Universal 320R, 10min at 1800rcf). Next, 150µl of the supernatant were transferred to a sterile black 96-well plate (Appleton Woods), with the addition of 15µl of 10µM Sytox green (Invitrogen S7020) diluted 1/500 in RPMI medium (L0500 Biosera

supplemented with 0.5% glutamine), to quantify any free DNA within the supernatant. Fluorescence was read in arbitrary fluorescent units (AFU) by a fluorometer (Twinkle LB970, Berthold Technologies; Fluororepeated [x10], excitation filter F485, emission filter F535, lamp energy 2000) 10 times at 37°C. To investigate the process of NET quantification and the effect of MNase NET digestion and centrifugation upon the number of NETs quantified in the supernatant, further experiments were conducted with and without MNase and centrifugation and the NETs quantified as standard with Sytox green (Invitrogen S7020).

2.3.2 Blocking Buffer (1 litre):

1% BSA in PBS was produced by adding 10g BSA (Sigma A4503) to 1 litre PBS, which was aliquoted and stored at -20°C prior to use.

2.3.3 NET quantification plates

10µl of known concentrations of Calf Thymus DNA (Sigma 89370), ranging from 1.0 to 0.007µg (diluted in molecular biology quality water), were incubated with 175µl RPMI (L0500 Biosera supplemented with 0.5% glutamine) and quantified with 15µl of 10µM Sytox green (Invitrogen S7020) by a fluorometer (as described previously 2.3.1) to compare the influence of plate colour. The quantification of known DNA concentrations on the black plates was also used as a calibration curve, to be employed to determine the percentage of neutrophils producing NETs.

2.4 Long-term incubation of neutrophils

Being a terminally differentiated cell type with a short life expectancy *in vitro*, neutrophils are usually isolated from whole blood on the day of experiment and used immediately. To establish neutrophil *ex vivo* lifespan neutrophils were isolated and re-suspended in 5ml PBS (2.2.3) in a 50ml tube (Fisher Scientific), this was stored in the fridge (4°C) and cell viability determined every 24h with trypan blue dye exclusion in a 1:1 dilution using a haemocytometer (Naubauer, Reichart) and a light microscope (Leitz Laborlux s).

2.5 Storage of NETs

NETs were produced and selected wells quantified as described previously (2.3.1) and then discarded. Additional rows of control and HOCl-stimulated neutrophils were not quantified, but after the MNase digestion and centrifugation, were aliquoted and either refrigerated (4°C) or frozen: freezing conditions included -20°C, -80°C, slow freezing (Thermo Scientific Nalgene, -1°C/minute) or snap freezing with liquid nitrogen. After 24h, aliquots were removed from the fridge or defrosted at room temperature and quantified with MNase and Sytox green as described previously (2.3.1). Storage conditions were evaluated by comparing the quantity of NETs prior to storage to the number of NETs post 24h incubation.

2.6.1 Culture of *Fusobacterium nucleatum polymorphum* ATCC 10953 (*F. nucleatum)*

F. nucleatum (isolated from the inflamed gingiva of an adult male) was cultured by inoculating 6ml of brain heart infusion broth (10% serum, Oxoid BO0129E) with freeze dried bacteria. The *F. nucleatum* suspension was plated onto two blood agar plates (Base 2 with horse blood, Oxoid PB0114) using a flame-sterilised loop.

The inoculated broth and plates were incubated for 48h in an anaerobic chamber (9.97 CO_2 9.92% hydrogen 37°C Don Whitley Scientific, Modular Atmosphere Controlled System, CAL-3200, Shipley, UK). Additionally a third inoculated plate was incubated in aerobic conditions (37°C, 5% CO_2), to check for contamination. A gram stain was performed to indicate bacterial species, described below (2.6.4).

2.6.2 Culture of *Streptococcus intermedius* ATCC 27336 (*S. intermedius*) and *Streptococcus oralis* ATCC 35037 (*S. oralis*)

S. intermedius and *S. oralis* (kindly provided by Dr Mike Milward) were defrosted at room temperature and cultured by inoculating 10ml tryptone soya broth (OXOID) with 20µl of the bacteria suspension. Two blood agar plates (Base 2 with horse blood, OXOID PB0114) were also inoculated using a flame-sterilised loop. The inoculated broth and plates were incubated in a candle jar for 48h in an aerobic chamber (37°C, 5% CO_2).

2.6.3 Bacterial growth curves

Fresh broth was inoculated with bacteria; brain heart infusion broth (10% serum, Oxoid BO0129E) for *F. nucleatum* and tryptone soya broth (Oxoid) for *S. intermedius* and *S. oralis*. The OD of the suspension was measured by spectrophotometry at 570nm every hour to determine bacteria growth. Between measurements bacteria were incubated in the appropriate conditions; *F. nucleatum* in the anaerobic chamber (9.97 CO_2 9.92% hydrogen 37°C Don Whitley Scientific, Modular Atmosphere Controlled System, CAL-3200, Shipley, UK) and both *S. oralis* and *S. intermedius* in a candle jar in the aerobic chamber (37°C, 5% CO_2).

2.6.4 Gram Stain protocol

Gram staining differentiates Gram positive and Gram negative bacteria by detecting peptidoglycan, which whilst present as a thick outer layer in Gram positive cell walls, is thinner and surrounded by an outer lipid membrane in Gram negative species. These characteristic cell wall properties enable species distinction as Gram positive bacteria stain purple and Gram negative stain pink (McClelland 2001). Drops of saline were deposited onto a microscope slide, prior to the addition of a bacterial colony, forming an emulsion, which was then passed through a flame to fix. Crystal violet was added to the slide for 30sec, rinsed with water, followed by the addition of Lugol iodine (Sigma L6146) for a further 30sec and rinsed again. Acetone was then added and immediately rinsed with water; finally the slide was flooded with carbol fuchsin for 30sec and rinsed with water. The slide was blotted dry and visualised under oil immersion under a microscope at x100 magnification (Leitz Dialux 22) and digital images captured (Nikon Coolpix 1990).

2.6.5 Penicillin-Streptomycin (Sigma P0781) (Pen-strep)

Pen-strep was incubated with bacteria to determine the minimum inhibitory concentration (MIC) needed to inhibit bacterial growth. This was achieved by preparing a serial dilution of the penicillin-streptomycin antibiotic (pen-strep) ranging from 8µg/ml to 0.01µg/ml (diluted in PBS [2.2.3]) and incubating with 10^2 bacteria per ml in a 96-well clear plate (SARSTEDT) for 24h. Bacterial growth was assessed by turbidity using a microplate reader (BIO-TEK Elx800), whereby absorbance was read immediately after the addition of pen-strep and 24h later at 570nm. Bacterial growth was determined by subtracting the initial turbidity reading from the 24h measurements.

2.7 Bacteria and NET incubation assay

Following neutrophil isolation and stimulation for NETs with HOCl, the 96-well plate was treated with 15µl of 14.3units/ml MNase diluted in PBS (2.2.3) and incubated at room temperature for 10min. This was followed by centrifugation (10min at 1800rcf, Hettich Universal 320R), to separate neutrophils from the NETs; leaving NETs free in the supernatant. Selected wells containing NETs were quantified as described previously (2.3.1), however NETs to be incubated with bacteria were not, as Sytox green supplementation may have influenced bacteria growth. Post centrifugation, 150µl of the supernatant were transferred to a sterile clear 96-well plate (SARSTEDT), followed by the addition of 100µl bacteria to selected wells. Bacteria used in the assays were freshly cultured for 4h prior and used at 10^2 cells per ml, determined by OD measurements (turbidity) at 570nm and diluted in broth accordingly. Each well was thoroughly mixed by pipetting and the plate incubated in the appropriate chamber for 24h. Incubation conditions included bacteria incubated alone, which was made up to the correct volume with the neutrophil incubation medium (RPMI, L0500 Biosera supplemented with 0.5% glutamine). To act as a negative control, selected wells contained bacteria incubated with the supernatant derived from control neutrophils (neutrophils which did not receive HOCl). Finally, selected wells contained bacteria incubated with the supernatant of HOCl-stimulated neutrophils (NETs present). Turbidity measurements with a microplate reader (BIO-TEK Elx800) were used to determine changes in bacterial number; measurements were taken immediately after bacteria were mixed with the neutrophil supernatant and again post 24h incubation. Bacterial growth was identified by subtracting the initial reading from the measurements post 24h incubation. Bacterial growth inhibition was assigned to NET-mediated killing by comparing the growth of bacteria

incubated with the supernatant of unstimulated neutrophils (no NETs) with bacteria incubated with the supernatant of HOCl-stimulated neutrophils (NETs present).

Chapter 3: Results

All statistical analyses were performed using the InStat3 statistical software package (Graphpad Instat 3). Statistical tests employed for the purpose of this study were at a significance of 0.05, parametric and paired when appropriate. A 1-way ANOVA was used to analyse NET quantification methods, followed by a Tukey test to determine where the significance was. A 1-way ANOVA was employed to examine the NET storage data and paired t-tests to analyse the influence of NETs on bacterial growth.

3.1 Neutrophil *ex vivo* viability

To determine neutrophil *ex vivo* survival post isolation, cells were re-suspended in 3ml PBS and refrigerated (4°C). Cell viability was determined every 24h using trypan blue exclusion staining which revealed that cell viability remained high for the first 48h, following which a marked reduction was noted. At approximately 64h, the number of trypan positive cells (non-viable) overtook the number of trypan negative cells (viable), indicating >50% cell death (figure 6, n=1).

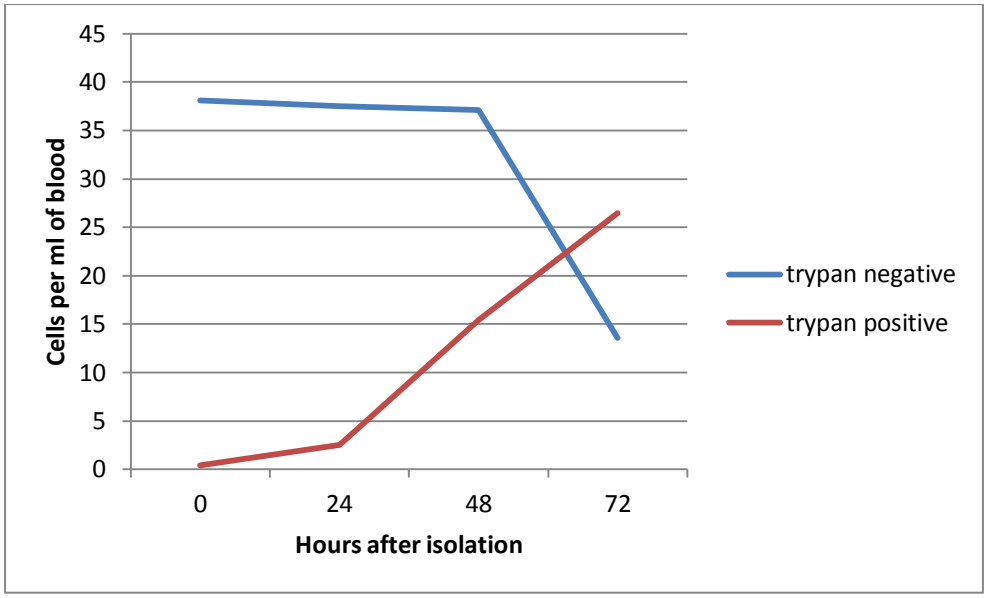

Figure 6: Trypan blue counts performed at 24h intervals to determine neutrophil *ex vivo* survival. Trypan negative (blue) = viable, trypan positive (red) = non-viable. n=1.

3.2.1 Effect of multi-well plate colour upon fluorescence

Previous work has quantified NETs using white microwell plates by measuring fluorescence, however many other studies employ black plates, which is believed to reduce background fluorescence. To compare the effect of plate colour (white or black) on fluorescence detected, known amounts of DNA were employed, with the plate colour representing the only variable. Results (figure 7, n=1 in triplicates) showed that the AFU for the amount of DNA quantified in white plates gave on average a 96% higher readout than when quantified in black plates. Notably an empty white well without Sytox green produced a background reading of approximately 6000 AFU, compared with 10 AFU for black wells (data not shown), confirming that black plates generate less background signal.

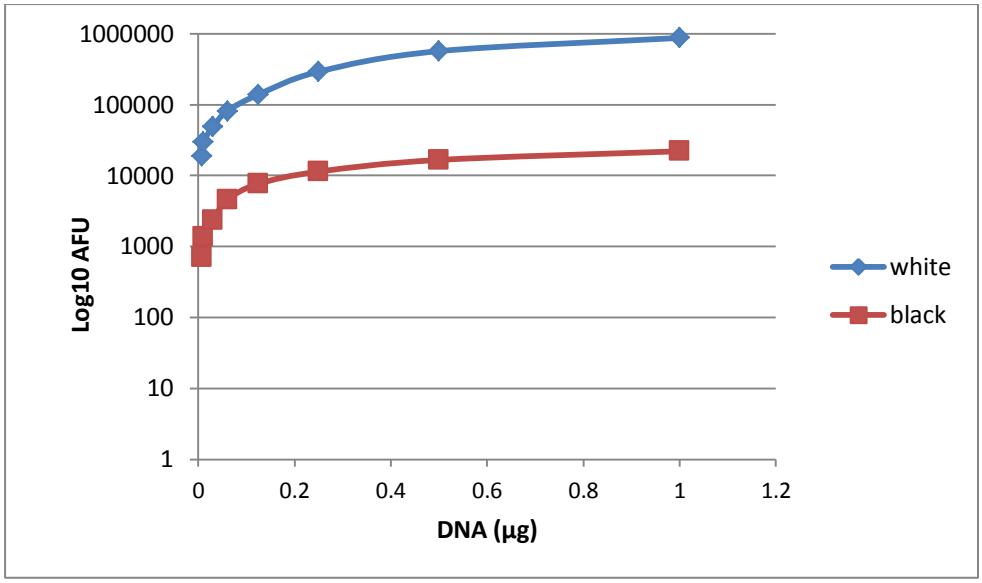

Figure 7: Known concentrations (µg) of Calf Thymus DNA quantified in black and white plates. AFU values were significantly higher when quantified in white plates. Fluorescence measured in arbitrary fluorescent units (AFU). (n=1, in triplicates).

3.2.2 NET quantification Calibration curve

Quantification of known DNA concentrations was used to generate a calibration curve, which was used to ascertain the percentage of neutrophils producing NETs. The average number of NETs produced by HOCl-stimulated neutrophils, based on quantification with Sytox green, equates to a signal of 12000 AFU. According to the standard curve (figure 8, n=1), this corresponds with approximately 0.25µg of DNA, which is the average amount of NET-DNA produced. The literature indicates each human cell contains around 5 picograms (pg) DNA (Mee & Adelstein 1978), which when adjusted for 10^5 neutrophils which were added to each well, provides a total DNA content of 0.5µg. This suggests that 50% (0.25µg/0.5µg*100) of the neutrophils in each well are producing NETs when stimulated with HOCl.

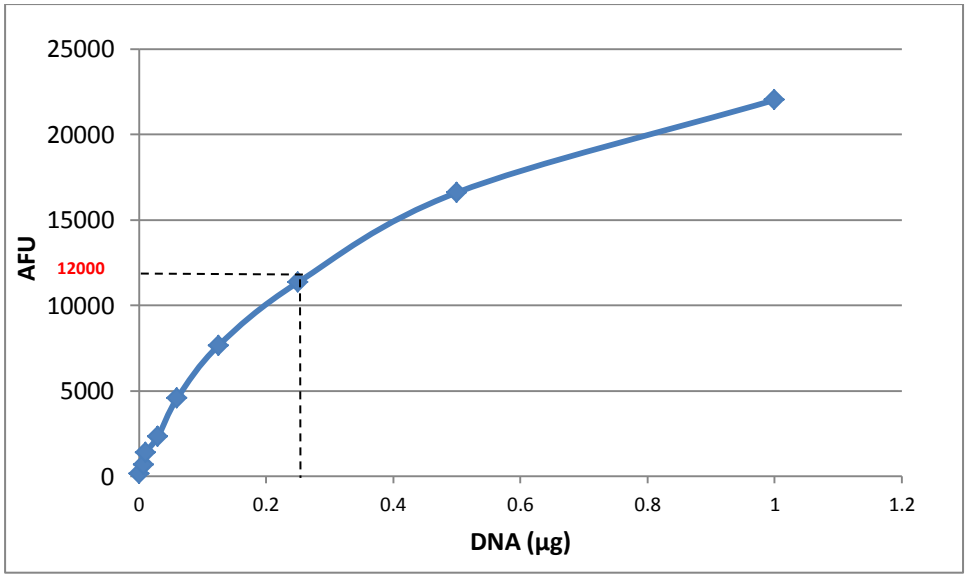

Figure 8: Known concentrations (µg) of Calf Thymus DNA quantified in black plates to produce a calibration curve. Average number of NETs produced by HOCl-stimulated neutrophils was 12000 AFU, which corresponds to 0.25µg DNA. Fluorescence measured in arbitrary fluorescent units (AFU). (n=1 in triplicates).

3.2.3 NET quantification evaluation

Experiments were performed with and without MNase and centrifugation and the NETs quantified as standard with Sytox green. Measurements revealed (figure 9, n=5 in quadruplicates) that both MNase digestion with centrifugation are required to quantify the optimal amount of NETs released, compared with the 3 alternative methods; MNase with no centrifugation, no MNase with centrifugation or no MNase with no centrifugation. Quantification without MNase and with centrifugation produced the fewest number of measureable NETs. A one way ANOVA revealed that the different methods to quantify NETs were indeed significant ($p=0.0365$), with a Tukey test identifying the significant difference to lie between "MNase with centrifugation" (standard quantification method) and "no MNase with centrifugation" ($q=4.24$ therefore $p=<0.05$) (columns A and C, n=5 in quadruplicates, figure 9).

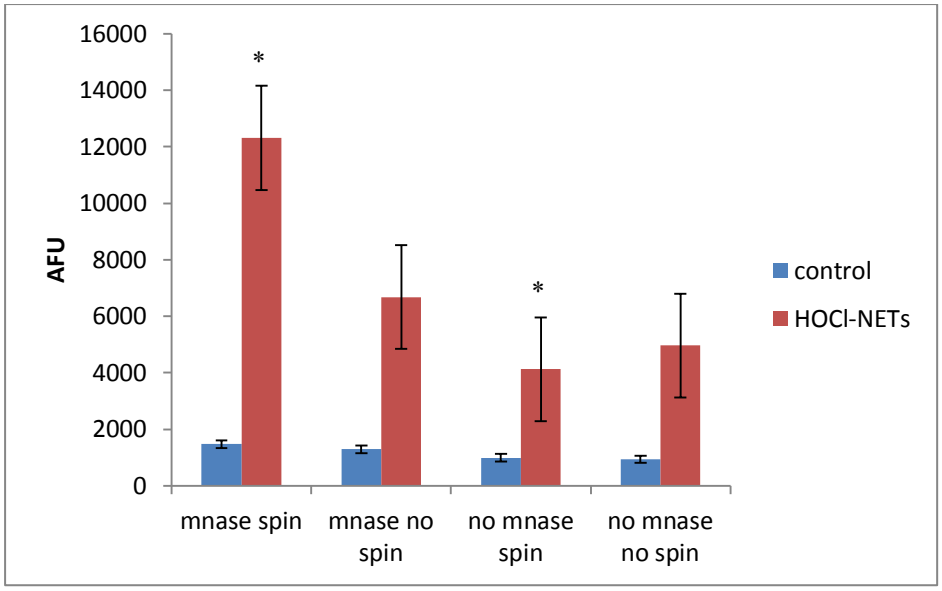

Figure 9: The effect of MNase and centrifugation upon NET quantification. Quantification was highest following MNase digestion and centrifugation. There was a significant difference in NET quantity between "MNase with centrifugation" and "no MNase with centrifugation" (Tukey q=4.24 therefore p=<0.05) Fluorescence measured in arbitrary fluorescent units (AFU). (Data plotted as mean ± SEM, n=5 in quadruplicates).

3.3 Storage of NETs

To potentially provide a stock source of NETs, which can be utilised for bacteria incubation assays, NETs were initially quantified and subsequently stored at a range of temperatures and conditions. Quantification results (figure 10, n=5 in quadruplicates) revealed that storage for 24h increased the quantity of DNA measured and snap freezing with liquid nitrogen was most representative of the fresh quantification results. However a one way ANOVA showed the differences between storage conditions were not significant for the supernatant derived from HOCl-stimulated or control neutrophils (p=0.988 and p=0.0627 respectively). Notably control neutrophils, which received no HOCl stimulation, were also found have increased DNA measurements following storage; however this increase produced AFU readings still considerably lower than HOCL-stimulated neutrophils.

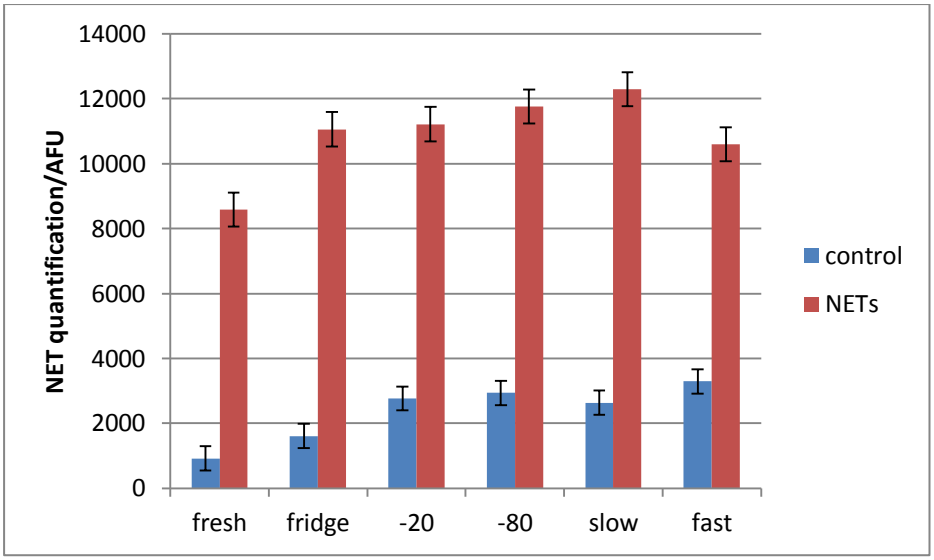

Figure 10: NET quantification following 24h incubation in different conditions: fridge (4ºC), -20ºC, -80ºC, slow freezing (1 degree/minute) or fast freezing (liquid nitrogen). Results show an increase in DNA quantification following storage and liquid nitrogen freezing producing the smallest change, compared with the fresh HOCl-stimulated neutrophils measurements, however this was not significant (p=0.988 ANOVA) (data plotted as mean ± SEM, n=5 in quadruplicates).

3.4.1 Gram stains

Gram stains were performed to indicate that the bacteria culture was likely to be the correct species. Microscopy analysis also indicated whether the cultures were consistent with the specie/sub-specie morphology. Figure 11.

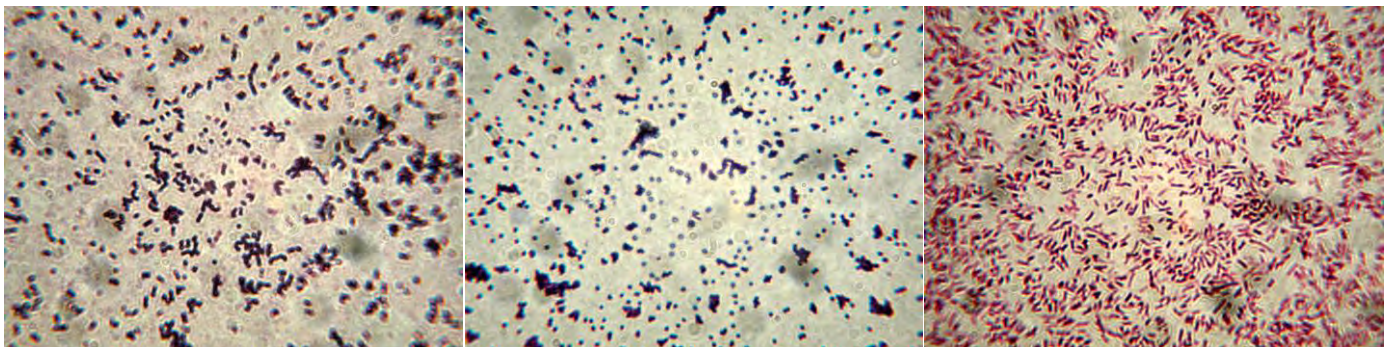

Figure 11: Gram stains demonstrated the colony growth of *S. intermedius* and *S. oralis* as gram positive cocci species (blue), whilst *F. nucleatum* is a gram negative rod species (pink). Gram stain slides were captured by visualising under oil immersion on a microscope (Leitz Dialux 22) and digital images were taken (Nikon Coolpix 1990, x100 magnification).

3.4.2 Bacteria growth curves

Fresh broth was inoculated (*F. nucleatum* in brain heart infusion broth, *S. intermedius* and *S. oralis* in tryptone soya broth) and OD measurements (570nm) were obtained every hour to produce bacteria growth curves. *S. intermedius* was found to have the shortest log phase of around 2h, after which point bacteria were rapidly reproducing, whilst *F. nucleatum* had the longest (3h). All bacteria reached their stationary phase at approximately 6h (figure 12, n=1)

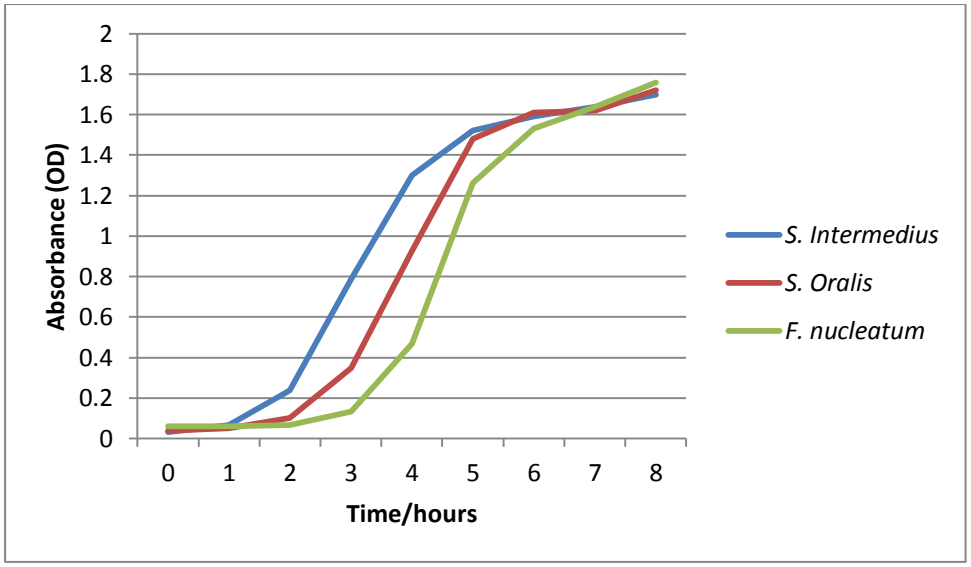

Figure 12: Growth curves of *S. intermedius, S. oralis* and *F. nucleatum.* Absorbance (570nm) readings obtained every hour post inoculation for 8h. n=1.

3.4.3 MIC of bacteria incubated with penicillin-streptomycin (pen-strep)

MIC determines the minimum concentration of pen-strep required to inhibit bacterial growth and was employed to act as a positive control for bacterial growth inhibition. The MIC of pen-strep against *S. intermedius, S. oralis* and *F. nucleatum* was determined utilising doubling dilutions. At 24h, turbidity was measured to determine growth levels and to determine the MIC. Absorbance measurements (figure 13, n=1) showed 2μg/ml pen-strep were required to inhibit the growth of *S. intermedius* and *S. oralis*, whilst inhibition of *F. nucleatum* needed 4μg/ml. All bacteria reached maximum turbidity at pen-strep concentrations of 0.25μg/ml and lower.

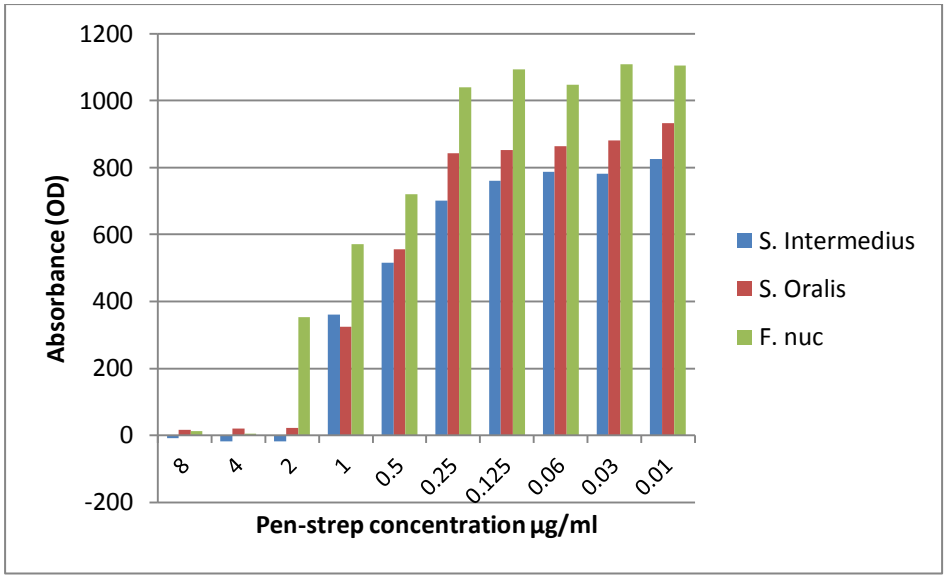

Figure 13: The minimum concentration of pen-strep required to inhibit the growth of *S. intermedius, S. oralis* and *F. nucleatum.* 2µg/ml pen-strep were required to inhibit the growth of *S. intermedius* and *S. oralis* (MIC=2µg/ml), whilst 4µg/ml were needed for *F. nucleatum* (MIC=4µg/ml). Concentrations lower than those stated (i.e. below the MIC) did not prevent bacterial growth. (n=1 in triplicates).

3.5 Bacteria and NET incubation assay

Subsequent to a 24h incubation period, turbidity was measured and initial turbidity measurements subtracted from this. Bacterial growth inhibition was assigned to NET-mediated killing by comparing the growth of bacteria incubated with the supernatant of unstimulated neutrophils (no NETs) with bacteria incubated with the supernatant of HOCl-stimulated neutrophils (NETs present). This revealed only a marginal difference, with a paired t-test confirming NETs did not significantly inhibit bacterial growth (*S. intermedius* p=0.2677, *S. oralis* p=0.3267 and *F. nucleatum* p=0.6033. n=5 in quadruplicates, figure 14). As expected, bacterial growth was highest for bacteria incubated with RPMI, with each bacteria reaching approximately 1200 OD.

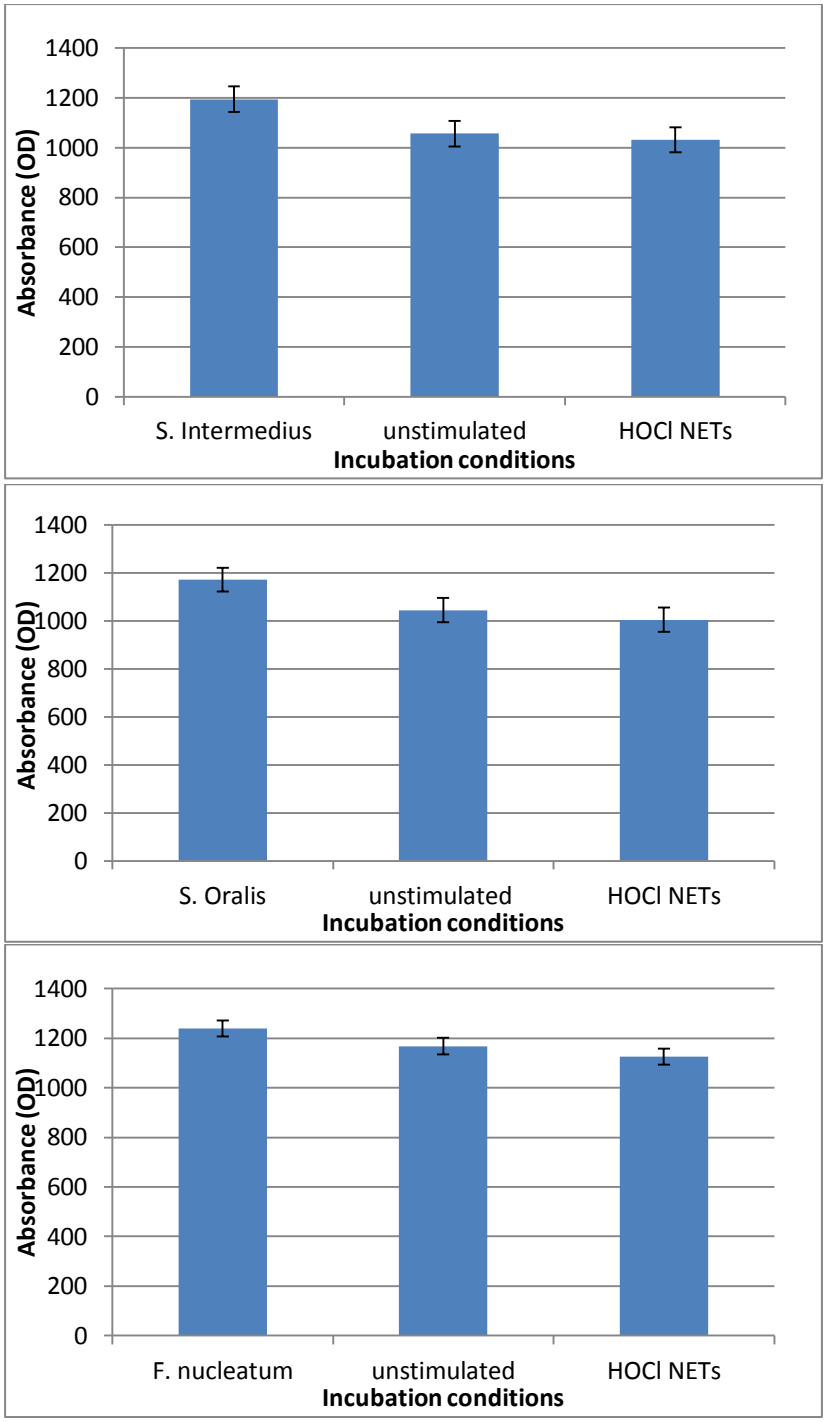

Figure 14: Turbidity readings (absorbance 570nm) of bacteria (*S. intermedius*, *S. oralis* or *F. nucleatum*) incubated with RPMI, the supernatant of control unstimulated neutrophils (no NETs) or the supernatant of HOCl-stimulated neutrophils (NETs). Results show that bacteria growth was not significantly impeded when incubated with NETs (paired t-tests: *S. intermedius* p=0.2677, *S. oralis* p=0.3267 and *F. nucleatum* p=0.6033). (Data plotted as mean ± SEM, n=5 in quadruplicates).

Chapter 4: Discussion

This project aimed to investigate the influence of NETs on the growth and survival of oral species implicated in periodontitis. Despite NETosis often being referred to as an additional means of neutrophil-mediated defence, it has been proposed that NETs can cause deleterious effects to the host (Palmer *et al.,* 2012). This may be of particular relevance in diseases such as periodontitis, which although initiated by bacterial pathogens, disease progression results primarily from the host response. Currently there is nothing in the literature investigating the responses of NETs if they encounter periodontopathogenic bacteria, specifically whether NETs are capable of immobilising bacteria and preventing further growth and/or survival. To investigate this, a method whereby NETs are co-incubated with bacteria needed to be established. Preliminary experiments considered NET survival, bacteria growth, incubation conditions and isolation of NETs from neutrophils. Thus far these preliminary data have provided a robust foundation for future study and the co-incubation assay, under the conditions applied, indicated that NETs do not prevent growth of bacteria, based on turbidity readings subsequent over a 24h incubation period.

Data indicated that under the assay conditions applied, the percentage of neutrophils estimated to produce NETs with HOCl stimulation was ~50%, based on there being 5 pg DNA per cell (Mee & Adelstein 1978) and the standard curve with known DNA concentrations being quantified by fluorescence. Pilsczek *et al.,* (2010) reported 10-15% of neutrophils produce NETs in response to *Staphylococcus aureus* (*S. aureus*) over 10min. Notably this study employed bacterial stimulants and quantified NET release from 100 neutrophils using microscopy and Sytox green. The percentage

difference in NET production between the published report and that detected here may be due to differences in the assay applied, for example a 10 minute incubation, 10^1 cells and *S. aureus* stimulation (Pilsceck *et al.,* 2012) compared with a 3h incubation period, 10^5 neutrophils and HOCl to stimulate for NETosis in the assay applied here. Indeed it has been shown that the number of neutrophils undergoing NETosis is influenced by the stimulant used, for example *F. nucleatum* was shown to stimulate production of a significantly higher number of NETs compared with *S. aureus* stimulation (reported in project 1, Phillipa Harris). Also HOCl is the principal trigger for NETosis (Palmer *et al.,* 2012), thus HOCl-stimulation of neutrophils is likely to release maximal NET-DNA Nonetheless, it may also be worthwhile in examining the number of neutrophils undergoing NETosis by fluorescence microscopy to support the percentage reached by the data here. A further concern regarding NET quantification is the effect of plate colour, with white plates producing 96% more fluorescence. Although NET quantification is relative between unstimulated and stimulated neutrophils, it is misleading when interpreting the absolute values. Based on these results and also the plates used in the literature (Bruns *et al.,* 2010), all further fluorometric NET quantification will employ black wells to reduce background fluorescence.

A further consideration is whether NETs can be stored post-isolation and latterly retrieved for assay, as this would better optimise time previously spent collecting blood, isolating neutrophils and stimulating for NETs. Quantification of NETs post storage actually increased, with liquid nitrogen freezing of HOCl-induced NETs producing the smallest increase in the quantity of NETs, compared with the fresh measurements, however this was not significant. Few reported studies have

indicated whether NETs can be stored, however Fuchs *et al.,* (2007) did report refrigeration (4ºC) storage of NETs, however they do not state the time period the NETs were stored for prior to use. The increase in DNA quantification compared with the fresh measurements was also observed in control neutrophils (supernatant derived from neutrophils which received no HOCl treatment). This increase in NETs detected for both stimulated and control neutrophils may be due to neutrophil necrosis, despite MNase treatment and centrifugation aiming to separate NETs from the neutrophils, there may still be some cells present within the supernatant. Freeze-thawing can reportedly cause the unprogrammed cell death of neutrophils, termed necrosis (Freire-de-Lima *et al.,* 2000, Kandil *et al.,* 2005). Notably, necrosing cells communicate with surrounding cells by releasing a necrotic signal, which is believed to be the cellular chromatin (Bianchi & Manfredi 2004). This would likely impact on the NET quantification reported here, as DNA released from the cell in necrosis will be stained, quantified and assumed to be the result of NETosis. In addition, it is also worth considering that when refrigerated/defrosted NETs are incubated with bacteria, other products released due to necrosis may also affect bacterial growth.

Neutrophil viability, determined by trypan blue exclusion, was found to be relatively stable up to 48h post-isolation (4ºC), after which survival rapidly decreased. Indeed the number of trypan blue positive cells (non-viable) equalled those of trypan negative cells (viable) at ~64h. Neutrophil *ex vivo* lifespan reported here is considerably higher than findings reported in the literature, for example Khwaja & Tatton (1999) reported that subsequent to the incubation of 10^6 neutrophils in RPMI, only 57% ± 4% neutrophils survived after 24h (Khawaja & Tatton 1999). However

these findings were based on annexin V binding and flow cytometry with slightly different incubation conditions, and of particular interest is that the neutrophils were isolated with Histopaque, which has been found to activate cells more than Percoll during the isolation process (please see project 1, Phillipa Harris). Combined these factors may significantly influence neutrophil survival.

The incubation of NETs with *S. intermedius, S. oralis* or *F. nucleatum* did not appear to impede bacterial growth or survival, based on the turbidity measurements 24h post co-incubation. It is difficult to ascertain whether this uninhibited growth is a result of NETs not being capable of immobilising and killing these bacterial species, or currently whether the assay applied was not sensitive enough. The three bacteria were selected because of their known characteristics, for example whilst *S. intermedius* releases DNase, *S. oralis* is not reported to (Palmer *et al.,* 2012). Considering this, co-incubation of NETs with *S. oralis* was expected to inhibit growth more so than with *S. intermedius,* as no such differences were observed it is likely the assay was not representative of the *in vivo* bacterial-NET relationship. This could be due to multiple factors, such as MNase digestion of NETs prior to co-incubation, incubation conditions, measuring growth by turbidity or the bacteria and NET concentrations (MOI).

Subsequent to HOCl-stimulation and incubation, NETs were treated with MNase to liberate NET-DNA from neutrophils. MNase cuts DNA in multiples of 200 base pairs, which is believed to be sequence dependent, for example there is a preference for multiple contiguous A-T residues. The size of the NET once extruded and the subsequent MNase cutting positions means it is very difficult to determine the actual

size of the NETs following MNase digestion. Therefore one explanation as to why the NET-bacteria incubation assay may be unrepresentative of the *in vivo* NET response is the MNase digestion, as the NETs may be too fragmented to immobilise bacteria. Preliminary work included the addition of EDTA subsequent to MNase, as this is known to halt digestion (Wang *et al.,* 2009). However later co-incubations of bacteria with just EDTA revealed EDTA has antimicrobial properties, consistent with the reports in the literature (Al-Bakri *et al.,* 2009), thus growth inhibition cannot be attributed to NETosis alone if EDTA were employed to halt MNase digestion.

To establish an assay that is representative of the *in vivo* NETs-bacteria encounter, further work needs to develop an assay without using MNase digestion, and thus an alternative means of separating NETs from the neutrophils needs to be applied. Co-incubation of neutrophils with live bacteria can trigger phagocytosis, for example Hampton *et al.,* (1994) showed incubation of neutrophils with *S. aureus* led to bacteria ingestion, which prevented further bacterial growth. Any contaminating neutrophils present alongside the NETs may therefore misrepresent the effect of NETs on bacterial survival, especially considering that only 50% neutrophils complete NETosis, the remaining neutrophils may still respond to bacterial stimulation. Keshari *et al.,* (2012) investigated the influence of NETs on bacterial growth, but rather than using MNase to liberate NETs, PMA-stimulated neutrophils were centrifuged and the supernatant carefully removed. This approach should mean NETs are still attached to the neutrophils and the NETs will precipitate with the cells, thus aspirating off the supernatant should leave behind neutrophils with NETs adhered to the well. Cytochalasin D, an actin polymerisation inhibitor, was added to prevent neutrophil phagocytosis of bacteria. Bacteria were then added to the well

and combined with the NETs by centrifugation, thus if bacteria growth/survival is impeded it should not be due to phagocytosing neutrophils and would therefore assay NET antimicrobial activity. A further mechanism worthy of consideration is that neutrophil extracellular degranulation is still likely to arise resulting in the release of neutrophil AMPs. Whilst cytochalasins inhibit phagocytosis, they do not prevent microtubule assembly and it has been reported that cytochalasin treatment increases granule transport to the plasma membrane and this may accelerate degranulation (Pryzwansky & Merricks 1998). Microtubules are key components of the cell cytoskeleton and are involved in many cellular processes, such as intracellular vesicle transport and degranulation (Mollinedo *et al.,* 1989). Neutrophil granules contain antimicrobial peptides and if co-incubated with bacteria may influence bacteria growth. Therefore it may be necessary to add other inhibitors alongside cytochalasin D, such as nocodazole which causes microtubule depolymerisation. To implement Keshari's (2012) bacteria-NET incubation assay, preliminary experiments have been conducted to establish the number of NETs which precipitate to the bottom of the well with the neutrophils following centrifugation. Figure 9 demonstrated the NET quantification in the supernatant once subjected to no MNase digestion but with centrifugation, which showed the number of NETs in the supernatant is approximately 66% less, compared with MNase treatment. This data suggests the significant decrease in supernatant NETs is indeed due to these NETs precipitating with the neutrophils to the bottom of the well upon centrifugation; thus the addition of bacteria to the well may enable a more sensitive NET-bacteria incubation assay without the need for MNase digestion.

Further explanations as to why the NET-bacteria incubation assay appeared to lack sensitivity may be due to the incubation conditions, for example the MOI and a 24h incubation period. Neutrophils were prepared at 10^5 cells per well, however considering only ~50% of the neutrophils produced NETs (according to the standard curve), the absolute number of NETs incubated with bacteria is difficult to determine. The bacteria however were used at 10^2 per ml, which was determined by optical density, as preliminary experiments revealed that even at concentrations as low as 10 per ml the bacteria reached maximum turbidity following 24h incubation (data not shown). Young *et al.,* (2011) employed a similar bacteria killing assay to investigate the response of NETs to *Pseudomonas aeruginosa* in cystic fibrosis, this study suspended neutrophils at 10^6 cells per well and incubated with bacteria at an MOI of 0.1, 1, 10 or 100, notably their results found that NET-mediated killing was most efficient at the lowest MOI.

A further consideration is turbidity readings to determine the influence of NETs on bacteria, subsequent to a 24h incubation period. Spectrophotometric turbidity indirectly measures bacterial growth by absorbance, which means it does not differentiate between live and dead bacteria. Therefore an alternative measure would be to perform serial dilutions of the NET-bacteria supernatant post incubation, instead of measuring turbidity, colony counts could be used to accurately quantify viable bacterial numbers at 24h. There is also scope to investigate the NET-bacteria incubation period, which the literature suggests could be as little as an hour for NETs to affect bacteria growth (Keshari *et al.,* 2012). If NETs could be frozen by liquid nitrogen prior to the NET-bacteria incubation assay, NETs could be defrosted on the day of experiment, meaning the incubation period would be more adaptable as time

would not be spent isolating neutrophils. This would also circumvent problems of interpersonal variation between neutrophil donors, as the same NETs could be employed for a range of bacteria-NET co-incubations. Further experiments are needed to ensure that freeze/thawed NETs remain representative of NET function, however the present data suggests NETs most representative of the fresh are those frozen with liquid nitrogen.

Chapter 5: Conclusion

This project has investigated approaches for the study of NETs on the growth of oral bacteria species. Since the discovery of NETs by Brinkmann in 2004, it is still not fully understood if NETs are simply immobilising bacteria, or whether they have antimicrobial properties capable of killing foreign pathogens. Recently there have also been suggestions that NETs can be detrimental to the host, for example the accumulation of NETs in small vessel vasculitis tissue has been suggested to trigger vasculitis and facilitate autoimmunity (Kessenbrock *et al.,* 2009). It is also possible that NETs may cause unfavourable effects in periodontitis, as the three most pathogenic periodontal bacteria were not found to produce DNase; implying uninhibited NETs cause a more severe manifestation of the disease (Lisa Palmer PhD Thesis). Despite this possibility, NETs may still play a role in containing oral bacterial species during infection, but the mechanism involved and the extent to which they do this is as yet undetermined. There are very few studies investigating the effect of NETs on bacterial growth, and this project is one of the only studies exploring oral bacterial species.

This project required a significant amount of preliminary work to develop an assay that was both sensitive and representative of *in vivo* NET-bacteria interactions. These experiments revealed that *ex vivo* neutrophils remain viable up to 24h post isolation, after which point the number of dead/dying neutrophils increases considerably, as confirmed with trypan blue exclusion. NETs are also present subsequent to 24h in the fridge or freezer, with liquid nitrogen producing the smallest change in NET quantification compared with the fresh measurements. This increase could be due to the presence of contaminating neutrophils in the

supernatant necrosing and releasing their cellular contents. If NETs are to be produced and stored for later NET-bacteria assays, the effect of neutrophil cellular contents upon bacteria growth will need to be considered, so that any growth inhibition is NET-mediated. The quantification of known DNA concentrations established that approximately 50% of neutrophils are producing NETs. This is likely to be stimulant dependent and it would be interesting to pursue this area and investigate the extent to which periodontal bacteria trigger NETosis.

For the purpose of this study, *S. intermedius, S. oralis* and *F. nucleatum* were cultured and employed for NET-bacteria incubation assays. To indicate the correct bacterial species were cultured, gram stains were performed prior to any work involving the bacteria; however further experiments may utilise polymerase chain reaction (PCR) assay for species confirmation. Results showed the growth of these bacteria was most accelerated at 2-3h, with growth slowing down after ~6h. If NETs could be defrosted on the day of experiment it would be possible to perform a 3h incubation, which may reveal growth changes more clearly than 24h incubations, as even if some bacteria were immobilised the remaining bacteria may proliferate and mask the effect.

The NET-bacteria incubation assay did not show a significant effect of NETs on bacterial growth, which could be due to NETs not having antimicrobial properties or because the assay was simply not sensitive enough. One explanation is the MNase digest of NETs prior to incubation with bacteria, which may render the NETs ineffective. Alternatively, incubation period, MOI or the means of measuring the influence of NETs may have all contributed. Moving forward there is scope to adjust

the methodology for isolating NETs from the supernatant, such as centrifugation, meaning that NETs are adhered to the bottom of well. However this approach would need several controls, such as nocodazole and cytochalasin D, to account for neutrophil degranulation and phagocytosis respectively. Once in place, the bacteria concentration and incubation period should be investigated further. The assay conditions may vary between bacteria species; thus a high-throughput method of investigating NET-bacteria interactions may still have to be tailored to recognise the differences in bacteria species growth.

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