Effect of adipokine priming upon neutrophil oxygen radical and extracellular trap release in response to stimulation by periodontal bacteria

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Abstract

Obesity is a major public health problem worldwide of increasing prevalence. It is characterised by a low grade chronic inflammatory state, which contributes to obesity-associated morbidity. A body of evidence suggests that obesity is associated with periodontitis, and is second only to smoking as a risk factor for periodontal tissue destruction, but underlying biological mechanisms for this association remain unclear. Adipose tissue functions as an endocrine organ, secreting cytokines (adipokines) that are reported to be associated with dysregulated/inflammatory immune responses to bacterial challenge. Adipokines include tumour necrosis factor alpha (TNFα), adiponectin, resistin, leptin and interleukin-6 (IL-6), all of which have been shown to prime/affect neutrophils for a secondary stimulus. Neutrophils are the major cell type involved in periodontal immunity to infectious agents. This study therefore aimed to elucidate neutrophil reactive oxygen species (ROS) and neutrophil extracellular trap (NET) responses to bacterial stimulation in an adipokine rich (obese) verses an adipokine normal (lean) environment. Blood was isolated from healthy male volunteers, with no history of diabetes and with normal body mass indices (BMI's) (range 20-25). Peripheral blood neutrophils were isolated by Percoll^{TM} discontinuous gradient centrifugation, primed with TNFα, adiponectin, resistin, leptin or IL-6, alongside control two control stimuli GM-CSF and IL-8, at concentrations reflective of lean and obese individuals. Neutrophils were subsequently stimulated with *Fusobacterium nucleatum* (ATCC 10953) for Toll-Like receptor (TLR)-4 activation, opsonised *Staphyloccocus aureus* (NCTC 6571) for FcγRII activation or phorbol 12-myristate 13-acetate (PMA) for direct activation via protein kinase C (PKC). Neutrophil ROS were measured by luminol enhanced and chemiluminsecence and NET release was by fluorometric assay of DNA and fluorescence microscopy. Adipokine concentrations representative of those found in obese individuals have been shown in this pilot study to enhance neutrophil ROS and

NET generation after exposure to bacterial stimulus. The effects are however stimulus dependent and require longer studies to validate any significant results.

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1. Introduction

1.1 Periodontitis

Inflammatory periodontitis is one of several diseases that affect the periodontal tissues. The disease is initiated by bacteria within a subgingival biolfilm but progresses because of aberrant host inflammatory immune responses to the biofilm organisms which fail to re-establish a "health-associated" biofilm. Both environmental lifestyle (Palmer *et al.* 2005) and genetic factors (Michalowicz *et al.* 1991) are thought to contribute to the dysregulation. Periodontitis lesions share many of the characteristics of the preceding gingivitis, which remains confined to the gingival tissues and is reversible inflammation (Armitage 2000), however destruction of the connective tissue attachment to the teeth, resorption of alveolar bone and tooth loss are specific to periodontitis (Listgarten 1986). Alveolar bone resorption occurs when levels of inflammatory mediators in the overlying soft tissues reach a certain threshold at a critical distance from the bone surface and activate pathways leading to the breakdown of bone (Graves and Cochran 2003). Figure 1.1 illustrates the effects of periodontitis.

The accumulation of a plaque biofilm within the oral cavity elicits an inflammatory immune response by the host which leads to the formation of an "initial lesion" (Löe *et al.* 1965: Theilade *et al.* 1966), neutrophils and other immune cells are recruited to the lesion by chemokines, cytokines and other stimulatory molecules secreted by cells within the affected tissues. Enhanced accumulation of neutrophils has been reported in the gingival tissues of patients with periodontitis (Liu *et al.* 2001). Lesions that become "established" are accompanied by changes in the dominant immune cell infiltration, such as the transition of T cells to B cells, which is also mediated by cytokines (Gingivitis, 1999).

Chronic periodontitis is a major cause of tooth loss and is significantly associated with other diseases including type II diabetes (Herring and Shah 2006), rheumatoid arthritis (Pischon *et al.* 2008) and cardiovascular diseases (Pihlstrom *et al.* 2005).

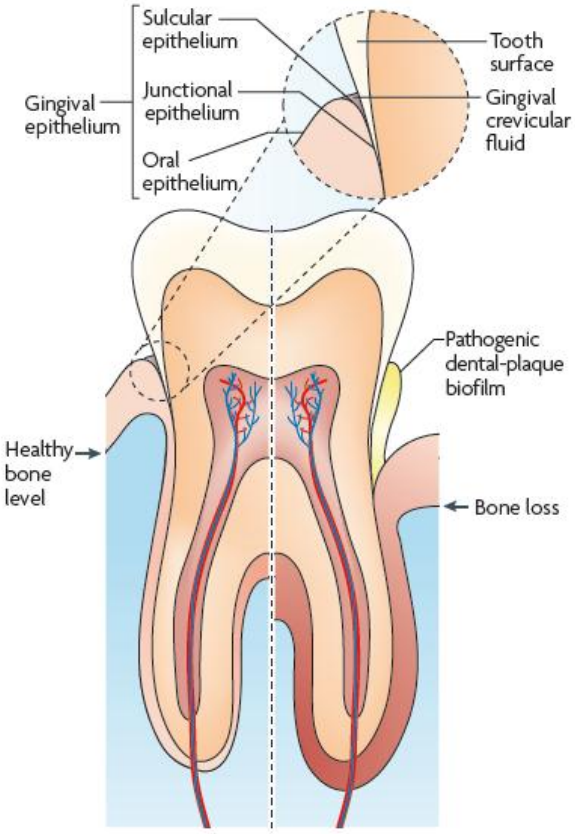


Figure 1.1 The differences between healthy and periodontitis tissues (Darveau 2010). The tissue to the left represents a healthy individual, with the oral epithelium covering the supportive connective tissue which is bound to the tooth surface by junctional epithelium. The right side of the diagram represents a periodontitis sufferer; the accumulation of plaque bacteria results in the destruction of the connective tissue resulting in eventual tooth loss.

1.2 Bacteria in periodontal disease

Oral tissues are constantly exposed to bacteria and their products; the soft and hard tissues in such close proximity create a niche in which bacteria can survive, multiply and colonise (Bolstad *et al.* 1996). More than 400 bacterial species have been cultured from periodontal pockets and plaque biofilms and 1,200 phylotypes have been identified by molecular methods (Dewhirst *et al.* 2010), however few are implicated in disease (Signat *et al.* 2011). Characteristics of pathogenic periodontal bacteria include the ability to colonise, evade the host immune system and release virulence factors that inadvertently induce an inflammatory response. This response can initiate tissue destruction and also provide an environment for more virulent microorganisms to colonise. From the early to late stages of plaque formation within the gingival crevice there is a shift from Gram positive to Gram negative colonisers (Ritz 1967) such as *Porphymonas gingivalis* and *Fusobacterium* species (Socransky *et al.* 1998).

1.3 The host immune response in periodontal disease: the neutrophil

The immune response to the pathogenic bacteria that colonise periodontal tissues involves both the adaptive and innate systems. Along with eosinophils and basophils, neutrophils form the granulocyte family of innate white blood cells (Ehrlich 1880) comprising 50-60% of total circulating leukocytes. Neutrophils are the primary major defence cell mobilised during an immune response against bacterial pathogens within the periodontal tissues and they comprise 50% of the inflammatory cell infiltrate within the junctional epithelium and 90% of the infiltrate within gingival extracellular fluid (Kowashi *et al.* 1980). Neutrophils are generated in the bone

marrow at a rate of 10^{11} cells per day but this can increase tenfold during infection (Cannistra and Griffin 1988).

Under normal physiological conditions neutrophils flow in mid-stream within the capillaries, however during an inflammatory episode they are the first cells to be recruited to sites of infection or injury. Recruitment is initiated by expressed selectin and integrin receptors on vascular endothelium in response to chemokines secreted by inflamed tissues such as interleukin-8 (IL-8), which bind to chemokine receptors on the surface of the cell (Rollins 1997). The neutrophils follow what is known as the leukocyte migratory cascade resulting in exit from the blood vessel via diapedesis towards the site of inflammation. Neutrophil targets include bacteria, fungi, viruses and virally infected cells.

Periodontitis is driven by a diversity of bacterial pathogens, which secrete a range of microbe-associated molecular patterns (MAMPs) that are recognised by host cells through pattern recognition receptors (PRRs) present on their cell surface, the result of which is the production and secretion of cytokines. Some of the microbiocidal weaponry neutrophils are equipped with to combat invading pathogens that are relevant to this project are outlined below.

a) Phagocytosis

Neutrophils are involved in the killing and removal of bacterial pathogens by phagocytosis. This is achieved through binding by two different receptor classes, Fcγ receptors (FcγR) and complement receptors (Witko-Sarsat 2000). Opsonised bacteria i.e. those coated in antibody, bind to FcγRII which aggregate on the cell surface (Wilson *et al.* 1995; Baker and Wilson 1989; Wilson *et al.* 1989). This leads to the intracellular activation of Src-tyrosine kinases which trigger various signalling pathways that coordinate to phagocytose the bacterial particle forming a structure

known as a phagosome (Greenberg *et al.* 1996). The phagocytosed bacterium is destroyed by the merging of vesicles containing microbicidal molecules (such as proteolytic enzymes and binding proteins such as lactoferrin which are packed in granules formed from the Golgi apparatus) with the phagosome. The formation and release of Reactive Oxygen Species (ROS) also occurs within the phagosomal space within cell in what is commonly known as the "respiratory burst". Figure 1.2 shows an illustration of the different aspects of phagocytosis to combat *Staphylococcus aureus* which can colonise the oral cavity and cause infection.

b) Degranulation

Neutrophils contain many hydrolytic enzymes and antimicrobial peptides (AMPs) capable of digesting pathogens and their products via non-oxidative pathways. These are stored within specialised compartments known as granules within the cytosol and there are three types: specific and gelatinase, which contain lactoferrin, metalloproteinases and lysozyme, and azurophilic which contain myeloperoxidase (MPO), a haem containing protein which catalyses the formation of hypochlorous acid from hydrogen peroxide (Farschou *et al.* 2003).

c) Reactive oxygen Species (ROS) generation via NADPH-oxidase

The reduction of molecular oxygen to water results in the large free energy release that can give rise to Free Radicals (FR) and other ROS. These molecules are powerful oxidizing agents formed endogenously as bi-products in metabolism and as a form of defence against invading pathogens (Dröge 2002). The number of electrons oxygen is reduced by determines which species is formed such as superoxide (O_2^-), hydroxyl radicals ($^{\cdot}OH$), hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) (Battino *et al.* 1999). The importance of the oxidative

burst in immune defence is evident in people with severe impairments in this pathway, for example sufferers of chronic granulomatous disease, a hereditary condition in which defects in the NADPH-oxidase enzyme system result in the inability to generate ROS, leave the individual vulnerable to recurrent bouts of severe infections (Curnutte 1993).

Superoxide production in neutrophils is mediated by the NADPH oxidase system located on the plasma membrane as well as on the phagosome membrane. This system which is composed of cytosolic and membrane subunits, generates ROS/FRs at the expense of NADPH. ROS formation is stimulated by the binding to antigens such as bacterial products, cytokines and other immune factors such as opsonised particles (Chapple and Matthews 2007) to membrane receptors.

d) Neutrophil extracellular traps

Neutrophil extracellular traps (NETs) are stand-like extrusions composed of chromatin complexed with histones and granule proteins that are released by neutrophils into the extracellular space as an active and terminal defence mechanism to immobilise pathogens (Brinkmann *et al.* 2004; Fuchs *et al.* 2007; Urban *et al.* 2009). The process of NET formation is not yet fully understood, however activation has been shown to require ROS production through NADPH oxidase, MPO (Metzler *et al.* 2011) and HOCl (Palmer *et al.* 2012). NET formation is a precedent to apoptosis but is thought to be a controlled distinct cell death programme deployed by neutrophils to limit the spread of infection within the host (Fuchs *et al.* 2007).

e) Other roles of neutrophils in the host immune response

Neutrophils possess pattern recognition receptors (PRRs) and the most widely known and studied are Toll-like receptors (TLRs) which are responsible for

detecting pathogen-derived compounds such as lipopolysaccharide (LPS), an intergral component of the outer membrane of Gram negative bacteria (Erridge *et al.* 2002). In particular, TLR4 and TLR2 can be stimulated by bacteria and their products, including *F. nucleatum* (Wakelin *et al.* 2006; Kikkert *et al.* 2007). Upon binding, downstream signalling pathways are activated such as the NF-κB pathway, which results in the transcription of genes involved in the pro-inflammatory response, induction of the respiratory burst and the production of cytokines including IL-6, TNFα and IL-8 although at a much lower concentration that those released by monocytes (Cassatella 1995; Sheikhi *et al.* 2000).

Neutrophils are able to recruit other cell types including those that form part of the adaptive immune system. Dendritic cells and natural killer cells can be activated by neutrophils (Peters *et al.* 2008; Constantini *et al.* 2011). Neutrophils also express several T cell chemoattractants (Scapini *et al.* 2000) and B cell development and maturation factors (Scapini et al 2008).

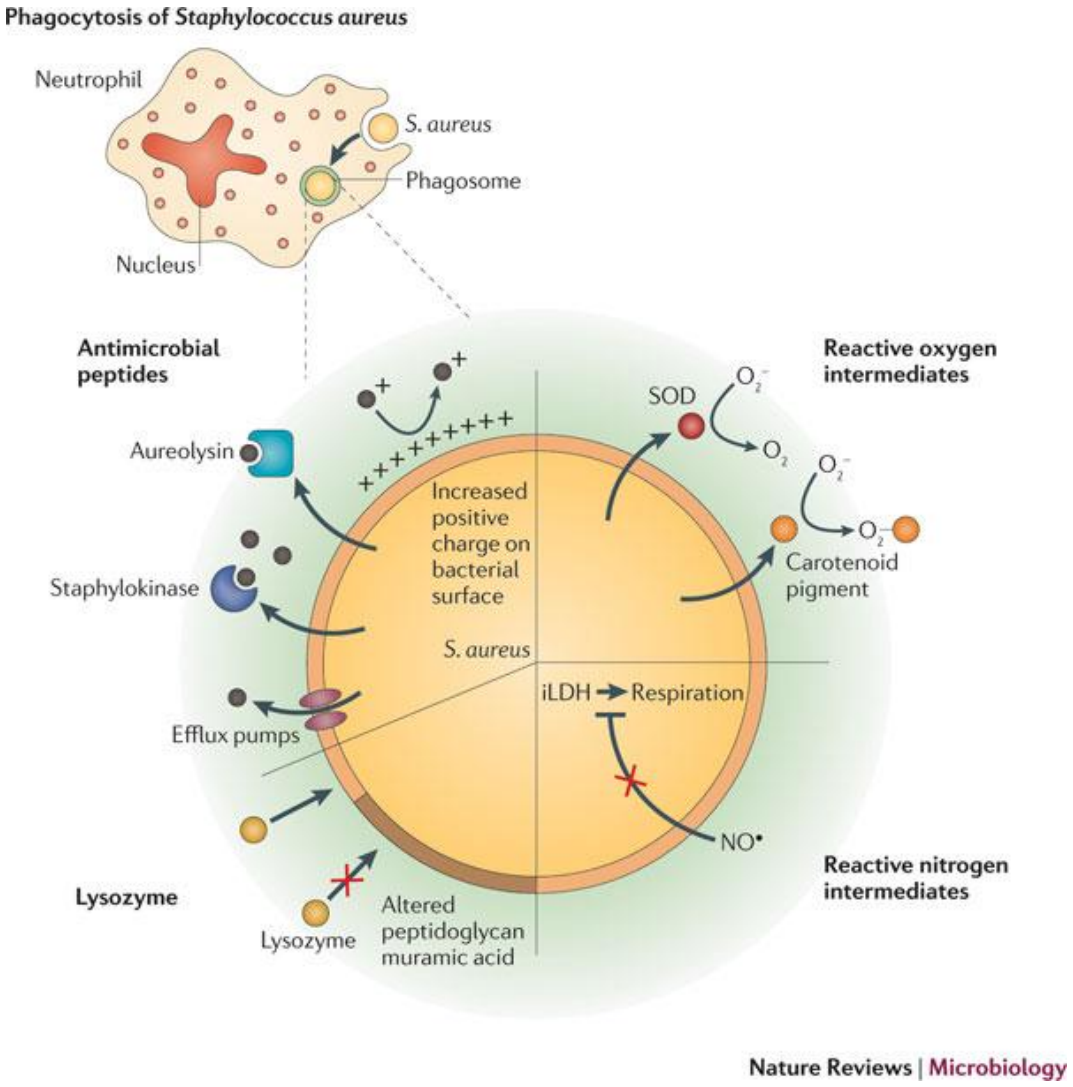


Figure 1.2. Phagocytosis by the neutrophil in response to bacterial infection. The phagosome forms when the neutrophil is activated through the engulfment of bacteria such as *S. aureus*, a common oral pathogen. There are a number of microcidal components to the phagosome that collectively kill the invading organism (Thwaites and Gant 2011).

1.4 Neutrophils and the pathology of chronic inflammation

While neutrophils are essential for host defence, they are also involved in the pathology of various inflammatory conditions. Elevated ROS production is implicated in a number of inflammatory disorders including rheumatoid arthiritis and periodontal diseases (Chapple 1997; Araujo *et al.* 1998). ROS can oxidise a number of structurally important host macromolecules when balancing its unpaired electronic state which can include proteins, lipids and carbohydrate resulting in irreversible cellular damage (Gutteridge *et al.* 1981; Halliwell 1994). Neutrophils are a rich source of ROS and in the absence of sufficient anti-oxidants within the periodontal tissues can lead to tissue damage (Waddington *et al.* 2000). Tissue damage occurs when neutrophil ROS are released extracellularly to such an extent that host antioxidants in the immediate vicinity are overwhelmed. Host tissue damage may arise due to premature neutrophil activation during migration, extracellular release of microbicidal products (e.g. elastase and ROS) during the killing of some microbes, or failure to resolve acute inflammatory responses (Smith 1994). There is also evidence that the production of ROS by phagocytic cells can activate osteoclasts, one of the major subtypes of cells found in bone involved in bone resorption (Hall *et al.* 1995), via FOX-O and WNT signalling pathways (Galli *et al.* 2012). The cytokines that are produced by host cells in response to the invading microorganisms can act alone or together stimulating inflammatory responses such as bone resorption and collagen destruction.

The regulation of adipokines is an essential part of inflammatory responses and immune functioning (Graves 2008). The imbalance of ROS and the antioxidant defence systems designed to minimise cellular damage is thought to be responsible for a significant component of non-resolving inflammation.

Chronic inflammatory conditions such as periodontitis are associated with increased oxidative stress, with neutrophil-mediated oxidant injury playing a major role (Chapple and Matthews 2007; Morel *et al.* 1991; Noguera *et al.* 2001). Peripheral blood neutrophils from periodontitis sufferers have been shown to generate significantly higher levels of ROS at baseline (no priming or stimulus) and also after *in vitro* FcγR stimulation compared with gender and age-matched healthy controls (Matthews *et al.* 2007a; Matthews *et al.* 2007b). The hyper-responsive neutrophil phenotype in chronic periodontitis could be an innate property of the neutrophils (hyperactivity) or involve sensitising or "priming" by cytokines or bacterial components (hyper-reactivity) (Matthews *et al.* 2007a). Priming of a cell increases its ability to respond to a second activating stimulus with enhanced functions including adhesion and the production of ROS and/or cytokine release (Gustafsson *et al.* 1997; Dias *et al.* 2011). Signal transduction processes are involved in neutrophil priming and involve calcium dependent and independent pathways (Thelen *et al.* 1993). A study by Matthews *et al.* (2007b) showed that non-surgical periodontal therapeutic intervention in patients with chronic periodontitis resulted in a reduction but not a complete removal of the hyper-reactive neutrophil phenotype with elevated extracellular ROS release remaining in patients versus controls. This suggests the role of peripheral priming of neutrophils may underlie the hyper-reactive neutrophil phenotype exhibited by patients with chronic periodontitis, but the neutrophil hyperactivity (ROS release in the absence of a stimulus) may remain, independent of priming.

Neutrophils can be primed by agents including TNF-α and LPS, which result in a dramatic increase in the response of the cells to activating agents both *in vitro* and *in vivo* (Condliffe *et al.* 1998). Neutrophils can be quiescent, primed or active. When primed there is not an overt increase in oxidase activity, rather the potential to

generate a greater oxidative response exists when the cells are provoked with the appropriate stimulus (Hallett *et al.* 1995). The increase in oxidative response can lead to "oxidative stress" which is "an imbalance between oxidants and antioxidants in favour of the oxidants, leading to a disruption in redox signalling and control and/or molecular demise" (Sies and Jones 2007). Oxidative stress is implicated in chronic periodontitis through neutrophil hyperactivity as a constitutional element; however peripheral priming of neutrophils by endogenous and exogenous factors may also have a significant part to play. Increased production of ROS has also been shown in peripheral neutrophils from patients with periodontitis by other groups (Asman 1988; Gustafsson and Asman 1996).

1.5 Obesity and inflammation

Obesity is defined as a body mass index (BMI) of more than 30 kg/m^2. It is a major public health problem prevalent in more than 30% of the US adult population (Flegal *et al.* 2010) and this is expected to rise. In obese individuals adipose tissue can constitute up to 50% of body mass. Over nutrition and increasingly sedentary lifestyles are the major causes of obesity. Increasing evidence links obesity to a low grade chronic inflammatory state (Hotamisligil 2006; Shoelson *et al.* 2006) which contributes to obesity-related diseases. The inflammatory state induced by metabolic surplus differs from "classical" inflammation which reflects the body's response to infection or injury (redness, swelling, pain and loss of function) (Hotamisligil 2006).

1.6 Adipose tissue as an endocrine organ for cytokines

Adipose tissue, which contains the fat storing cells of the body, has been shown to function as an endocrine organ that can secrete numerous factors that, with excessive adiposity, can cause disease through dysregulated immune responses (Ouchi *et al.* 2011). These factors are referred to as adipose-specfic cytokines or adipokines (Ouchi *et al.* 2003) and their expression in the body can vary depending on the site of adipose tissue deposition, in particular subcutaneous and visceral fat, which produce unique profiles of adipokines (Fried *et al.* 1998; Samaras *et al.* 2010). Adipokines are soluble proteins that bind receptors on target cells and initiate intracellular signalling cascades resulting in phenotypic changes to the cell through altered gene expression and regulation. They are effective at low concentrations and usually act on cellular receptors expressed by tissues within the surrounding environment of the cells they are produced by. They have a fundamental role in inflammatory disorders including periodontal disease (Hughes 1995). Adipokines, like other cytokines, are able to influence the secretion of other adipokines in networks, for example, IL-1β and TNFα can act synergistically in stimulating IL-6 secretion by human gingival fibroblasts (Kent *et al.* 1998). The accumulation of neutrophils within the tissues of periodontitis patients is associated with upregulated adipokine levels including IL-8 and TNFα (Liu *et al.* 2001).

Other cell types within adipose tissue include lymphocytes, macrophages and fibroblasts, the proportions of which can change with increasing adipocity. Adipose tissue is normally populated by 5-10% macrophages, but weight gain as a result of over nutrition can result in an increase in macrophage infiltration in adipose tissue of up to 60% (Weisberg *et al.* 2003). Conversely sustained weight loss in obese individuals is accompanied by a reduction in the number of adipose macrophages

and also concentrations of pro-inflammatory cytokines (Cancello *et al.* 2005) such as TNFα and IL-6 (Kern *et al.* 1995; Ziccadi *et al.* 2002). Table 1.1 lists the sources and characteristics of well-known adipokines.

Adipokine	Primary source(s)	Binding partner or receptor	Function
Leptin	Adipocytes	Leptin receptor	Appetite control through the central nervous system
Resistin	Peripheral blood mononuclear cells (human), adipocytes (rodent)	Unknown	Promotes insulin resistance and inflammation through IL-6 and TNF secretion from macrophages
RBP4	Liver, adipocytes, macrophages	Retinol (vitamin A), transthyretin	Implicated in systemic insulin resistance
Lipocalin 2	Adipocytes, macrophages	Unknown	Promotes insulin resistance and inflammation through TNF secretion from adipocytes
ANGPTL2	Adipocytes, other cells	Unknown	Local and vascular inflammation
TNF	Stromal vascular fraction cells, adipocytes	TNF receptor	Inflammation, antagonism of insulin signalling
IL-6	Adipocytes, stromal vascular fraction cells, liver, muscle	IL-6 receptor	Changes with source and target tissue
IL-18	Stromal vascular fraction cells	IL-18 receptor, IL-18 binding protein	Broad-spectrum inflammation
CCL2	Adipocytes, stromal vascular fraction cells	CCR2	Monocyte recruitment
CXCL5	Stromal vascular fraction cells (macrophages)	CXCR2	Antagonism of insulin signalling through the JAK–STAT pathway
NAMPT	Adipocytes, macrophages, other cells	Unknown	Monocyte chemotactic activity
Adiponectin	Adipocytes	Adiponectin receptors 1 and 2, T-cadherin, calreticulin–CD91	Insulin sensitizer, anti-inflammatory
SFRP5	Adipocytes	WNT5a	Suppression of pro-inflammatory WNT signalling

Table 1.1. Characteristics of well-known adipokines. ANGPTL2, angiopoietin-like protein 2; CCL2, CC-chemokine ligand 2; CXCL5, CXC-chemokine ligand 5; IL, interleukin; JAK, Janus kinase; NAMPT, nicotinamide phosphoribosyltransferase; RBP4, retinol-binding protein 4; SFRP5, secreted frizzled-related protein 5; STAT, signal transducer and activator of transcription; TNF, tumour necrosis factor (Ouchi *et al.* 2011)

1.7 Effect of adipokines on ROS activity

Many studies have reported on the effects of adipokines on ROS generation in neutrophils and a few examples are given here. Resistin has been shown to decrease the activation of the oxidative burst in neutrophils from healthy subjects (Cohen *et al.* 2008). Leptin can stimulate cells of the immune system and has been shown to directly stimulate neutrophils neutrophils at concentrations of up to 1000 ng/ml (Caldefie-Chezet *et al.* 2001). Jiang *et al.* (2011) showed that adiponectin can suppress the expression of NADPH oxidase in cultured vascular smooth muscle cells.

1.8 Periodontitis and obesity

Many studies have suggested that obesity is associated with oral diseases, second only to smoking as the strongest risk factor for periodontal tissue destruction (Nishida *et al.* 2005). The link between obesity and periodontitis disease was first shown by Perlstein *et al.* using a ligature-induced periodontitis in obese Zucker rats, where it was shown that bone resorption and periodontal inflammation were more severe in the obese cohort (Perlstein and Bissada 1977). Many epidemiological studies have demonstrated an association between obesity and periodontitis in humans (Saito *et al.* 1998; Saito *et al.* 2001; Wood *et al.* 2003; Saito *et al.* 2005) and also the reverse indication, that maintaining a normal weight by regular exercise is associated with lower periodontitis prevalence (Wakai *et al.* 1999; Karjalainen *et al.* 2002; Merchant *et al.* 2003).

The underlying biological mechanisms for the association between obesity and periodontitis are not known. Oxidative stress is thought to link periodontitis and metabolic syndrome (MetS), which encompasses a range of metabolic disorders

including high blood pressure and impaired glucose regulation (Eckel *et al.* 2005) and its root cause is linked to a chronic pro-inflammatory state. The bidirectional link between periodontitis and MetS is illustrated in figure 1.3.

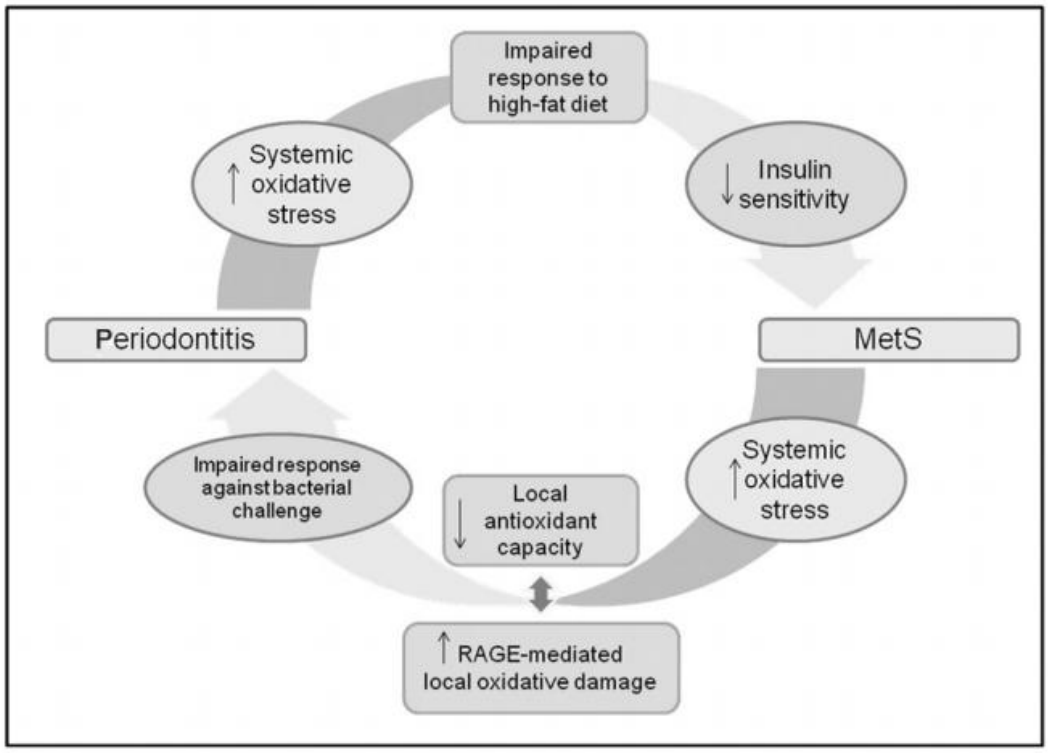


Figure 1.3. Potential role of oxidative stress linking periodontitis and metabolic syndrome (MetS) (Bullon *et al.* 2009). An individual suffering from periodontitis could be at risk of developing insulin resistance; an increase in ROS production has been shown to precede insulin resistance (Matsuzawa-Nagata *et al.* 2008). In contrast, an individual suffering from MetS or its components (obesity for example) and therefore exhibiting diminished anti-oxidant capacity of the periodontal tissues facilitating the pathology of infectious bacterial agents as a result of impaired immune responses (Bullon *et al.* 2009).
RAGE: receptor for advanced glycation end products. The presence of high RAGE expression in periodontal tissues supports their sensitivity to products derived from oxidative damage (Katz *et al.* 2005).

1.9 Methods of studying neutrophil activity

The focus of this project is the regulation of ROS and NETs by neutrophils, therefore selecting the most appropriate method to study them is important. Neutrophils are one of the shortest-lived cells in the body (5.4 days) (Pillay *et al.* 2010) abundant in the circulation and are therefore readily accessible for experimental investigation. Neutrophil cell lines that are cultured *in vitro* do not reflect the complex biological reality in tissues or the circulation. *In vivo* studies of neutrophils, such as those done in mice differ in important aspects from the human equivalents, for example the numbers of neutrophils in the circulation differ in mice and humans 30% versus 70% respectively (Amulic *et al.* 2012). The best available methods to study these cells is through *ex vivo* work, which involves isolating the cells from the peripheral blood of volunteers and their subsequent use in assays that measure their activity when exposed to appropriate stimuli.

The major activities of neutrophils involved in immune responses and linked to inflammation are ROS generation, protease release and NET formation. There are several methods of studying ROS activity and these are highlighted below:

i) Nitroblue tetrazolium (NBT) is a substrate for oxidases and ROS activity can be measured spectrophotmetrically by measuring the reduction of NBT (a yellow soluble substance) to formazan (a blue insoluble substance) (Seymour *et al.* 1986).

ii) Flow cytometry is a method for characterising single cells via labelling them with fluorescent dyes. ROS activity can be measured using this technique by evaluating 2,7-dichlorofluorescin (DCF), the fluorescent oxidative product of neutrophils upon stimulation with PMA (Battino *et al.* 1999).

iii) ROS can be detected spectrophotometrically at 550 nm by following Cytochrome C reduction.

iv) ROS produced upon stimulation can oxidise the cyclic hydrazine 5-amino-2,3-dihydo-1,4phthalazinedione (luminol) to 3-aminopthalate, which is accompanied by the emission of light as the anion relaxes from an excited to a ground state (Allen and Loose 1976). Luminol-dependent chemiluminescence assesses total ROS generation (intra and extracellular) as luminol has the ability to cross cellular membranes. Luminol is the most sensitive chemiluminescent substrate for detecting ROS generation by neutrophils in the laboratory environment (Kopprasch *et al.* 2003) and was therefore the chosen method in this study.

1.10 Methods of studying NET activity

NETs can be studied quantitatively and qualitatively. NET imaging techniques include Immunofluorescence microscopy, scanning electron microscopy (SEM), transmission electron microscopy (TEM) and live cell imaging (Brinkmann *et al.* 2010). The images generated provide a backdrop to quantitative analysis which involves fluorometric measurement of the NET DNA labelled with a nucleic acid stain, such as sytox green.

1.11 Project aims

The aims of this project were:

- To determine if adipokines at physiological levels reflective of lean and obese individuals are able to prime neutrophils sufficiently to generate differential ROS upon exposure to a second periodontally relevant bacterial stimulus.
- To measure the effect of different adipokine concentrations that have previously shown to positively prime neutrophils for enhanced ROS generation following their exposure to periodontally relevant bacterial stimuli.
- To determine if NET production by peripheral blood neutrophils is affected by neutrophil pre-incubation with lean or obese concentrations of adipokines.
- To compare the responses of neutrophils to activation of TLR or FcγRII receptors and to direct activation of PKC by PMA.

2. Methods

2.1 Adipokine concentrations utilised

A literature review of research publications that measured lean and obese concentrations of adipokines used in this project was performed. Key words were entered into search engines such as "Sciencedirect" and "PubMed" to find relevant studies that use or measure the adipokine concentrations of obese and lean individuals. From these studies the most appropriate concentrations were chosen based on the experimental technique used by a particular study and the medium in which the concentrations were taken, for example blood serum or plasma.

2.2 Blood collection

Healthy male volunteers were recruited for this project from staff and students of the Birmingham Dental Hospital. All had a BMI within the healthy range (19-24 kg/m^2) measured on the day of blood collection. Waist-hip ratio measurements were also taken. All were non-smokers, non-diabetic and were not taking anti-inflammatory drugs. Approximately 25ml of blood was collected into Vacutainer^{TM} (Greiner Bio-One Ltd, Stonehouse, UK) lithium heparin (17 IU/ml) tubes. Upon collection the tubes were inverted 8-10 times to prevent blood clotting. Approximately 20 minutes after collection the blood was processed for neutrophil isolation. Ethical approval was provided by the University of Birmingham (ERN_10-0518) and donors gave their informed consent. Table 2.1 shows the health characteristics of the volunteers.

Assay	Number of volunteers	Age (y)	BMI (kg/m^2)	Waist to hip ratio
ROS assays with TNFa and IL-6	10	23.6±0.23	22.06±0.22	0.87±0.005
ROS assays with priming concentrations of adipokines	5	23.2±0.52	21.00±0.32	0.86±0.005
ROS assays with priming concentrations of TNFa and IL-6 in combination	5	23.4±0.46	21.4±0.39	0.86±0.005
ROS assays with adipokines resistin, leptin, adiponectin, GM-CSF and IL-8	10	23.4±0.30	21.2±0.19	0.85±0.004

Table 2.1 Health characteristics of the volunteers. Waist to hip ratio signifiers: excellent <0.85; good 0.85-0.90; average 0.90-0.95; high 0.95-1.00; extreme >1.00. All volunteers were between average and excellent. BMI signifiers: 20-25 healthy, 25-30 overweight; 30-35 obese; 30-35 severely obese; >40 morbidly obese. All volunteers were within the healthy range.

2.3 Neutrophil isolation

Neutrophils were isolated from blood samples using percoll (GE Healthcare), a 1,130 g/ml colloidal silica suspension coated with polyvinylpyrolidone (PVP) (Pertoft *et al.* 1978). Two discontinuous gradients were used for neutrophil isolation. Densities of 1.079 and 1.098 were prepared as shown in table 2.2.

8ml of 1.098 density was layered under 8ml of 1.079 density percoll in a centrifuge tube. 8-9 ml of blood was then layered at the top of the tube. The cells were centrifuged for 8 minutes at 150 g (RCF), followed by 10 minutes at 1200 g.

Density	1.079	1.098
Percoll	19.708 ml	24.823 ml
Water	11.792 ml	6.677 ml
NaCl (1.5 M)	3.5 ml	3.5 ml

Table 2.2 Components of the percoll gradients.

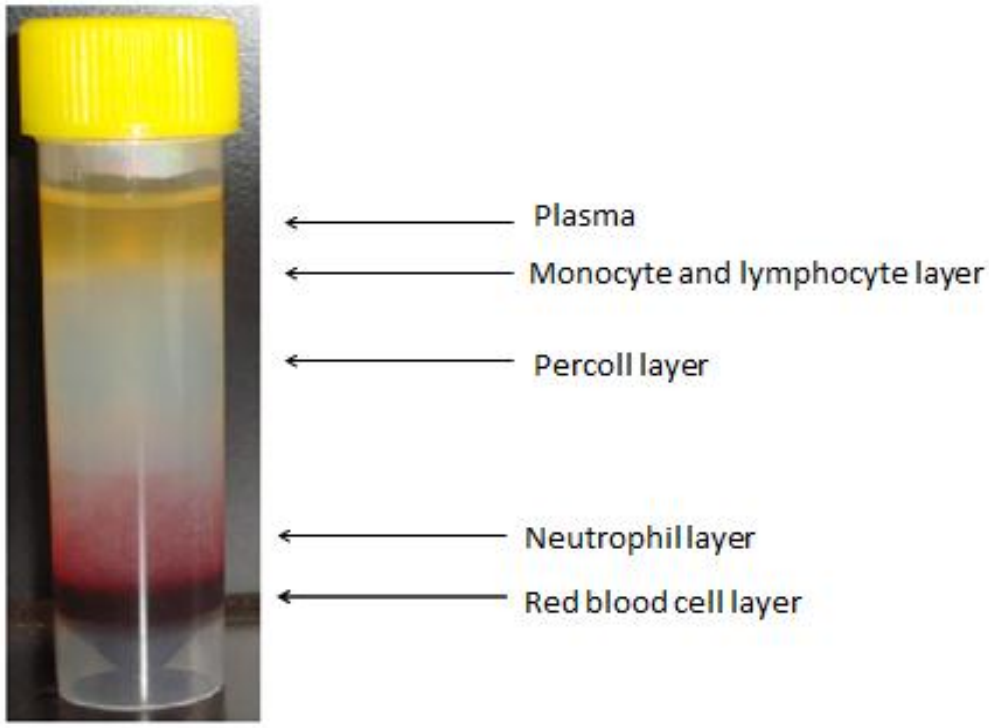


Figure 2.1 Photograph showing separation of the blood by differential percoll gradients.

The neutrophil layer within the supernatant was removed by gentle aspiration using a Pasteur pipette and added to another centrifuge tube containing 30 ml of buffer [0.83% NH_4Cl containing 1% $KHCO_3$, 0.04% Na_2 ethylendiamine tetraacetic acid (EDTA), 2_h2O and 0.25% bovine serum albumin (BSA)] for erythrocyte lysis. A final volume of 50 ml with additional lysis buffer was achieved. The tubes were gently inverted several times and left at room temperature for 10 minutes to allow for erythrocyte lysis. The cells in suspension were then pelleted at 350 g for 6 minutes. The supernatant was discarded and the pellet was resuspended in 3 ml lysis buffer. After a repeat centrifugation step, the cells were washed with 3 ml PBS, centrifuged again, the supernatant was removed and the cells were made up to a final volume of ~2-3 ml PBS.

The cells were counted using a haemocytometer (figure 2.2) which included duplicate total cell and trypan dye exclusion counts (which were diluted 1:2).

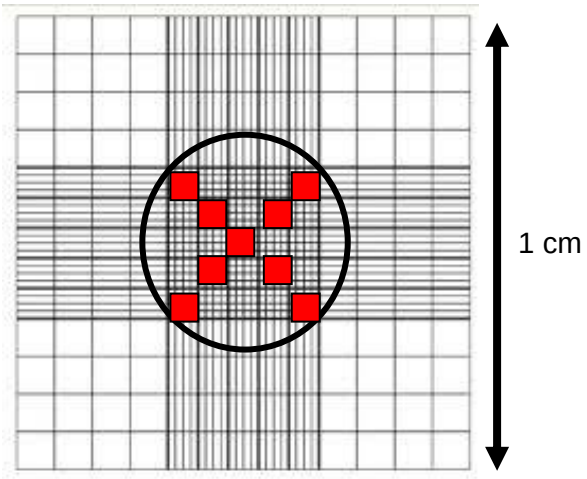


Figure 2.2 A haemocytometer grid. The red squares were the designated regions for counting neutrophils. This was scaled up using the equation described.

$1cm^3 = 10mm^3$. The small squares in red were counted. Each small square within the top right hand grid has a volume: 0.2 x 0.2 (width) x 0.1 (depth) = 0.004 mm^3.

Therefore the cells within the 9 squares were counted and the number of cells per ml was calculated as follows:

- Cell count /9 x 25 = no. of cells in the entire grid.
- No. of cells in the grid x 10^4 = no. of cells per ml.

This was repeated twice as were trypan blue counts (10 µl of cell suspension in 10 µl of trypan blue).

For both NET and ROS assays, 1 $x10^5$ cells/ml was required for each well (final volume 200 µl) and were diluted in gPBS accordingly.

For the NET visualisation assay the cells were made up to the same volume using RPMI (Biosera RM-R1640/500) enriched with L-glutamine.

2.4 Adipokine preparation

All adipokines were purchased from Sigma-Aldrich (Dorset, UK). The concentrations representative of lean and obese individuals for each is shown in table 2.1. The adipokines were diluted in PBS with 10% BSA and stored at -20 ºC prior to use.

2.5 Stimulus preparation

2.5.1 *Staphylococcus aureus*

S. aureus NCTC 6571, obtained from Fisher Scientific, was stored at -40 ºC in cryopreservative on beads (Prolab PL160). A single bead was retrieved and used to inoculate a mannitol salt agar plate and left to incubate at 37 ºC for 24 hours. The growth of the bacteria discolours the agar from a red to a yellow appearance. Several colonies were picked from the agar plate to inoculate 500 ml sterile tryptone soya broth which was cultured for 24 hours (standing) at 30 ºC. The following steps outline the process of *S. aureus* opsonisation

1. Calculation of concentration of *S. aureus*

To approximate the number of bacteria from the broth culture, six 1ml serial dilutions (1:10) were made. 100 µl from the 5^{th} and 6^{th} dilution were inoculated onto Mannitol Salt Agar plates and incubated at 37 ºC for 24 hours. Optical density (OD) measurements were also taken of the 5^{th} and 6^{th} dilution. The following day the numbers of colonies were counted, this was scaled back to the original broth to give the approximate concentration of bacterial cells in the original 500 ml of broth.

2. Harvesting of bacteria

The bacteria grown in broth were pelleted in 10 x 50 ml tubes for 5 minutes at 6,000 rpm at 4 ºC in a Harrier 18/80 centrifuge (swing-out rotor). The supernatant was discarded and the pellets were re-suspended in 20 ml PBS and mixed by vortexing. The 20 ml PBS suspensions were pooled into 4 x 50 ml tubes and the washing step was repeated. After discarding the supernantant the pellets were re-suspended in PBS and pooled into 2 x 50 ml tubes and washed once more. The supernatant was discarded and the pellets were used for fixation.

3. Fixation

The harvested cells were re-suspended in 20 ml formyl buffered saline (strong formalin diluted 1:10 and 8.5 g NaCl per litre) for 1 hour at room temperature. The fixative was removed by centrifugation (same settings as during harvesting) and washed three times with PBS. The pellets were re-suspended in a final volume of 5 ml PBS and stored at 4 ºC.

4. Opsonisation with IgG

The isolated and fixed *S. aureus* was incubated at 80 °C for 20 minutes and allowed to cool. Vigam (Human IgG) 5 g/100 ml liquid (BPL) was added at 33 µl per 1 x 10^9 bacterial cells. The cells were left to incubate with a rotation device at room temperature overnight. The cells were then pelleted at 6,000 rpm for 10 minutes at 4 ºC, washed twice with PBS and re-suspended in a final volume of 5 ml PBS. The cells were diluted 1:100 with PBS and divided into aliquots of 500 µl and stored at -20 ºC prior to use. The number of bacteria required per well in all assays is 3 x 10^7 (300 bacteria per neutrophil) (Bergström and Asman 1993).

2.5.2 *Fusobacterium nucleatum*

F. nucleatum (ATCC 10953) was supplied by LGC Standards in freeze-dried format. 250 µl Brain heart infusion broth with 10% serum (Oxoid BO0129E) was added to the freeze-dried bacterial powder and re-suspended in a sterile test tube. A further 3 ml of broth was added. 100 µl of the suspension was streaked onto horse blood agar plates with 10% serum (Oxoid PB0114) and incubated for 24-48 hours in an anaerobic chamber at 37 ºC. A third plate was inoculated and left under aerobic conditions at the same temperature to test for purity.

A single colony was inoculated in the brain heart infusion broth and incubated anaerobically for 24-48 hours, after which OD measurements were taken and 1 ml of the bacterial suspension was diluted further in 1 litre of broth and streaked across new agar plates for bacterial quantification. The broth culture was pelleted and washed in PBS 3 x before re-suspending in a final volume of 20 ml PBS. The bacteria were heat killed by incubating at 80 ºC for 20 minutes. The number of bacteria present on the agar plates was used to approximate the number of bacteria present in the broth culture. Approximately 100 bacteria are required per neutrophil. The bacteria were stored at -20 ºC prior to use.

2.5.3 Phorbol 12-myristate 13-acetate preparation

PMA was supplied by Sigma (P8139). It was dissolved in 1 ml 20 mM DMSO before being diluted in PBS and stored at -20 ºC prior to use. A concentration of 0.1 µM was used.

2.6 ROS assays

White microwells (Microlite 2, VWR, Lutterworth, UK, 8 wells/strip for 96 well plate format) were pre-blocked overnight using filter sterilised 1% BSA (Sigma A4503) in PBS. 200 µl was added to each well and stored overnight at 4 ºC. The plate was then washed three times with PBS using a microplate washer (BioTek ELx50).

Different concentrations of adipokines were added to the cells in combination with each stimulus (*F. nucleatum*, opsonised *S. aureus*, PMA or negative control) as shown in table 2.3.

Adipokine	Lean concentration	Obese concentration	Priming concentration
TNFα	2 pg/ml	4 pg/ml	3 ng /ml and 5 ng /ml
IL-6	0.5 pg/ml	1.5 pg/ml	100 ng/ml and 1000 ng/ml
Adiponectin	13.3 µg/ml	8.6 µg/ml	10 µg/ml
Leptin	6.4 ng/ml	23.45 ng/ml	250 ng/ml
Resistin	12.8 ng/ml	33 ng/ml	30 ng/ml
IL-8 (positive control)			10 ng/ml
GM-CSF (positive control)			100 ng/ml

Table 2.3 Lean and obese concentrations and priming concentrations of adipokines used in this study.

All samples were assayed in triplicate (representative of one individual). Each microwell had a final volume of 200 µl. The cells and luminol were loaded into the microwells and luminescence was recorded using a Berthold microplate luminometer (LB96v) and Winglow software for 30 minutes at 37 ºC. Adipokines and PBS supplemented with glucose and cations (1.8 g glucose, 0.15 g $CaCl_2$ and 1.5 g $MgCl_2$) (gPBS) as a control, were then added and luminescence was recorded. A further 30 minutes following this, the test stimulus (bacteria or PMA) was added or gPBS for controls and luminescence was recorded for a further 60 minutes. Triplicate measurements were recorded per condition per individual. Table 2.4 shows the components and volumes of wells for all ROS assays in this project.

	Luminol (µl)	**Adipokine (µl)**	**Stimulus (FN/PMA/SA) (µl)**	**Neutrophils diluted in in gPBS (µl)**	**Additional gPBS**
Control	30	0	0	100	70
Adipokine only	30	10	0	100	60
Stimulus only	30	0	25	100	45
Adipokine and stimulus	30	10	25	100	35

Table 2.4 Components of all the ROS assays. Total volume in each well was 200 µl.

2.7 Control ROS assays

2.7.1 Cell free ROS assay

To ensure no background luminescence activity was occurring an assay was completed with all components of the ROS assay but excluding neutrophils. After washing pre-blocked microwells all components of the ROS assay (priming agents, stimuli, luminol) as displayed in table 2.5 were added with the exception of neutrophils and placed into the luminometer. Luminescence was recorded over a 12 second period, after 2 seconds 30 µl 1% H_2O_2 diluted in water and 0.0008 Units/µl of horseradish peroxidase was used to generate luminescence activity by oxidation of luminol. Measurements were recorded using the Berthold Microplate Luminometer LB 96V and Winglow software.

2.7.2 Cell metabolic activity assay

To estimate the metabolic activity of neutrophils within the ROS assays the cellular ATP content was measured using Cell Titre Glo (Promega), a reagent used to determine the quantity of ATP within a cell by use of a luminescent substrate. Neutrophils were isolated as described previously and the same protocol for the ROS assay was adopted (adipokines, neutrophils and gPBS) however no stimulus (bacteria/PMA) or luminol was added. The plate was measured for luminescence activity using the same programme for the same length of time as the ROS assay. At three time points (0 minutes, 30 minutes and 80 minutes) 80 µl CellTitre-Glo luminescent substrate (Promega) was added. This substrate indicates the number of viable cells in culture based on quantification of the ATP present, which signals the presence of metabolically active cells.

2.8 NET assays

2.8.1 Micrococcal nuclease (MNase) assay

96 well micro plates were pre-blocked with 200 µl filter sterilised 1% BSA in PBS at 4 ºC overnight. After washing the plate, neutrophils were suspended in 100 µl with RPMI to give a concentration of 1 x 10^6 cells/ml. Neutrophils (diluted in RPMI) and respective adipokines or RPMI were added and left to incubate at 37°C with 5% CO_2 for 30 minutes. Stimulus was then added and the plate was left to incubate for a further 2 hours. 15 µl of 14.3 units/ml MNase (Worthington LS004797) diluted 1:100 with PBS was added and the plate was incubated at room temperature for 10 minutes. The microplate was centrifuged at 1800 RCF for 10 minutes. 150 µl of the supernatant from each well was placed into a new black 96 well microplate. 15 µl of 10 µM SYTOX green (Invitrogen S7020) diluted 1/500 in RPMI was added to each well. DNA fluorescence was read 5 times using a Berthold Twinkle LB970 fluorometer with excitation at 485 nm and emission at 525 nm. Data was obtained using Mikrowin software. Table 2.5 shows the template for the MNase assay.

	Cells in RPMI (µl)	Cytokine (µll)	Stimulus (FN/PMA/SA) (µl)	MNase (µl)	Sytox (µl)
Control	170	0	0	15	15
Adipokine only	160	10	0	15	15
Stimulus only	145	0	25	15	15
Adipokine and stimulus	135	10	25	15	15

Table 2.5 Components of the MNase assay.

2.8.2 Control MNase assay

To demonstrate that the fluorescence activity was recorded from NET-derived DNA an assay was completed with all components of the NET assay except neutrophils. Various concentrations of DNA were added to show that DNA can be detected and quantified in a concentration-dependent manner. Calf thymus DNA was supplied by Sigma (89370) and diluted in molecular biology quality water to 0.5 µg. This was further diluted 1:2 down to 0.03 µg. 10 µl of each dilution was added. The total volume per well was made up to 200 µl with RPMI. DNA fluorescence was read five times using a Berthold Twinkle LB970 fluorometer as previously described.

2.8.3 NET visualisation assay

Costar 24-well plates were blocked with 500 µl PBS with 1% BSA and incubated at 4 ºC overnight. The liquid was removed by vacuum aspiration and the cells (1 x 10^5/ml, 200 µl/well) suspended in RPMI were added. The plate was incubated in a 37 ºC at 5% CO_2 enriched environment for 30 minutes, after which adipokine (20 µl) was added and the cells were incubated for a further 30 minutes. Stimulus (25 µl) and further RPMI (50 µl) was then added and the plate was incubated at 37 ºC for 2 hours.

After the incubation 25 µl of 100 nM SYTOX green (Invitrogen S7020) diluted in RPMI was added to each well. The plate was imaged using a fluorescence microscope (Nikon Eclipse TE300, Surrey, UK) using a x20 objective lens and images captured by digital camera (Nikon CoolPix 450, Surrey, UK).

3. Results

3.1 Adipokine concentrations

Table 3.1 shows the different physiological adipokine concentrations found in human subjects. Highlighted in green is the lean or obese concentrations used in this project, in red are the priming concentrations also used. Table 2.3 gives a refined summary of the adipokine concentrations.

Many studies demonstrate the potential for GM-CSF and IL-8 to enhance the respiratory burst in neutrophils when exposed to the appropriate secondary stimuli (Nathan 1989; Weisbart *et al.* 1985).

	Serum lean	Reference	Serum obese	Reference	Plasma lean	Reference	Plasma Obese	Reference
Resistin	4-9 ng/ml	Hiroshima et al. 2012			3.8-7.8 ng/ml	Hiroshima et al. 2012	3.6 ng/ml	Vendrell et al. 2004
	8 ng/ml	Saito et al. 2008			21.5 ng/ml	Silha et al. 2003	28.8 ng/ml	Silha et al. 2003
	3.6 ng/ml	Degawa-Yamauchi et al. 2003	5.3 ng/ml	Degawa-Yamauchi et al. 2003	7 ng/ml	Kunnari et al. 2006		
	12.8 ng/ml	Cohen et al. 2008	33 ng/ml	Cohen et al. 2008				
	12.6 ng/ml	Janowska et al. 2006	14.5 ng/ml	Janowska et al. 2006				
	12.8 ng/ml	Azuma et al. 2003	24.6 ng/ml	Azuma et al. 2003				
Other research on resistin	Study by Kusminski et al. 2007 found 30 ng/ml had an affect an adipocytes. Another study by Kawanami et al. 2008 found 30nm of resistin was a sufficient concentration to induce the expression of adhesion molecules on endothelial cells.							
Adiponectin	12.4 ug/ml	Saito et al. 2008			13.3 ug/ml	Silha et al. 2008	8.6 ug/ml	Silha et al. 2008
	9.64 ug/ml	Onat et al. 2008	8.09 ug/ml	Onat et al. 2008	10 ug/ml	Arita et al. 1999	4 ug/ml	Arita et al. 1999
					8.9 ug/ml	Arita et al. 1999	3.7 ug/ml	Arita et al. 1999
					16.7 ug/ml	Yu et al. 2002	12.1 ug/ml	Yu et al. 2002
							16.2 ug/ml	Vendrell et al. 2004
					16.68 ug/ml	Illan-Gomez et al. 2012	5.82 ug/ml	Illan-Gomez et al. 2012
Other research on adiponectin	Study by Lovren et al. 2010 found 10 ug/ml was enough to prime monocytes to differentiate into anti-inflammatory M2 macrophages							
Leptin	6.4 ng/ml	Rosicka et al. 2003	23.45 ng/ml	Rosicka et al. 2003	5.9 ng/ml	Silha et al. 2003	26.9 ng/ml	Silha et al. 2003
	5.7 ng/ml	Murdolo et al. 2008	18.4 ng/ml	Murdolo et al. 2008				
			24 ng/ml	Gavrila et al. 2003				
			30.8 ng/ml	Vendrell et al. 2004				
	9.6 ng/ml	Caro et al. 1996	40.2 ng/ml	Caro et al. 1996				
Other research on leptin	Study by Zarkesh-Esfahani et al. 2004 found 250 ng/ml leptin can stimulate neutrophils. A study by Caldefie-Chezet et al. 2001 found optimal stimulation at 250 ng/ml and non significant at 5ng/ml.							
TNFa	2 pg/ml	Murdolo et al. 2008	4 pg/ml	Murdolo et al. 2008				
Other research on TNFa	Gustaffson et al. 1997 found 3 ng/ml sufficient to prime neutrophils. Wright et al. 2007 found 5ng/ml was sufficient to prime neutrophils.							
IL-6	0.5 pg/ml	Murdolo et al. 2008	1.5 pg/ml	Murdolo et al. 2008				
Other research on IL-6	Kharazami et al. 1989 found 100ng was sufficient to prime neutrophils. Mulklen et al. 1995 showed 1000 ng/ml was sufficient.							
IL-8	A study by Wozniak et al. 1993 found 10 ng/ml stimulated neutrophil production							
GM-CSF	Weisbart et al 1988 and 1987, showed that GM-CSF can prime neutrophils at 100 pmol/L Balazovich et al. 1991 1ng/ml was enough to prime neutrophils. Dang et al. showed 500 pM can prime neutrophils. Nathan 1989 showed 100 ng/ml of GM-CSF was enough to prime cells for enhanced respiratory burst.							

Table 3.1 Adipokine concentrations identified through a literature search. Highlighted in green are the concentrations reflective of lean and obese people which were used. Red highlights concentrations shown to positively prime/activate neutrophils.

3.2 Control assays

3.2.1 Cell free ROS assay

The cell free ROS assay demonstrated that in the absence of neutrophils there was no background oxidative activity arising from any of the other assay components. The addition of hydrogen peroxide is measured as an increase in luminescence and demonstrated that none of the components block the luminescent signal. The results of the cell free assay are shown in Figure 3.1 a-e. The addition of all components in all combinations did not generate any luminescence and therefore no NADPH oxidase independent ROS. When hydrogen peroxide was added there was a sharp increase in luminescence that fell back down to baseline seconds after its addition. Hydrogen peroxide is a highly reactive oxygen species and a powerful oxidising agent. When added to the wells the horseradish peroxidase catalyses the reaction of the hydrogen peroxide with the luminol which results in energy release in the form of light. The hydrogen peroxide is then decomposed to form oxygen and water. Figure 3.1 shows that the various adipokines and positive controls used in this project do not prevent luminescence from being detected or inhibit peak signals over the blank (control). The data is representative of one individual with repeats in triplicate for each condition.

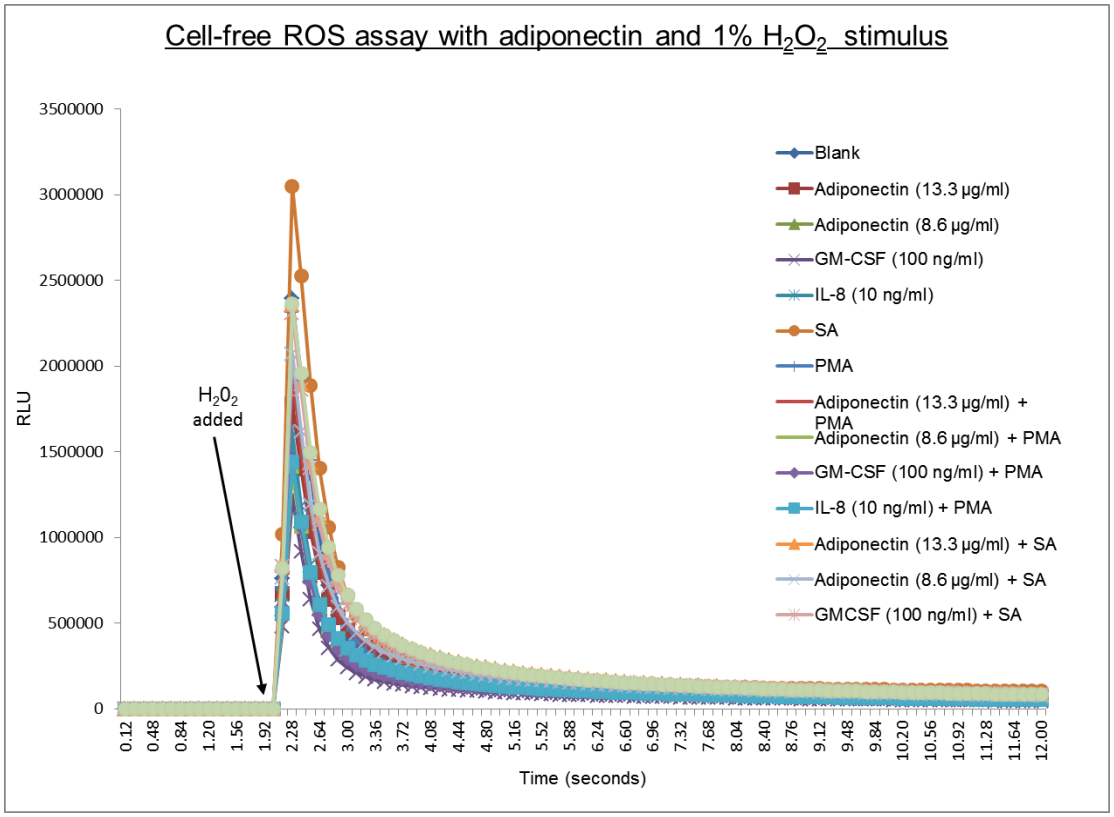


Figure 3.1 a) Cell free ROS testing luminescent signal activity with adiponectin and stimuli.

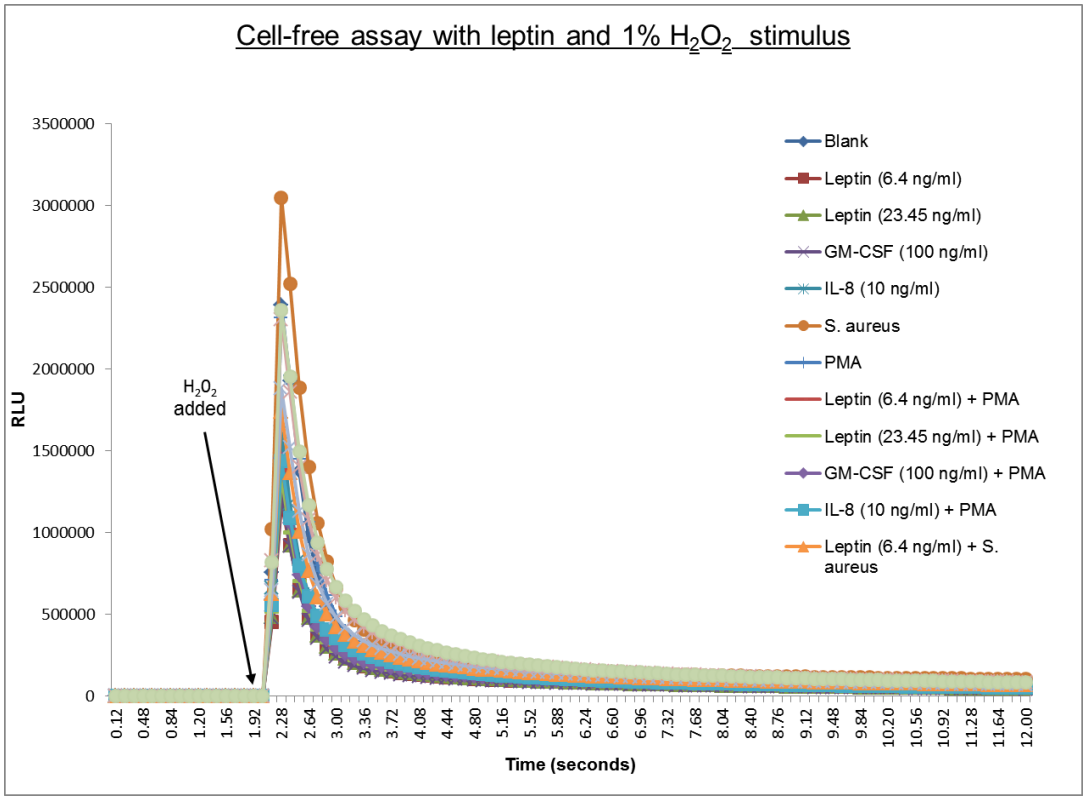


Figure 3.1 b) Cell free ROS testing luminescent signal activity with leptin and stimuli.

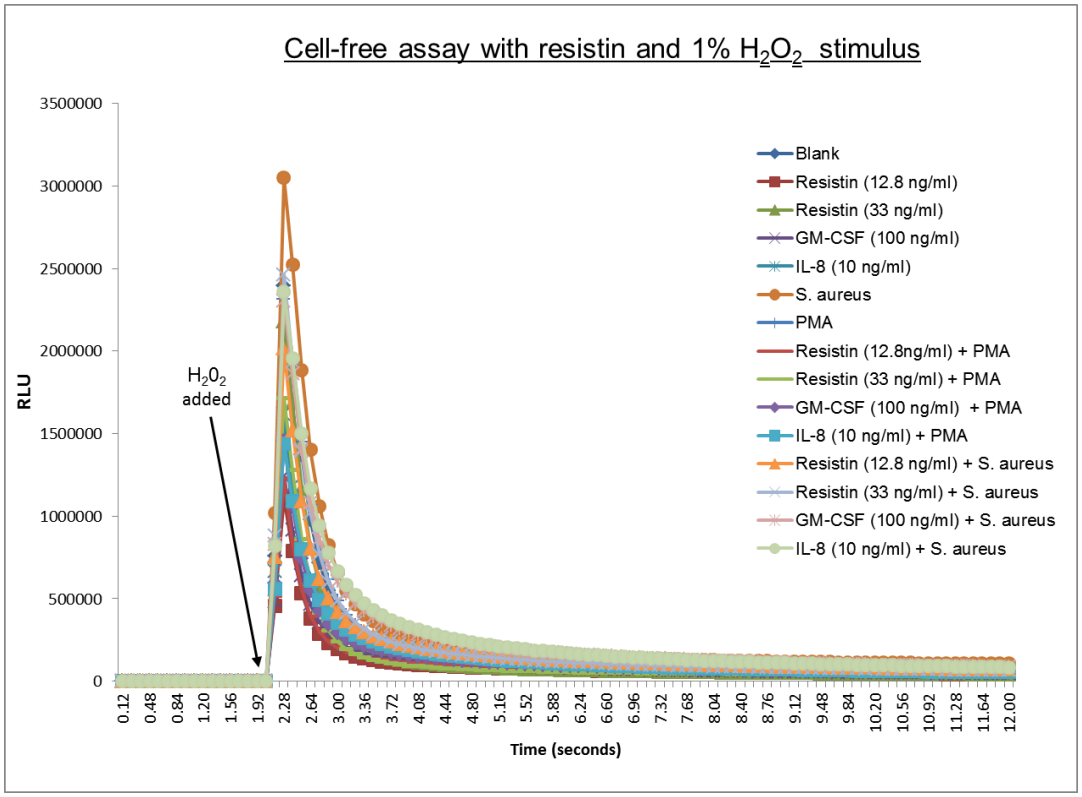


Figure 3.1 c) Cell free ROS testing luminescent signal activity with resistin and stimuli.

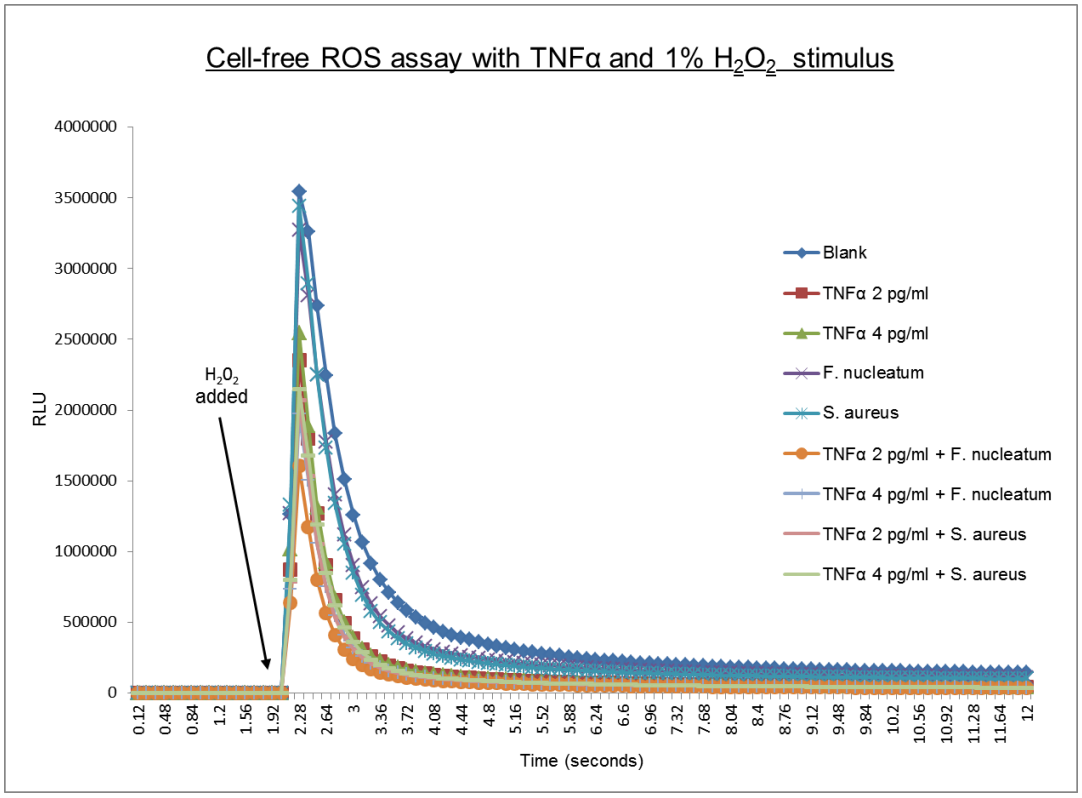


Figure 3.1 d) Cell free ROS testing luminescent signal activity with TNFα and stimuli.

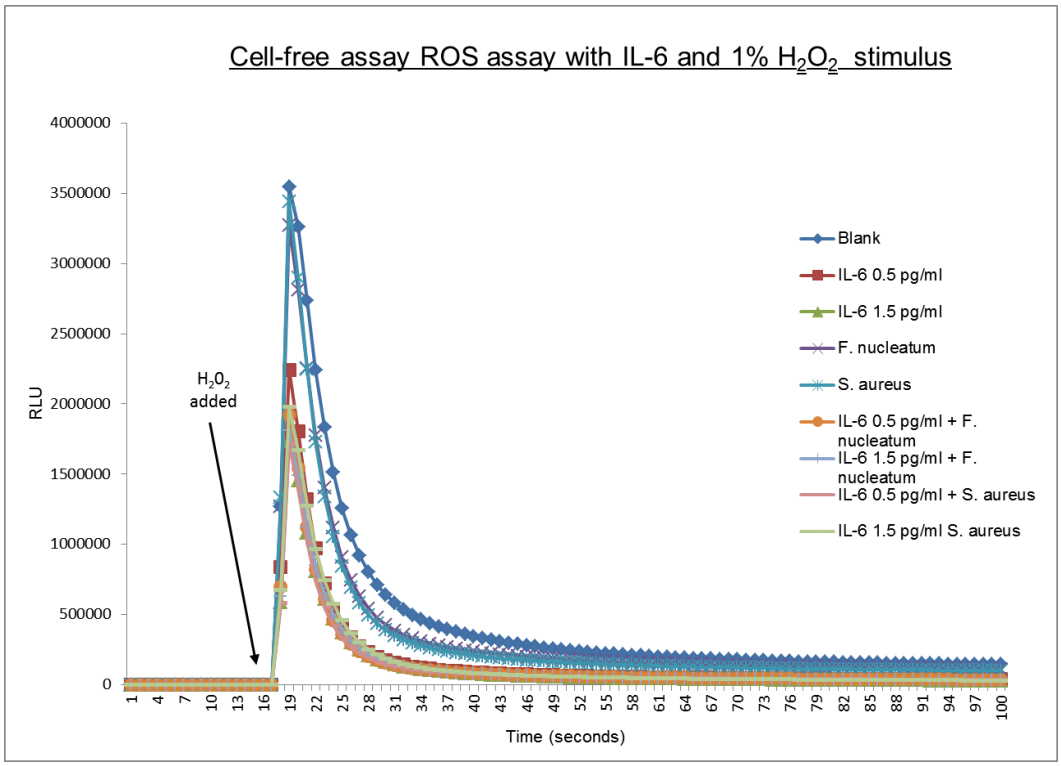


Figure 3.1 e) Cell free ROS testing luminescent signal activity with IL-6 and stimuli.

3.2.2 Cell metabolic assays

Figure 3.2 shows the decay of luminescence over the time course of the cell metabolic assay. Cell Titer Glo was added at 0, 30 or 80 minutes. Cells followed from time 0 demonstrate the same decay of ATP quantity as those with substrate added at 30 and 80min. This demonstrates the decreasing metabolic activity of the short-lived neutrophils and that the addition of the substrate did not affect the activity of the cells. Figures 3.3a-e shows the influence of adipokines on the cell metabolic activity over the 120 minute time period, which is the total duration of the ROS assay to show that the cells are still metabolically active. Here little difference was seen for TNFa, IL-6, adiponectin or leptin. Resistin showed an altered pattern in cell metabolic activity, which should be further examined, however it should also be noted that this experiment has only be completed with one volunteer, as is therefore preliminary in nature.

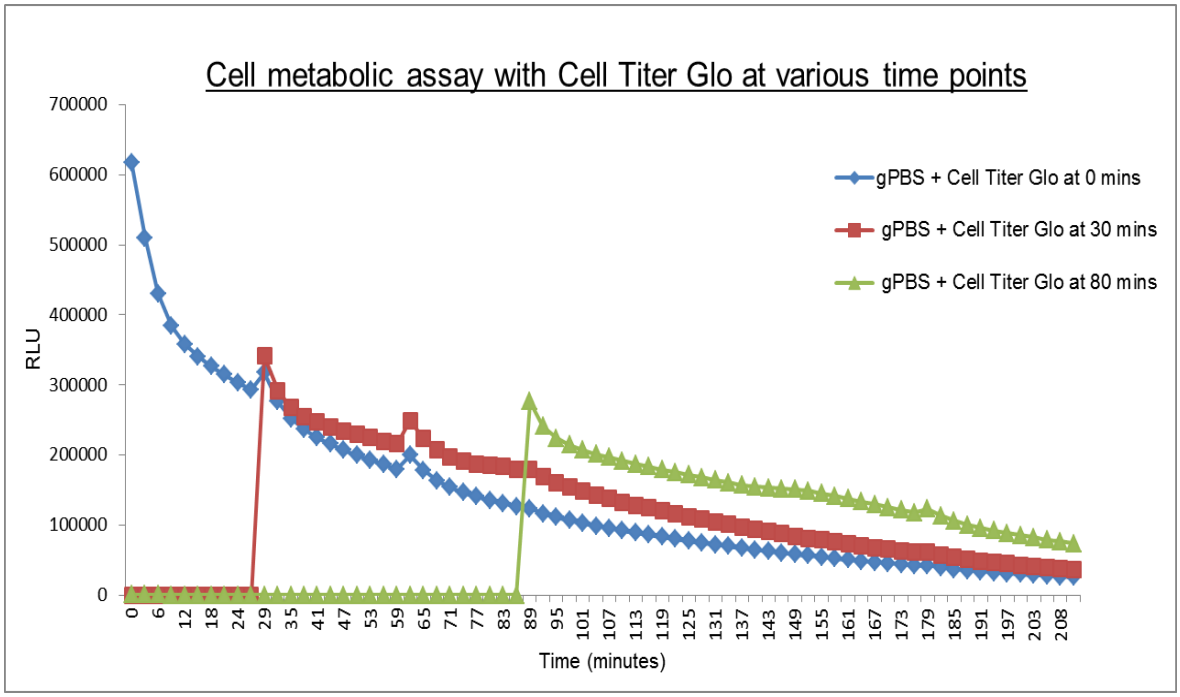


Figure 3.2 Cell metabolic assay with cells in gPBS at various time points that represent the addition of adipokine, and stimulus and the extent of cell metabolic activity after 2 hours.

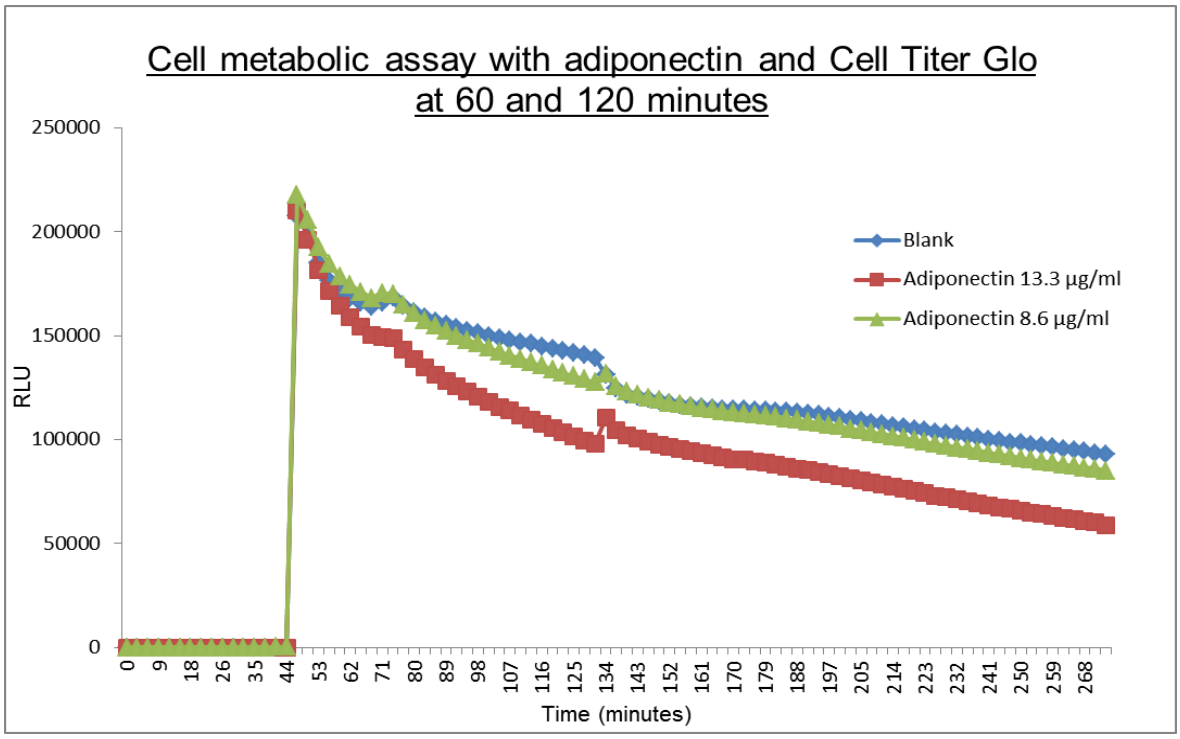


Figure 3.3 a) Cell metabolic assay with adiponectin and Cell Titer Glo added at the point the stimulus would be added (60 minutes) and at the end point of the assay (120 minutes) to show that the cells are metabolically active.

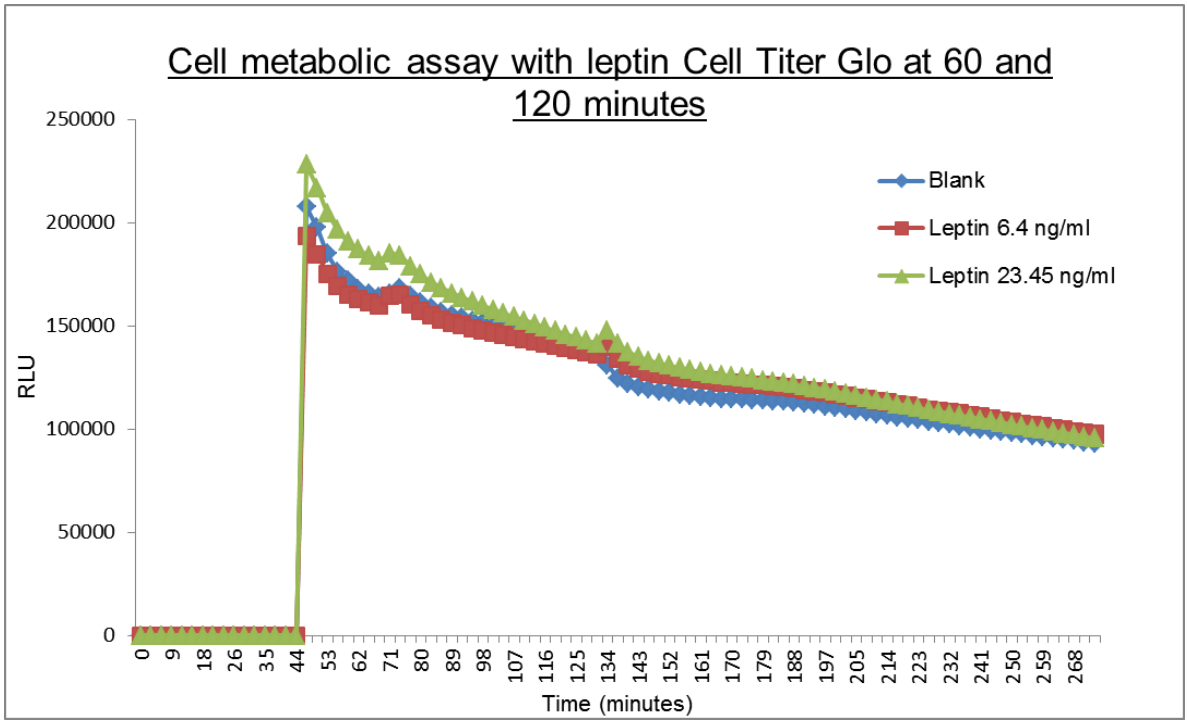


Figure 3.3 b) Cell metabolic assay with leptin.

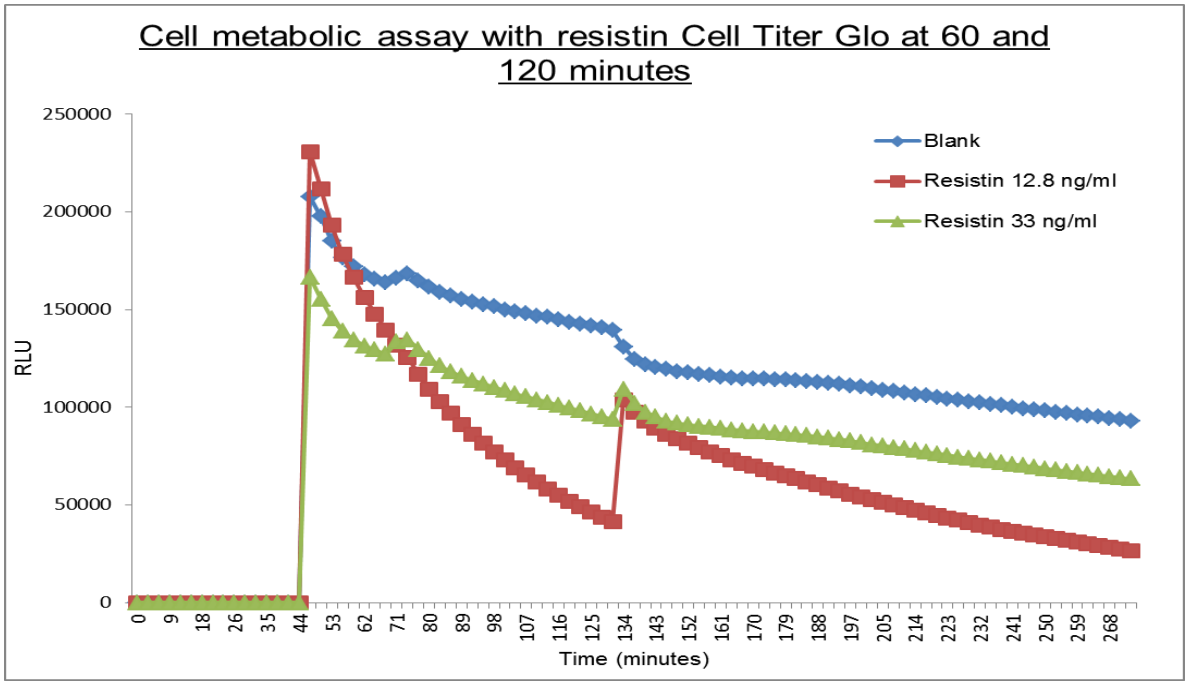


Figure 3.3 c) Cell metabolic assay with resistin.

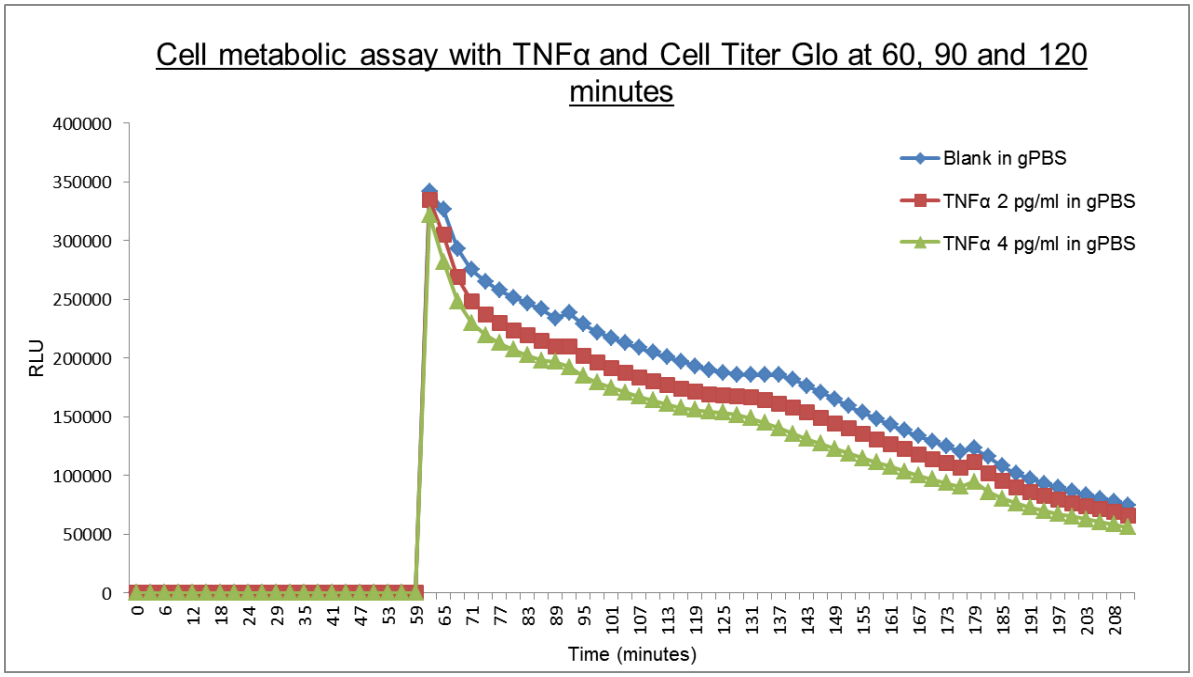


Figure 3.3 d) Cell metabolic assay with TNFα.

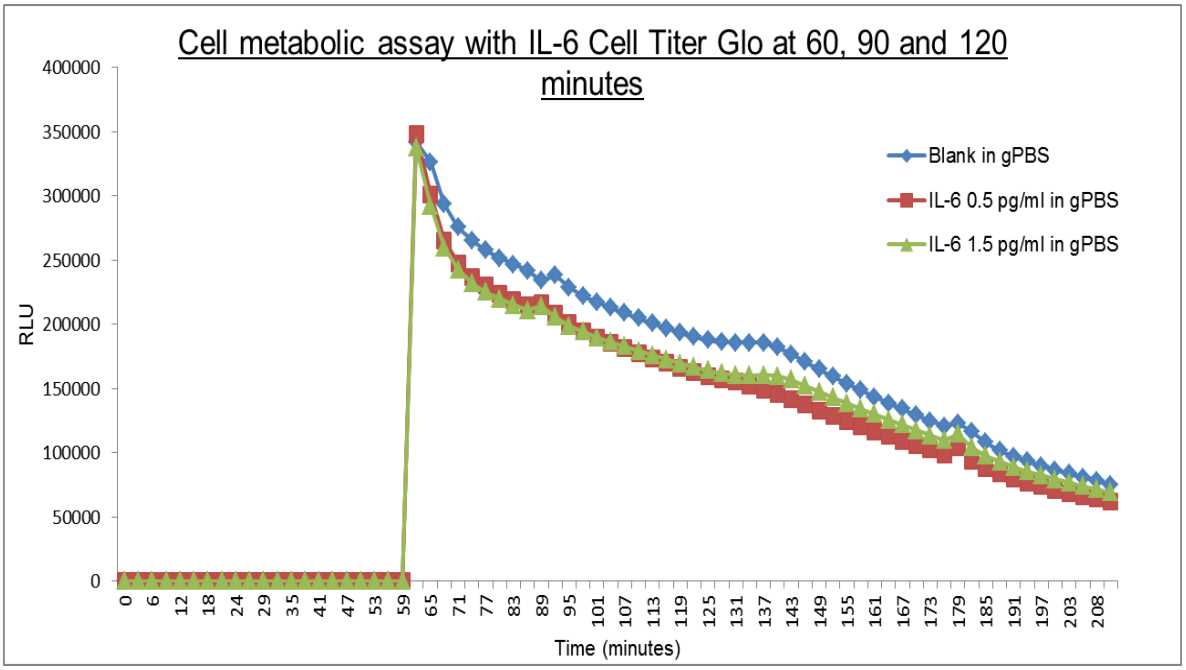


Figure 3.3 e) Cell metabolic assay with IL-6.

3.3 ROS assays

3.3.1 ROS assay results with lean and obese concentrations of adipokines

The data collected from the ROS assays included peak signal and area under the curve data (also known as integration) for the 30 minute (prior to addition of any adipokine), 60 minute (addition of adipokine) and 90 minute (addition of stimulus) time points. The peak signal gives the highest output score in relative light units (RLU) whereas integration provides the area under the curve between specified X-axis time points.

The data was analysed using Prism 5.0. Control values were subtracted from all other conditions, after which control adipokine values were subtracted from the corresponding adipokine with stimulus added. For example TNFα lean concentration alone was subtracted from TNFα lean concentration with PMA. The lean and obese peak and integration results for each adipokine were then compared graphically and statistically. A Wilcoxon matched-pairs signed rank test was performed on all ROS results.

Figures 3.4 and 3.5 show mean ROS activity of all volunteers (n = 10) with adipokines and stimuli. Tables 3.2 and 3.4 provide a summary of the integration and peak data for ROS assays with lean versus obese adipokine concentrations and stimuli respectively, which are derived from figures in the supplementary data (figure S1-S16). Tables 3.3 and 3.5 provide summaries of the positive priming controls, GM-CSF and IL-8, versus stimulus alone.

It should be noted that for both peak and integration results at the 30 minute time point the neutrophils are at baseline (i.e. no adipokine or stimulus present) and no increase in luminescence is expected. At the 60 minute time point adipokines have been added, and again there should not be any significant ROS generation (unless the

adipokines directly stimulate ROS rather than prime the neutrophils) until the 120 minute time point, at which the stimulus has been added. Any ROS generation at 60 minutes implies neutrophil stimulation, rather than priming, for a secondary stimulus.

Priming agent ↓	Stimulation →	*F.nucleatum*		Opsonised *S. aureus*		PMA	
Time point (mins) →		60’	120’	60’	120’	60’	120’
TNFa		-	-	-	-		
IL-6		-	-	-	↑P=0.006		
Adiponectin				-	-	-	↑P=0.001
Leptin				↑P= 0.01	-	-	-
Resistin				-	-	-	-

Table 3.2 Summary of integration data for ROS production after priming concentrations (lean & obese) of adipokines and various stimuli. - Denotes no significant difference between lean and obese adipokine-induced ROS production; ↑ denotes a significant increase between of obese over lean neutrophils for the stimulant highlighted (obese > lean). All data were normalised by the subtraction of the blank and/or adipokine alone signal.

Priming agent ↓	Stimulation→	Opsonised *S. aureus*		PMA	
Time point (mins) →		60’	120’	60’	120’
GM-CSF		-	-	↑ P = 0.002	↑ P = 0.02
IL-8		-	↓ P = 0.003	-	↑ P = 0.01

Table 3.3 Summary of integration data for ROS production after priming with positive controls GM-CSF and IL-8 and stimuli. - denotes no significant difference between lean and obese adipokine-involved ROS production; ↑ denotes a significant increase between lean and obese treated neutrophils for the stimulant highlighted (obese > lean). ↓ denotes a significant decrease in ROS generation in priming concentration of adipokine compared to stimulus-only treated neutrophils (stimulus alone > adipokine + stimulus).

Priming agent ↓	Stimulation→	*F.nucleatum*		Opsonised *S. aureus*		PMA	
Time point (mins) →		60’	120’	60’	120’	60’	120’
TNFa		-	-	-	-		
IL-6		-	-	-	↑P=0.025		
Adiponectin				-	-	-	↑P= 0.002
Leptin				-	-	-	↑P= 0.00371
Resistin				-	-	-	↓ P = 0.002

Table 3.4 Summary of peak data for ROS production after priming concentrations (lean & obese) of adipokines and various stimuli. - Denotes no significant difference between lean and obese adipokine-induced ROS production; ↑ Denotes a significant increase between lean and obese treated neutrophils for the stimulant highlighted (obese > lean). ↓ denotes a significant decrease in ROS generation in obese compared to lean treated neutrophils (lean > obese).

Priming agent ↓	Stimulation→	Opsonised *S. aureus*		PMA	
Time point (mins) →		60’	120’	60’	120’
GM-CSF		-	-	-	-
IL-8		-	-	-	↑ P = 0.01

Table 3.5 Summary of peak ROS production after priming with positive controls GM-CSF and IL-8 and stimuli. - Denotes no significant difference between adipokine-induced and stimulus-only ROS production. ↓ denotes a significant decrease in ROS generation in priming concentration of adipokine compared to stimulus-only treated neutrophils (stimulus alone > adipokine + stimulus).

TNFα

There were no significant differences between 2 pg/ml (lean) and 4 pg/ml (obese) priming concentrations of TNFα for the integration signal following a secondary stimulus with *F. nucleatum* (figure S.1-2; table 3.2). The same was true of peak data (figure S.3; table 3.4). When *S. aureus* was used as a stimulus, similarly, no signal differences were found for lean or obese concentrations of TNFα as a priming agent (figure S.2 and S.4).

IL-6

Tables 3.2 and 3.4 highlight a statistically significant difference between IL-6 obese concentrations (1.5 pg/ml) in enhancing ROS production over IL-6 lean concentrations (0.5 pg/ml) when stimulated with opsonised *S. aureus* from integration and peak data ($P = 0.006$ and $P = 0.03$ respectively) (figure S.2 and S.4).

Adiponectin

Adiponectin priming showed a statistically significant increase in obese (8.6 μg/ml) over lean (13.3 μg/ml) concentrations when stimulated with PMA for both integration ($P = 0.001$) and peak data ($P = 0.002$) as shown in figure S.5 and S.8 and tables 3.2 and 3.4. There were no significant differences between lean and obese concentrations of adiponectin when assessed for priming for a secondary *S. aureus* stimulation.

Leptin

There was no significant difference for integration data at the 120 minute time point with leptin with either PMA or *S. aureus* stimuli, however the peak data show a significant difference between lean (6.4 ng/ml) versus obese (23.45 ng/ml) at 120 minutes with PMA stimulus ($P = 0.004$) (figure S.8).

Resistin

The only statistically significant result with resistin was a decrease in peak ROS activity between lean (12.8 ng/ml) and obese concentrations (33 ng/ml) (P = 0.002), following PMA stimulation (figure S.9 and table 3.4).

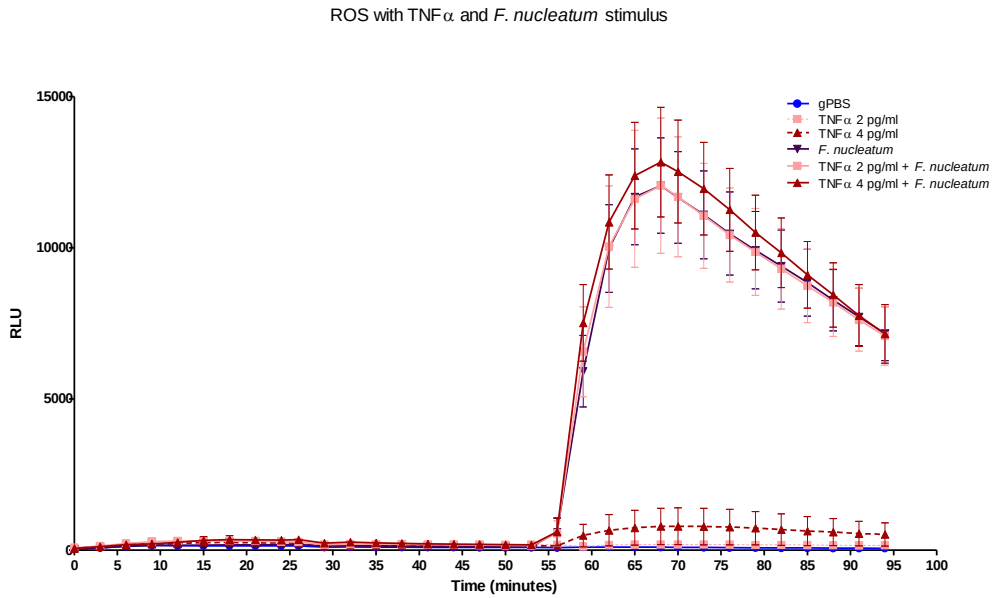
GM-CSF

The integration results showed a clear increase in ROS generation when the neutrophils are pre-incubated with GM-CSF at both 60 minutes (pre-incubation) and 120 minutes (addition of PMA stimulus) (P = 0.002 and 0.02 respectively) (figure S.6 and table 3.3) . There was no statistically significant differences in the peak data, which highlights the importance of analysing both peak and integration results; the effect of an agent on ROS activity can differ with some causing a sharp increase (PMA) while others causing a more pronounced long-term ROS generation (*S. aureus*).

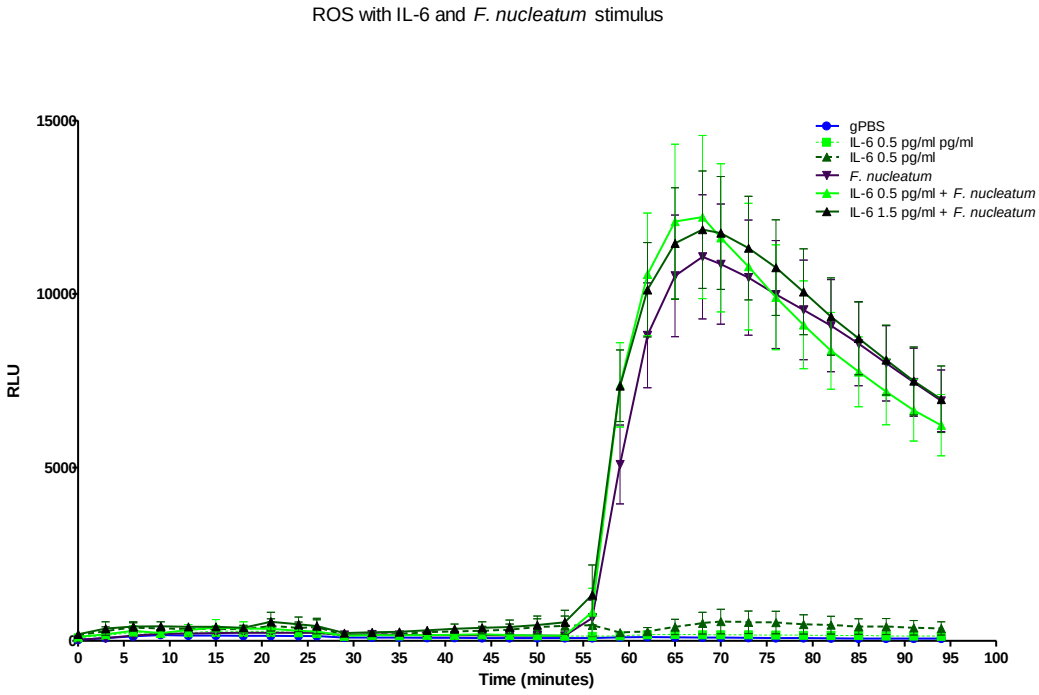
IL-8

IL-8 shows a differential response for integration and peak results which appear dependent on the chosen stimuli. There were statistically significant responses at integration (P = 0.01) and peak (P = 0.01) over PMA stimulus alone (figures S.7 and S.10, tables 3.3 and 3.5). Stimulation with opsonised *S. aureus* gave a significantly lower response when the neutrophils were pre-incubated with IL-8 at integration (figure S.13 and table 3.3).

ROS assay results with lean versus obese concentrations of TNFα and IL-6 and *F. nucleatum* stimulus



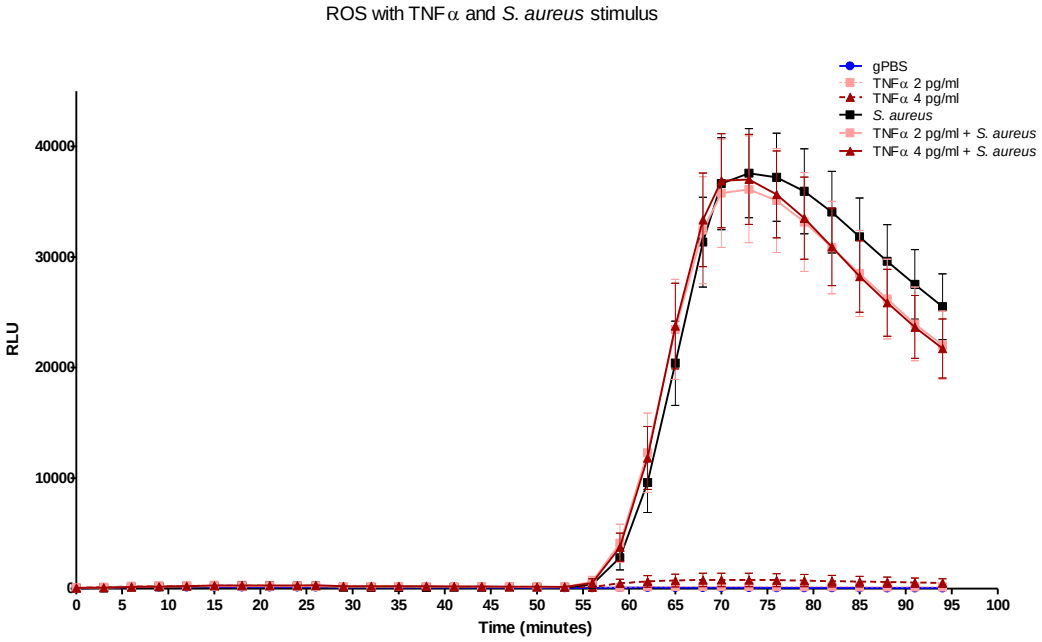
3.4a)



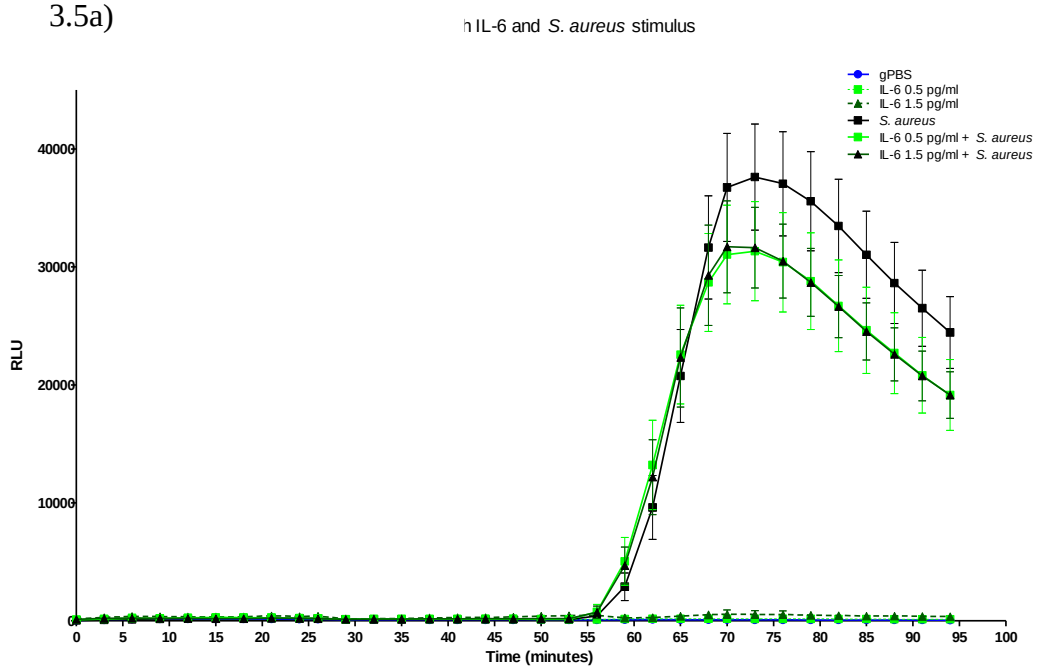
3.4b)

Figure 3.4a and 3.4 b) ROS assay result with lean versus obese concentrations of TNFα and IL-6 with *F. nucleatum* stimulus respectively.

ROS assay results with lean versus obese concentrations of TNFα and IL-6 with *S. aureus* stimulus



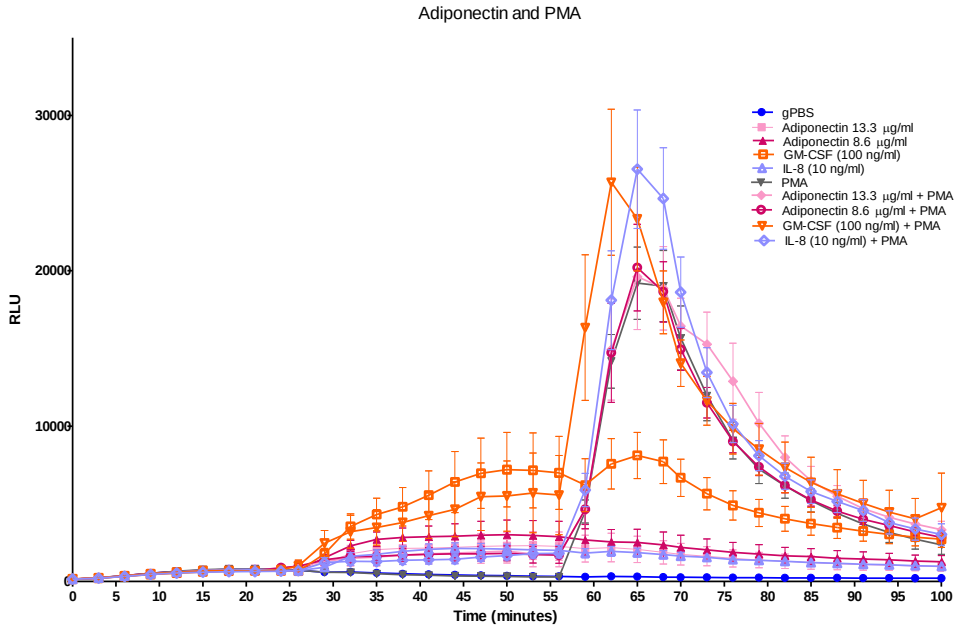
3.5a)



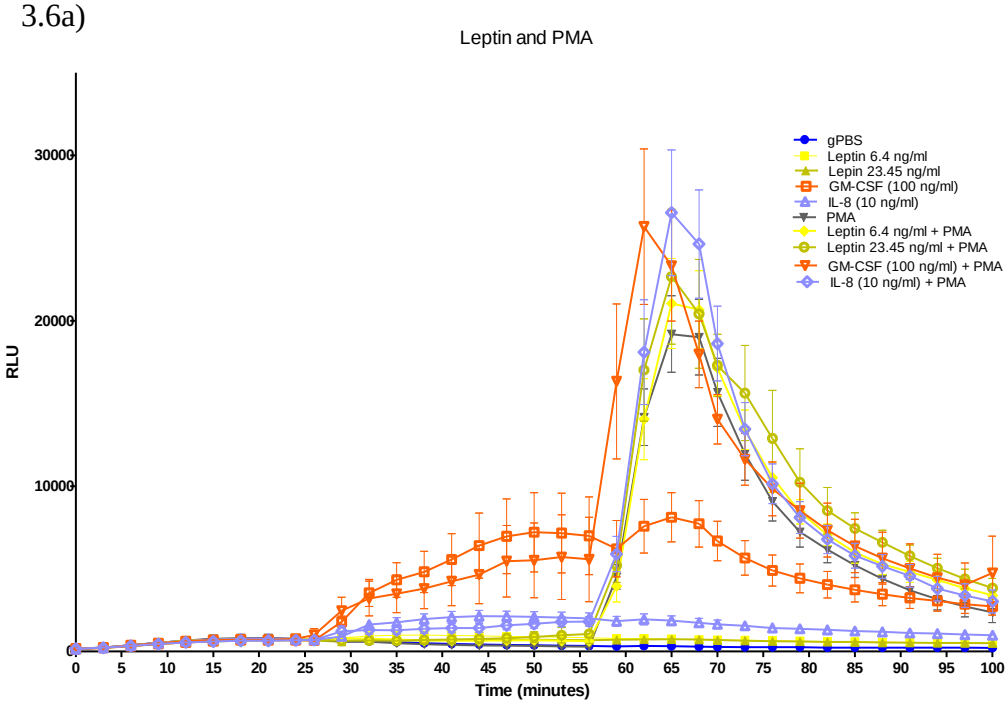
3.5b)

Figure 3.5 a and 3.5b ROS assay result with lean versus obese concentrations of TNFα and IL-6 with *S. aurues* stimulus.

ROS assay with lean versus obese concentrations of adiponectin and leptin with controls IL-8 and GM-CSF and PMA stimulus

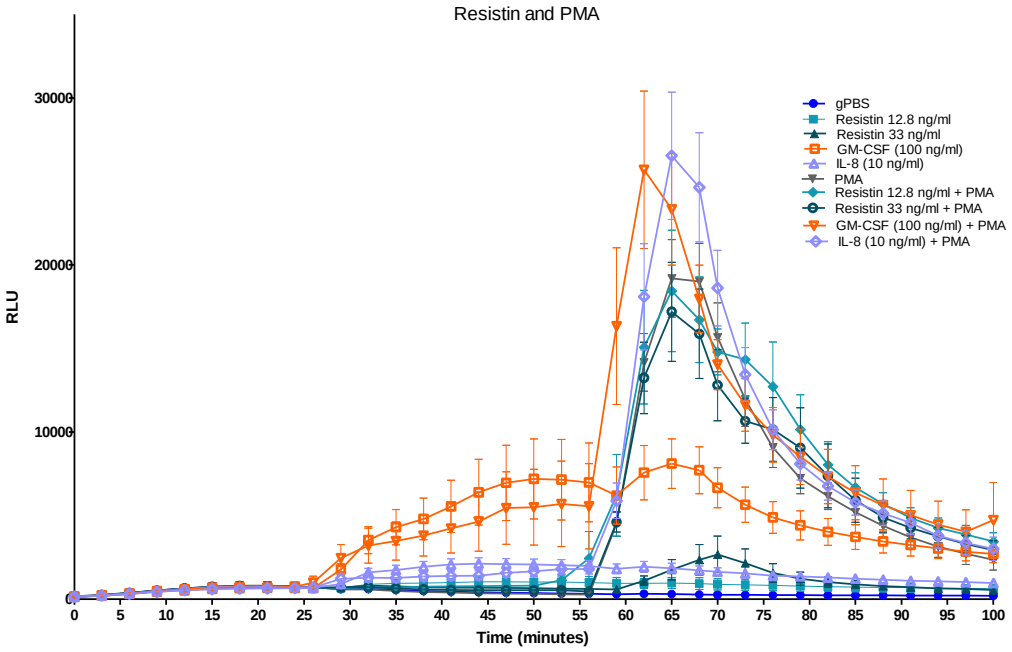


3.6a)



3.6b)

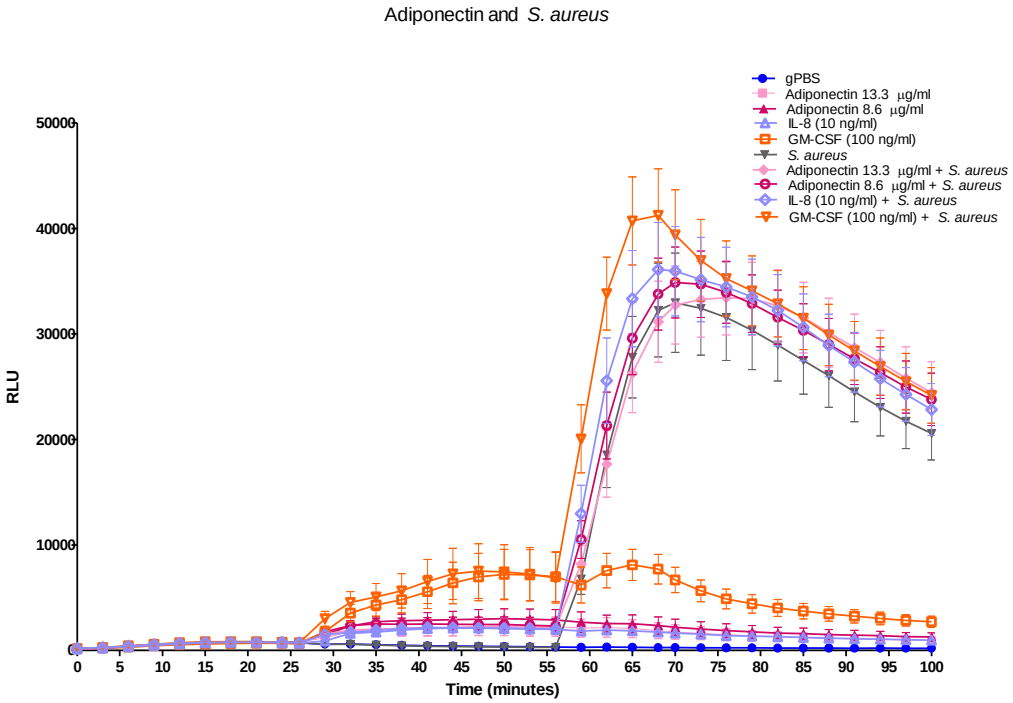
ROS assay with lean versus obese concentrations of resistin with controls IL-8 and GM-CSF and PMA stimulus



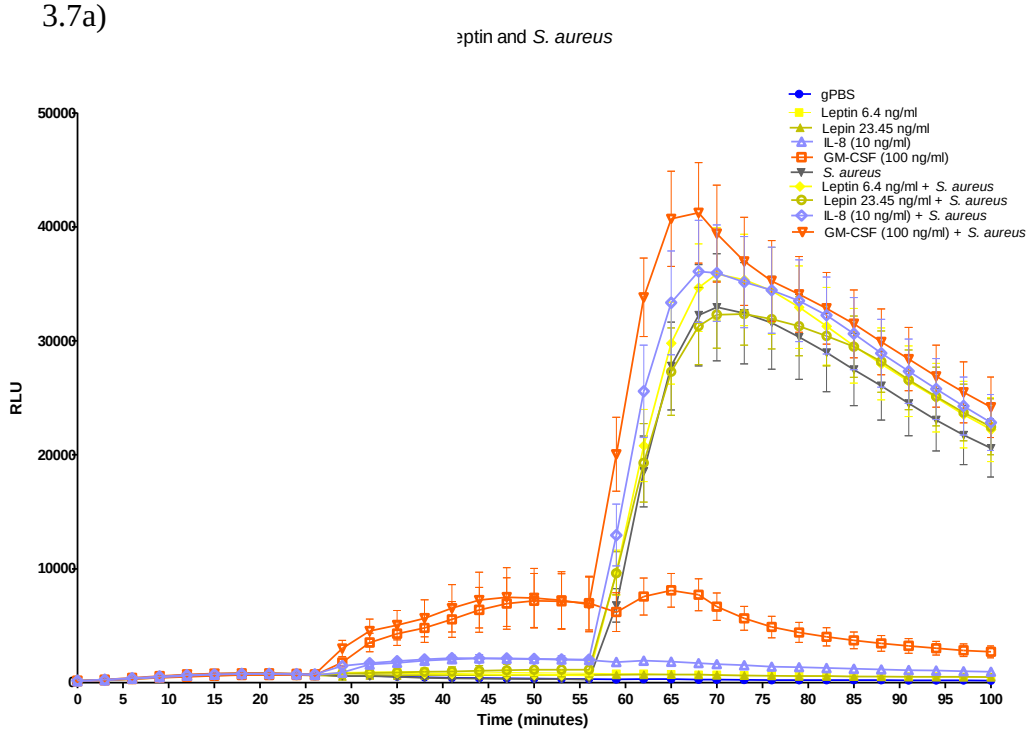
3.6c)

Figure 3.6 a,b,c ROS assay results with adiponectin, leptin, resistin, GM-CSF and IL-8 with PMA stimulus

ROS assay with lean versus obese concentrations of adiponectin and leptin with controls IL-8 and GM-CSF and *S. aureus* stimulus

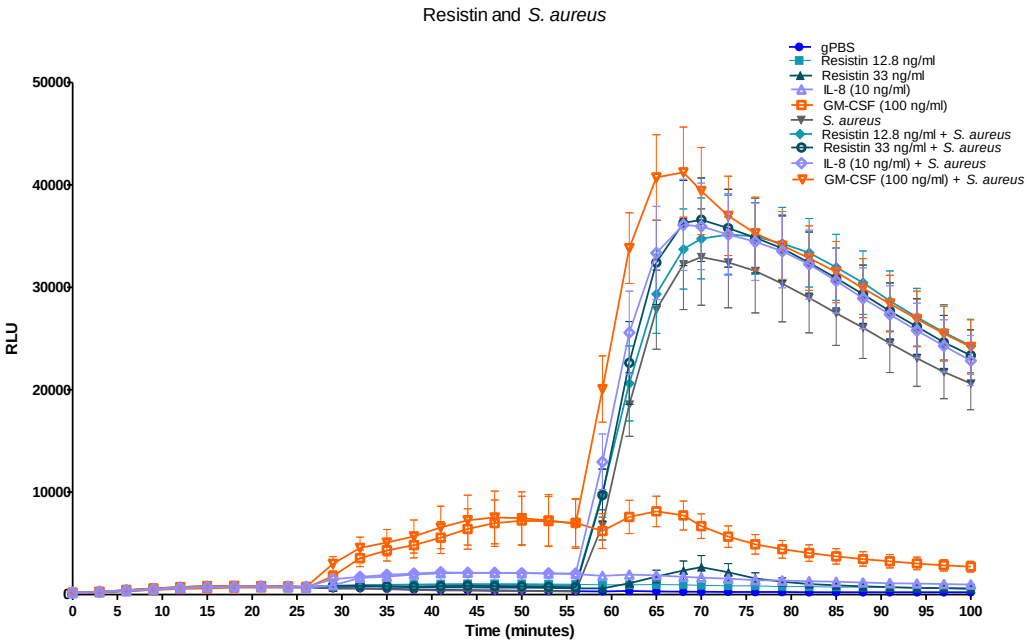


3.7a)



3.7b)

ROS assay with lean versus obese concentrations of resistin with controls IL-8 and GM-CSF and *S. aureus* stimulus



3.7c)

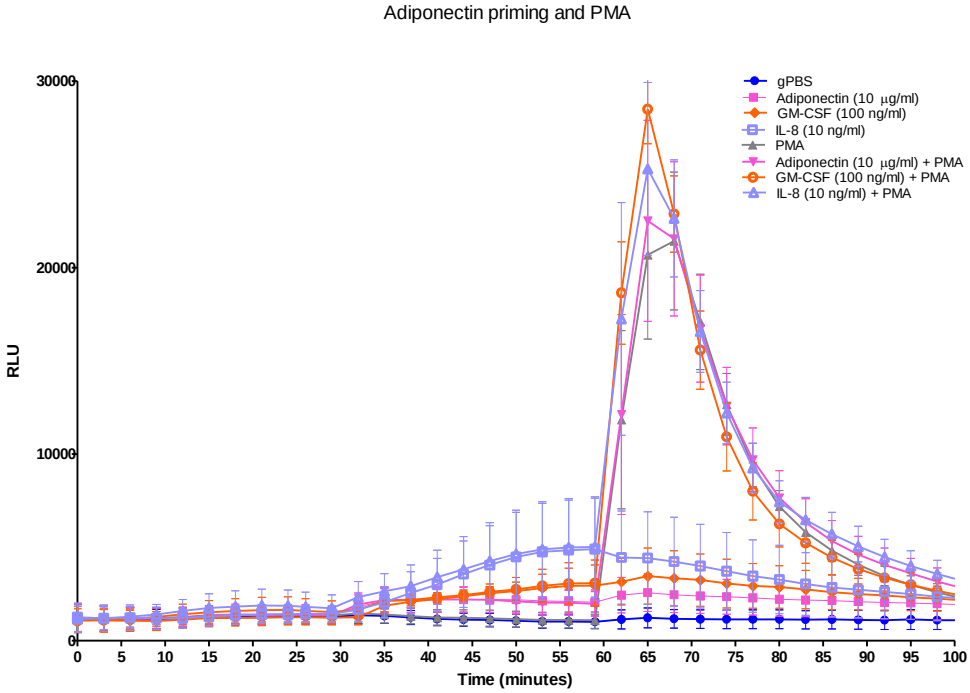
Figure 3.7a, b and c. ROS assay results with adiponectin, leptin, resistin, GM-CSF and IL-8 with *S. aureus* stimulus.

3.3.2 ROS with priming concentrations of adipokines

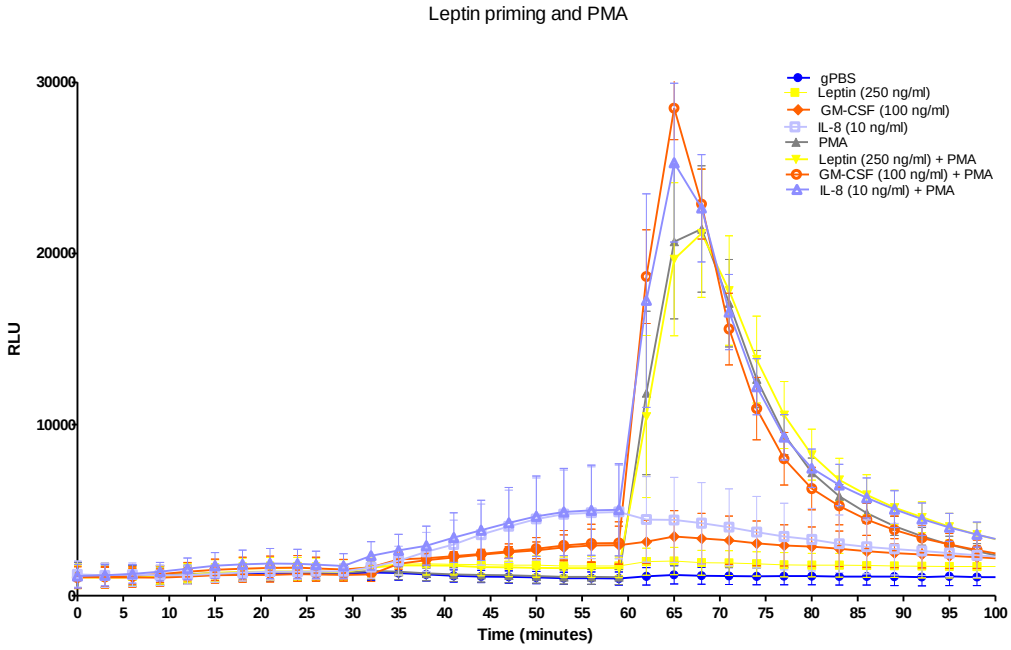
Figures 3.8, 3.9 and 3.10 show ROS assay results with the adipokines at "priming concentrations" i.e. the concentration at which the cell is affected according to the scientific literature. Adiponectin, resistin and leptin do not show an increase in ROS activity upon stimulation with PMA (figures 3.8a-c). Stimulation with *S. aureus* shows an increase for leptin and resistin over the control but not for adiponectin (which would be expected due to its anti-inflammatory properties) (figures 3.9 a,b and c). GM-CSF and IL-8 enhances the respiratory burst in all the ROS assays (figures 3.8 and 3.9). Priming with TNFα and IL-6 show enhanced ROS activity with increasing concentrations compared to stimulus alone (3.10 a-b).

The peak and integration data were also analysed from the priming ROS assays, however there were no statistically significant differences at any of the three times points (data not shown). This is probably due to the low numbers of individuals used in the assays (n = 5). In some cases an aberrant result, probably due to pipetting errors, meant exclusion of that result. This demonstrates the importance of larger numbers of individuals in these experiments to normalise any outlying data and provide a more accurate reflection of what may be affecting neutrophil activity.

ROS assay with priming concentrations of adiponectin and leptin with controls IL-8 and GM-CSF and PMA stimulus

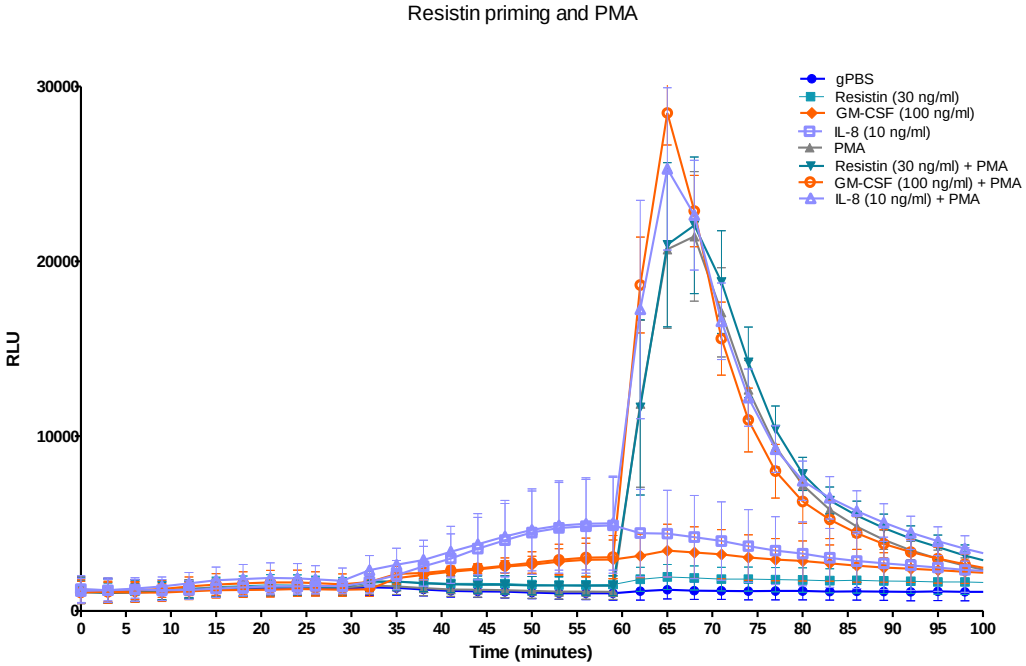


3.8 a)



3.8 b)

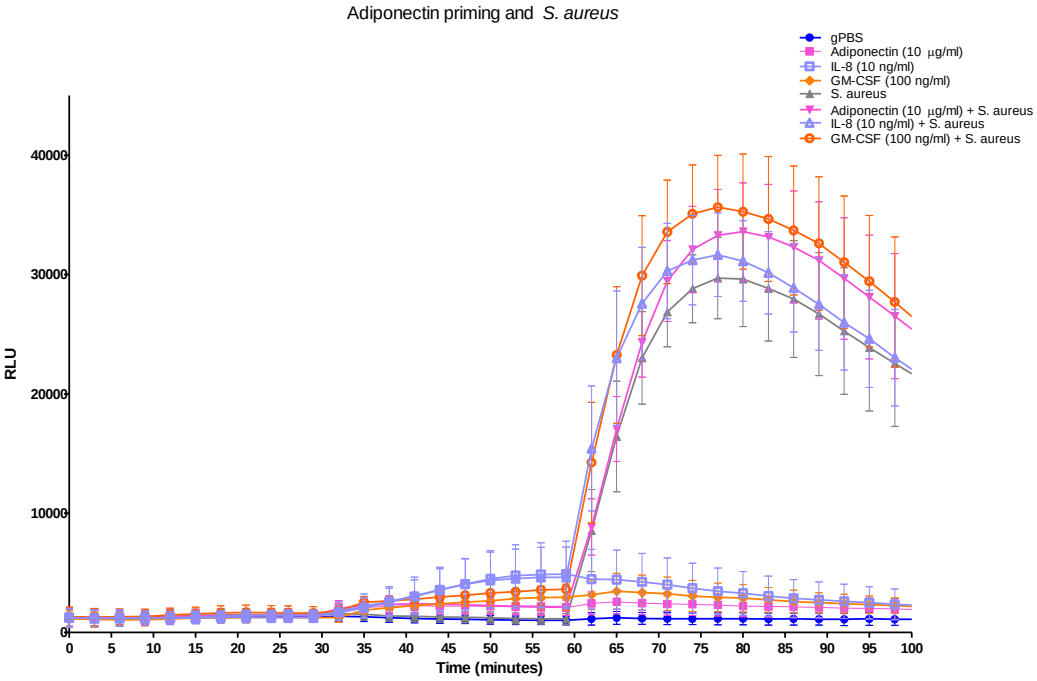
ROS assay with priming concentrations of adiponectin and leptin with controls IL-8 and GM-CSF and PMA stimulus



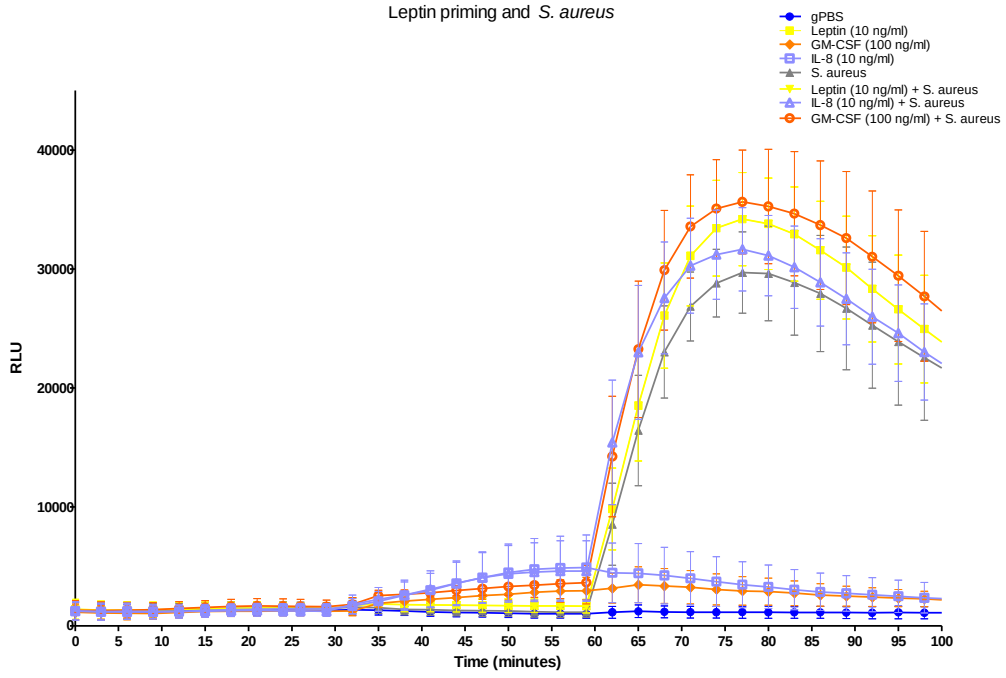
3.8 c)

Figure 3.8 a,b,c ROS assay results with priming concentrations of adiponectin, leptin and resistin, controls GM-CSF and IL-8, and PMA stimulus.

ROS assay with priming concentrations of adiponectin and leptin with controls IL-8 and GM-CSF and *S. aureus* stimulus

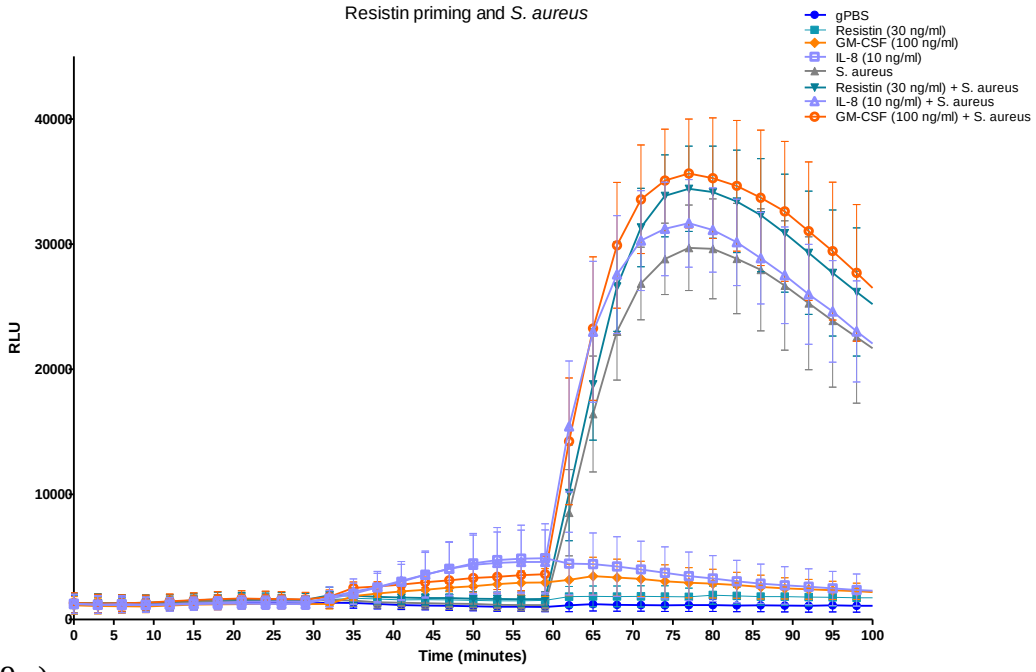


3.9 a)



3.9 b)

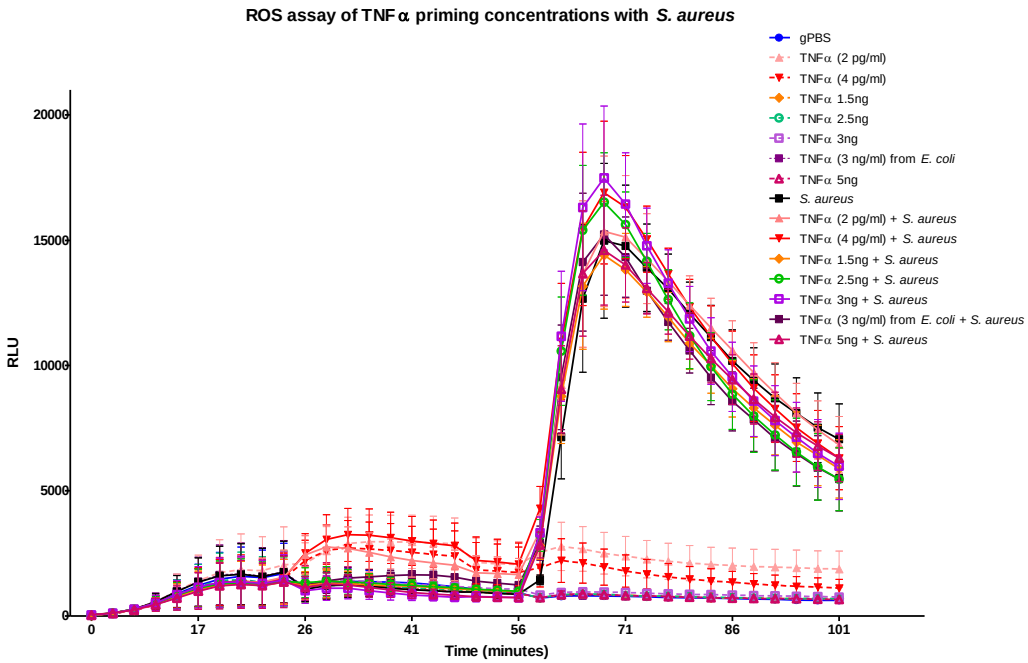
ROS assay with priming concentrations of resistin with controls
IL-8 and GM-CSF and *S. aureus* stimulus



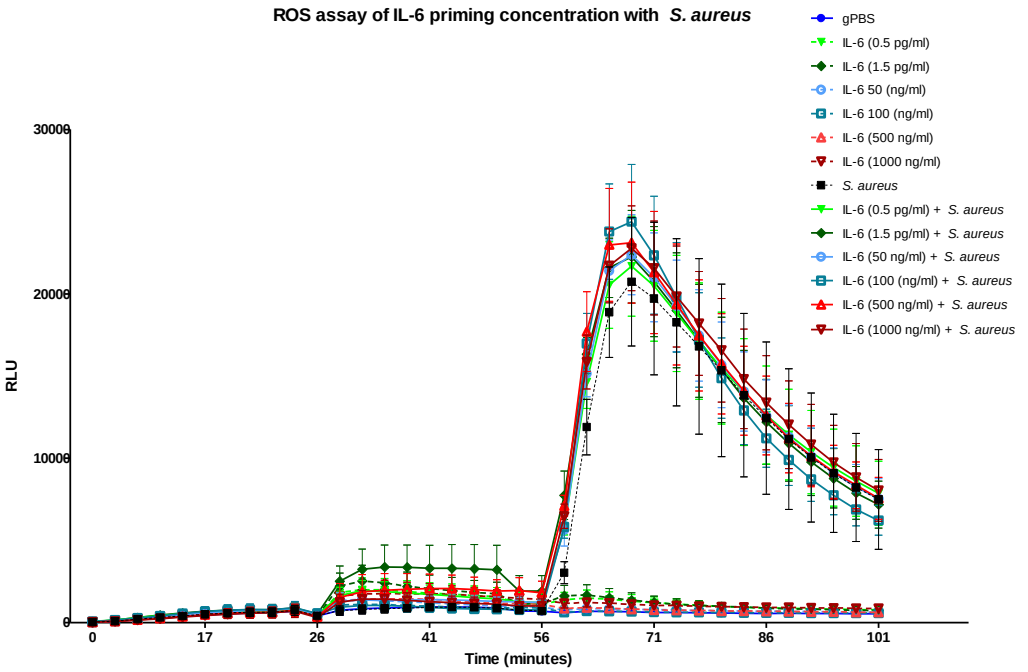
3.9 c)

Figure 3.9 a-c ROS assay results with priming concentrations of adiponectin, leptin and resistin, controls GM-CSF and IL-8, and *S. aureus* stimulus.

ROS assay with priming concentrations of TNFα and IL-6 with *S. aureus* stimulus



3.10 a)



3.10 b)

Figure 3.10a-b ROS assay results with priming concentrations of TNFα and IL-6 with *S. aureus* stimulus.

3.3 NET assays

3.3.1 Control MNase assay

To control for any interference of adipokines in the NET MNase assay experiments were run with all components except for neutrophils. Increases in fluorescence with varying quantities of DNA were proportional to the increasing DNA quantity added. Results are shown in figures 3.11a-c and 3.12a-b, representative of two separate experiments. Results show that *S. aureus* appears to interfere with AFU readings at lower DNA concentrations. This is also true when *S. aureus* is combined with leptin and resistin. Data is representative of one individual with each condition performed in triplicate.

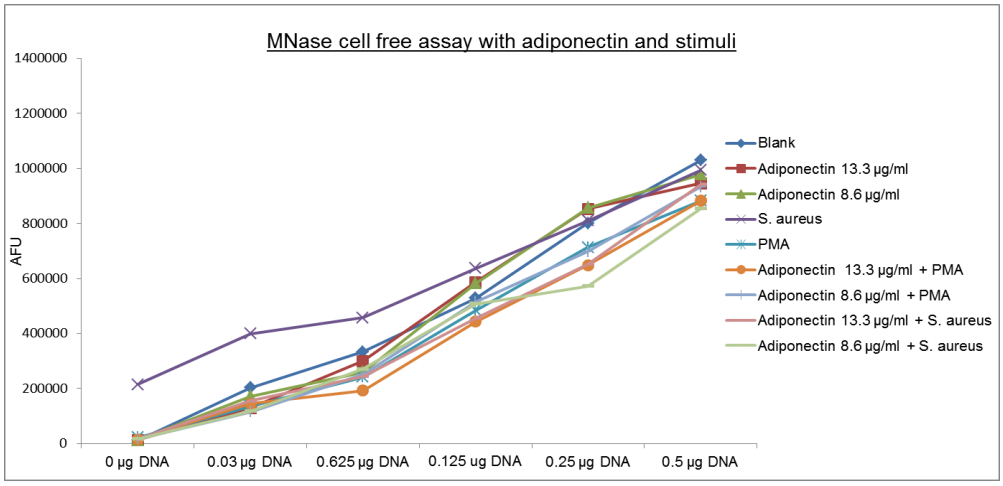


Figure 3.11a

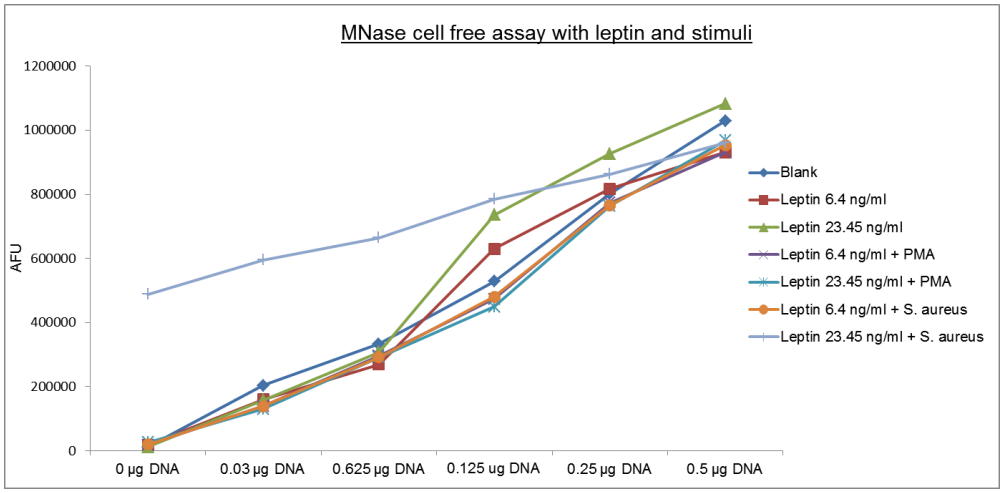


Figure 3.11b

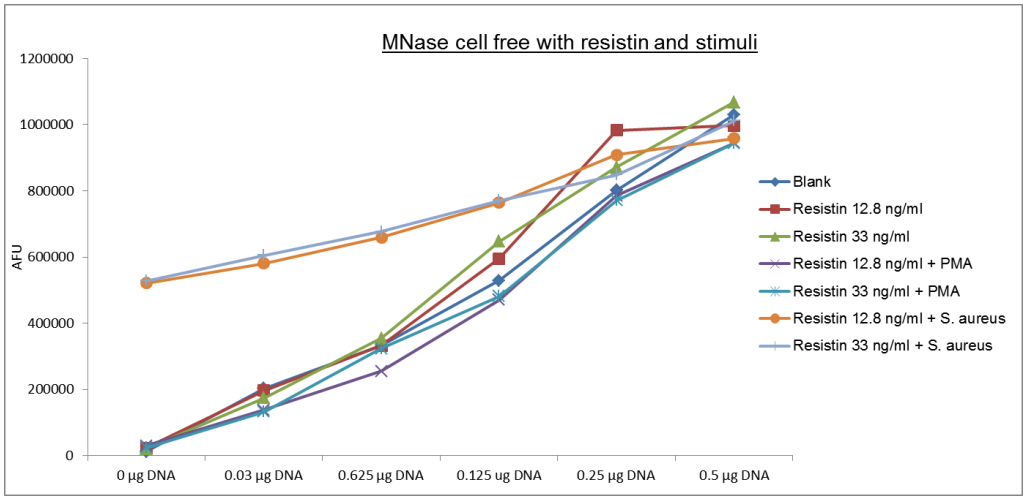


Figure 3.11c

Figure 3.11 MNase cell free assays with adipokines and stimuli

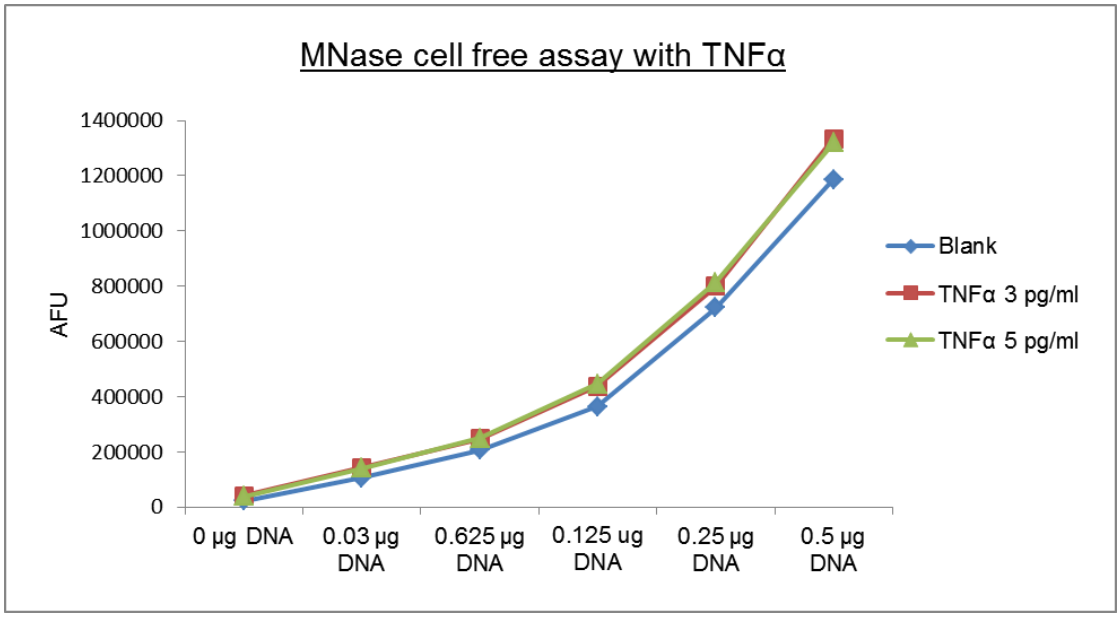


Figure 3.12a

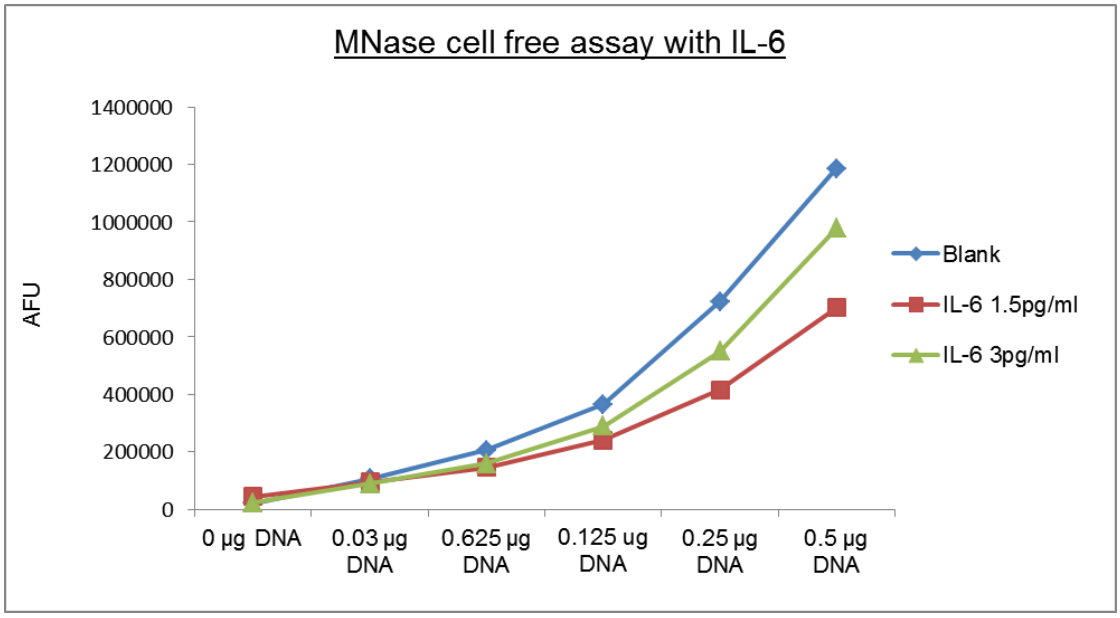


Figure 3.12b

Figure 3.12 Cell free MNase with adipokines.

3.3.2 MNase assay

Tables 3.6 and 3.7 summarise the NET MNase assay. The raw data is shown in figures 3.13- 3.15. Wilcoxon matched-pairs signed rank test was performed on for all NET results. Adiponectin and leptin showed no overall significant results. Interestingly resistin showed a decrease in NET production when incubated with the neutrophils at obese concentrations over lean in the absence of any stimulus ($P = 0.02$), which was unexpected as no NETs should be produced in the absence of stimuli, however after stimulation with PMA there was a significant increase in NET production at the obese concentrations over lean. Obese concentrations of TNFα increased NET production over lean when stimulated with *S. aureus* ($P = 0.03$). IL-6 obese concentrations enhanced NET production after stimulation with *F. nucleatum*.

Priming agent ↓	Stim-ulation →	No stimulation	*F.nucleatum*	Opsonised *S. aureus*	PMA
TNFa		-	-	↑ p=0.03	
IL-6		-	↑p=0.01	-	
Adiponectin		-		-	-
Leptin		-		-	-
Resistin		↓p=0.02		-	↑p=0.02

Table 3.6 Summary of NET MNase quantification after priming concentrations (lean & obese) of adipokines and various stimuli. - denotes no significant difference between lean and obese adipokine-stimulant ROS production; ↑ denotes a significant increase in obese versus lean neutrophils for the stimulant highlighted. ↓ denotes a significant decrease in lean versus obese treated neutrophils for the stimulant highlighted.

Priming agent ↓	Stim-ulation →	No stimulation	*F.nucleatum*	Opsonised *S. aureus*	PMA
GM-CSF		-		-	-
IL-8		-		-	-

Table 3.7 Summary of NET MNase quantification after priming concentrations of the controls GM-CSF and IL-8 versus stimulus in the absence of any priming agent.

MNase results with TNFa/IL-6 and *F. nucleatum/S. aureus* stimuli

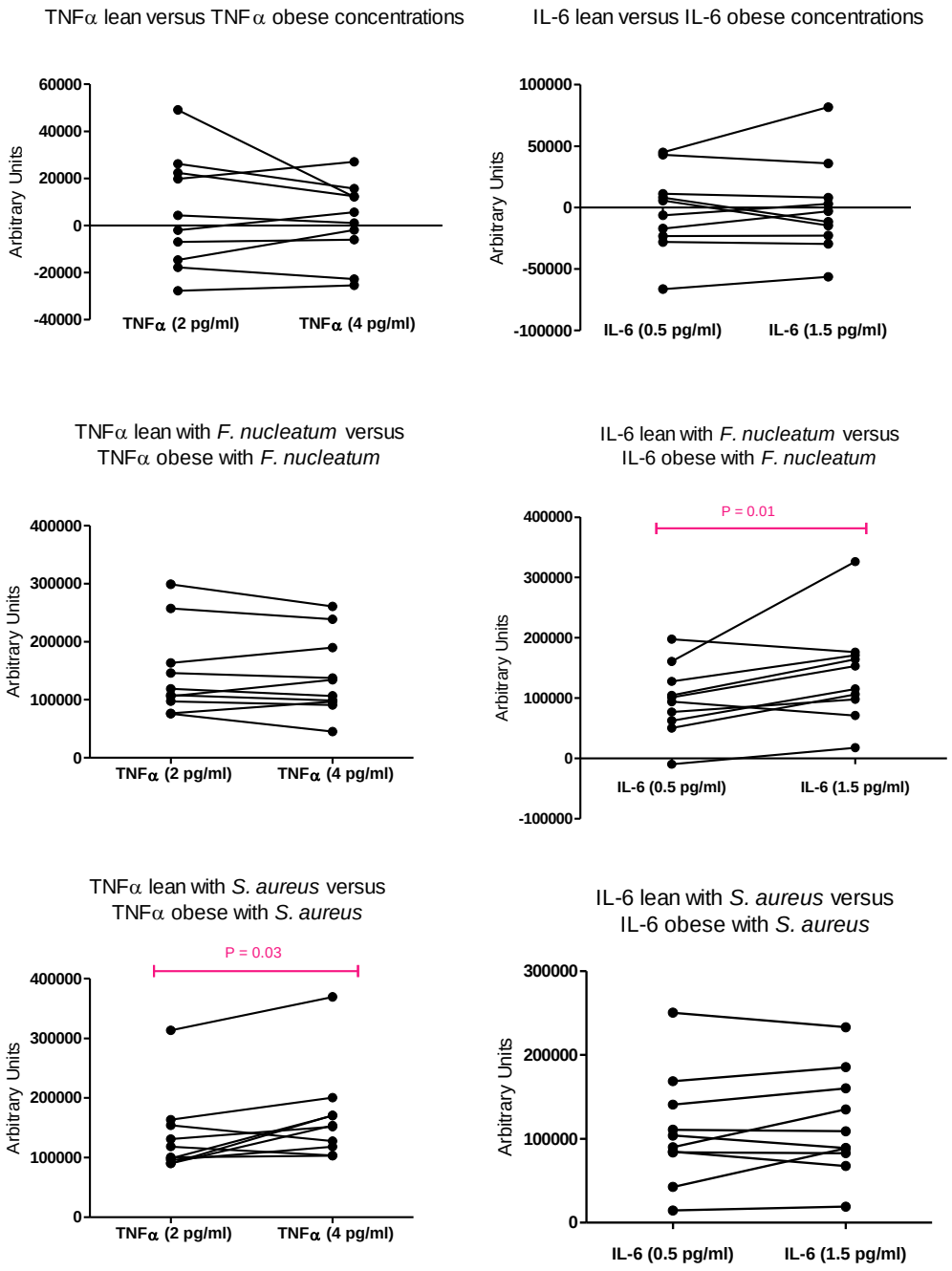


Figure 3.13 MNase results with TNFα and IL-6 lean versus obese concentrations alone, with *S. aureus* and with *F. nucleatum* stimulus.

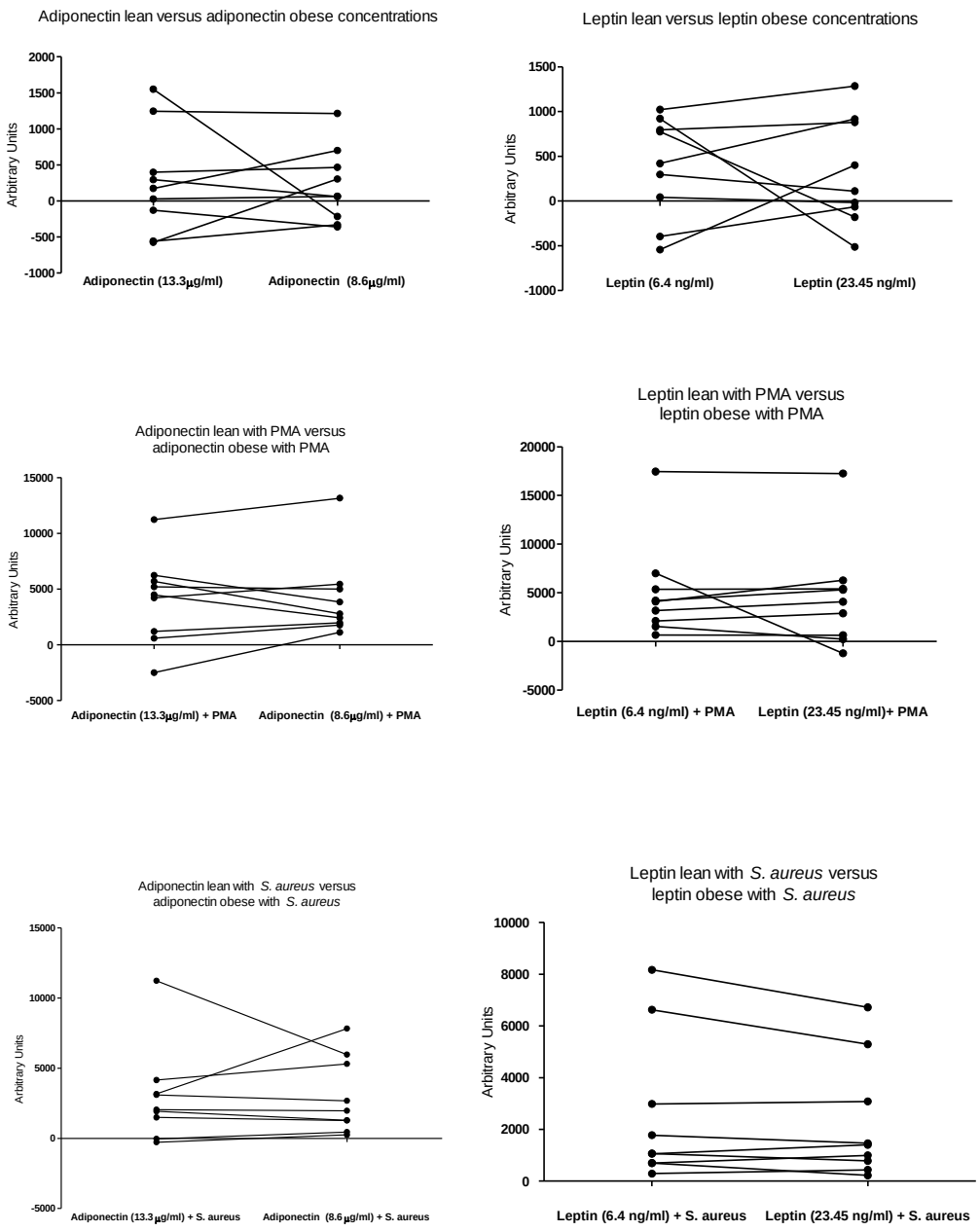


Figure 3.14 MNase results with adipopnectin and leptin lean versus obese concentrations alone, with PMA and *S. aureus* stimulus.

MNase results with resistin / GM-CSF and PMA / *S.aureus* stimuli

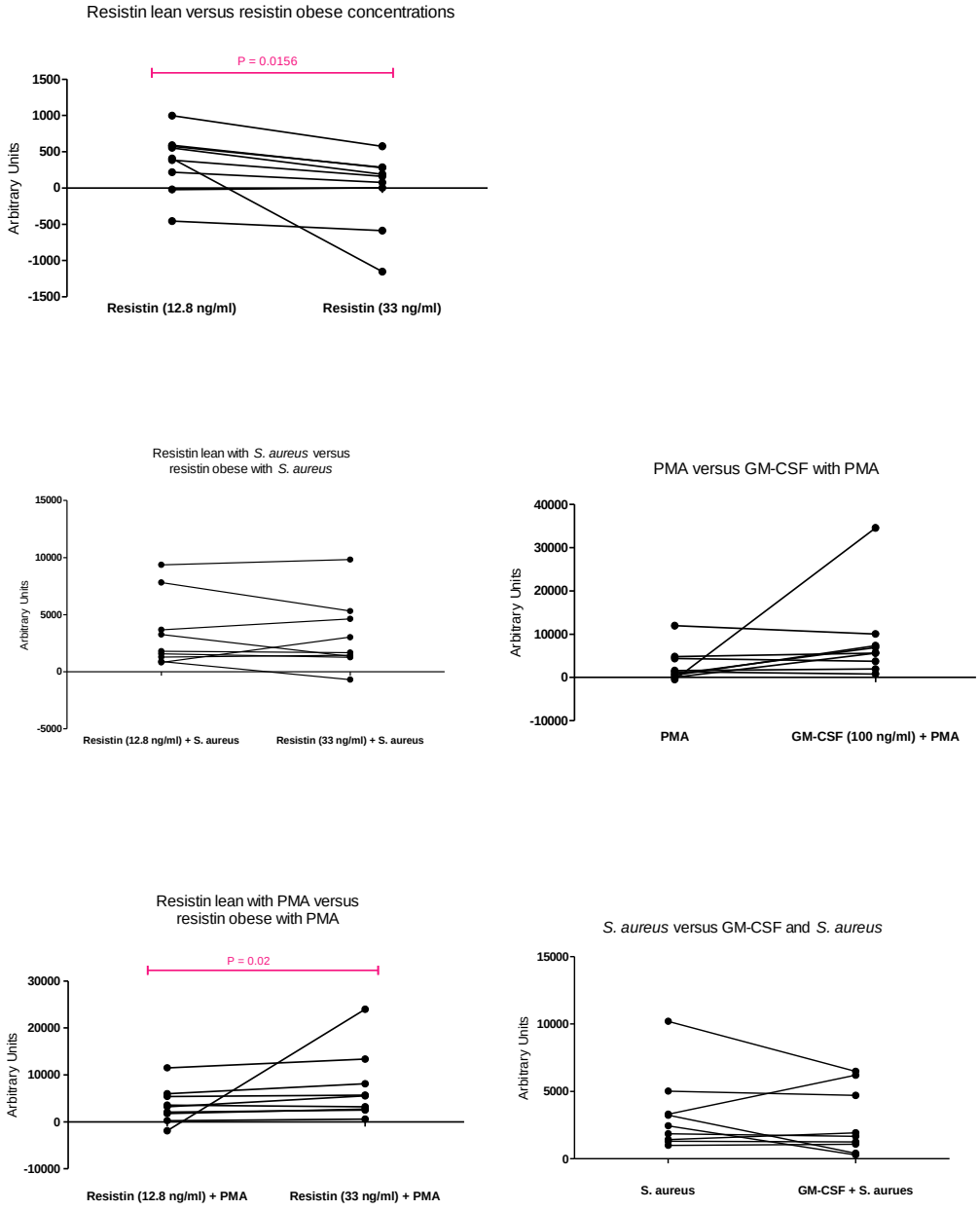


Figure 3.15 MNase results with resistin lean versus obese concentrations alone, with PMA and *S. aureus* stimulus. Results for the control GM-CSF is also shown.

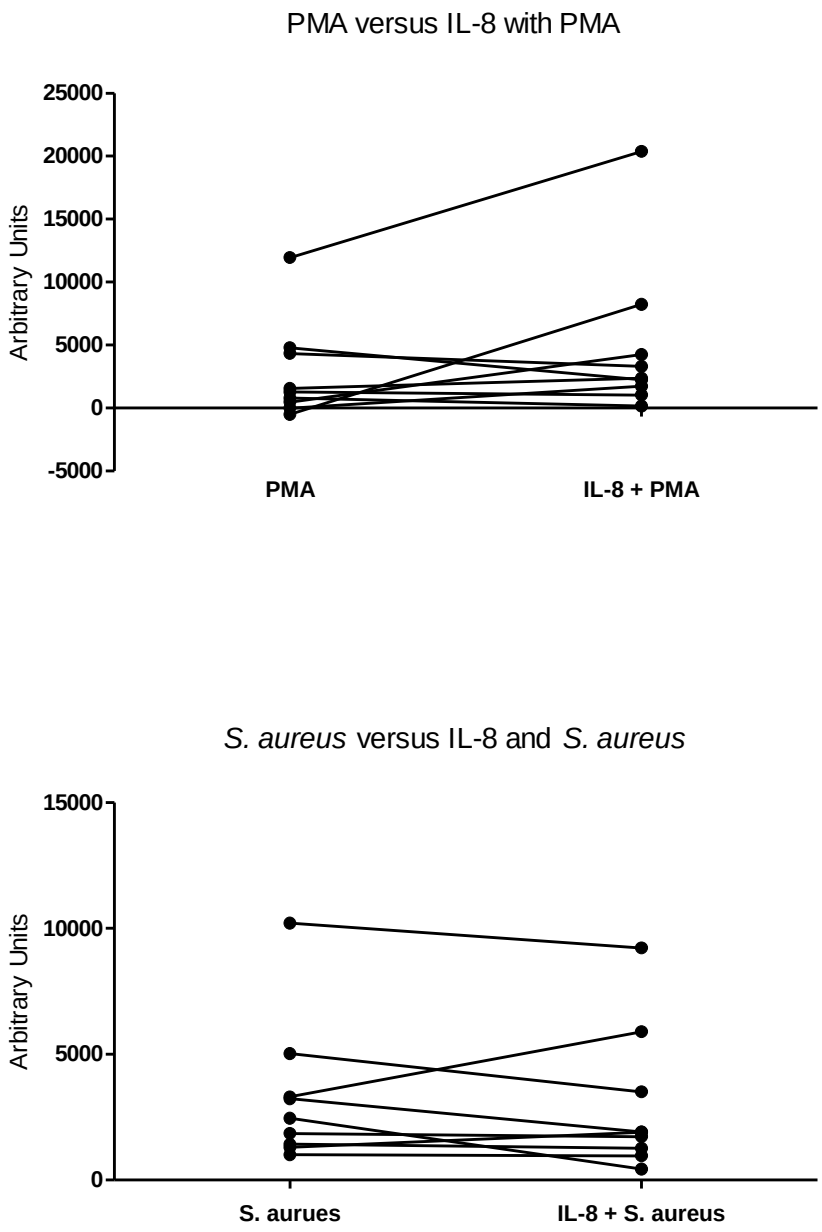
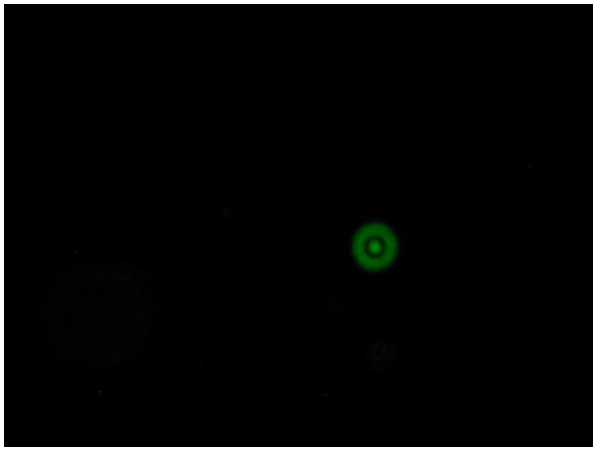


Figure 3.16 MNase results for the control IL-8 with PMA and *S. aureus* stimulus.

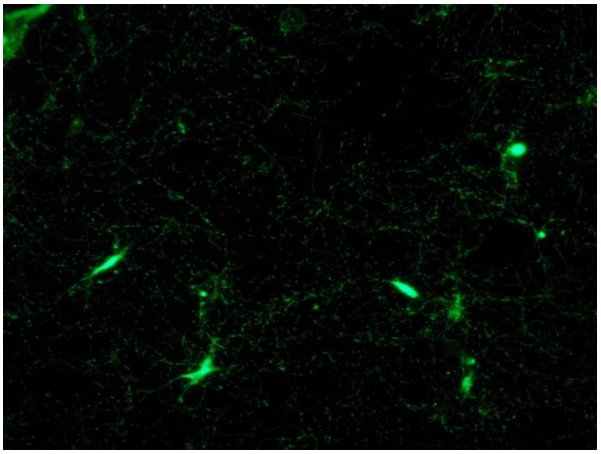
3.3.3 NET visualisation assay

The NET visualisation assay provides an image backdrop to the MNase assay, where NETs formed can be visualised but not quantified. There are clear differences in the photos between stimulus and blank but also the NETs formed between the different stimuli – *F. nucleatum* results in thinner extrusions and *S. aureus* is more rounded in appearance and PMA seems to generate long and rounded NETs (figure 3.17). Of the images taken with adipokine incubation prior to stimulus, some images reflect the quantified results and also the ROS result, for example TNFα lean versus obese – the NETs generated from incubation with the obese concentration appear thicker and longer than lean (figures 18-21).

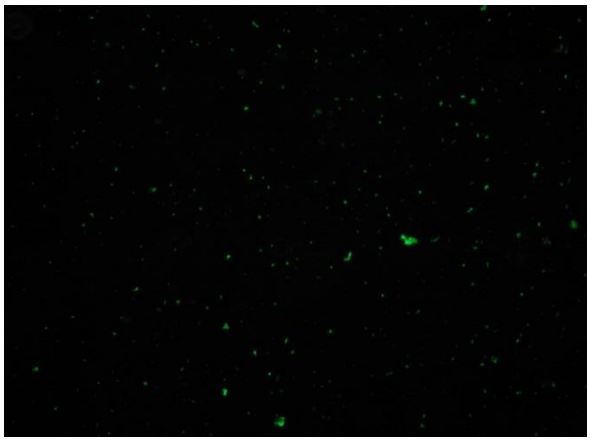
NET visualisation photos of blank and stimulus



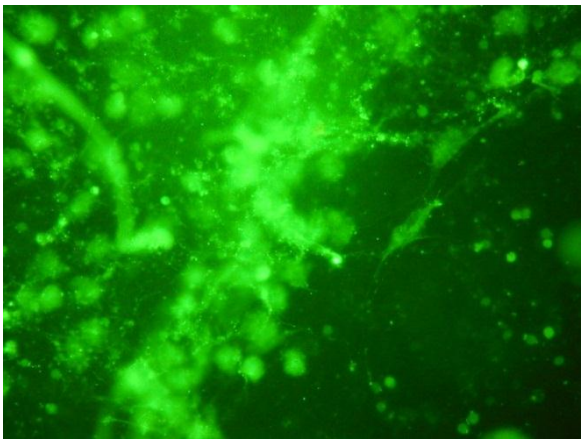
Blank



F. nucleatum



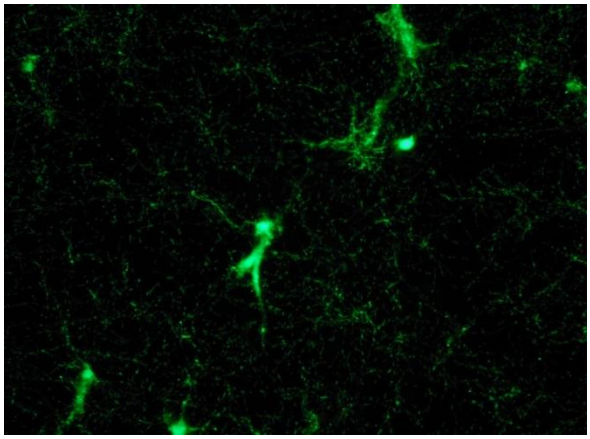
S. aureus



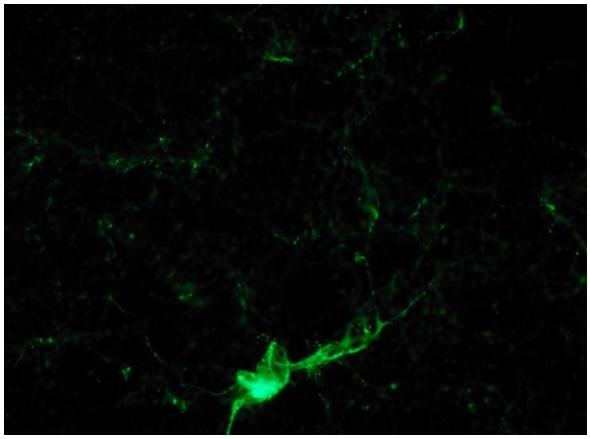
PMA

Figure 3.17 NET visualisation photos: controls

NET visualisation photos of IL-6 with *F. nucleatum*



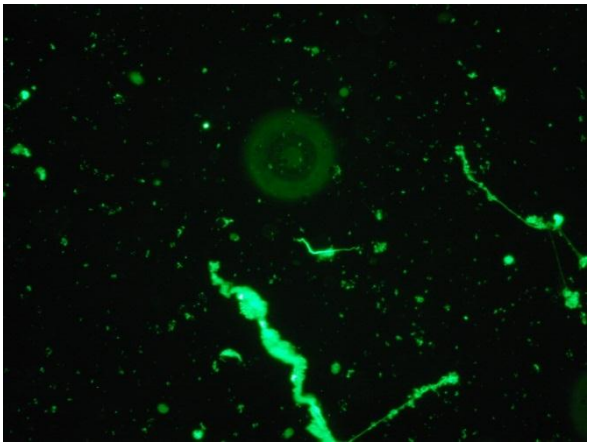
IL-6 (0.5 pg/ml) with *F. nucleatum*



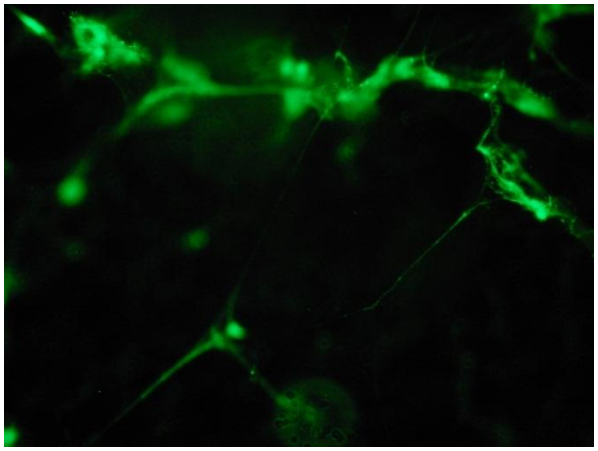
IL-6 (1.5 pg/ml) with *F. nucleatum*

Figure 3.18 NET visualisation photos: IL-6 with *F. nucleatum*

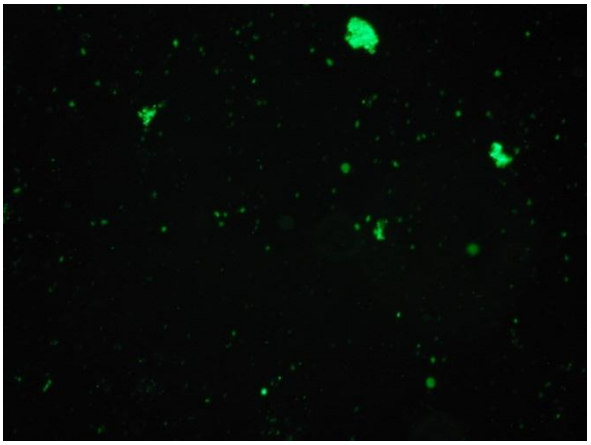
NET visualisation photos of TNfα and IL-6 with *S. aureus*



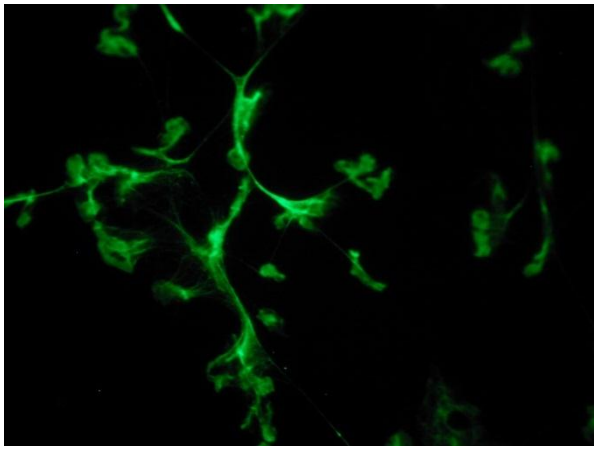
TNFα (2 pg/ml) with *S. aureus*



TNFα (4 pg/ml) with *S. aureus*



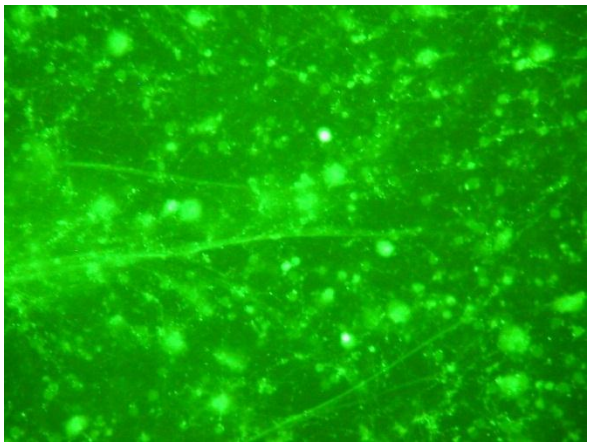
IL-6 (0.5 pg/ml) with *S. aureus*



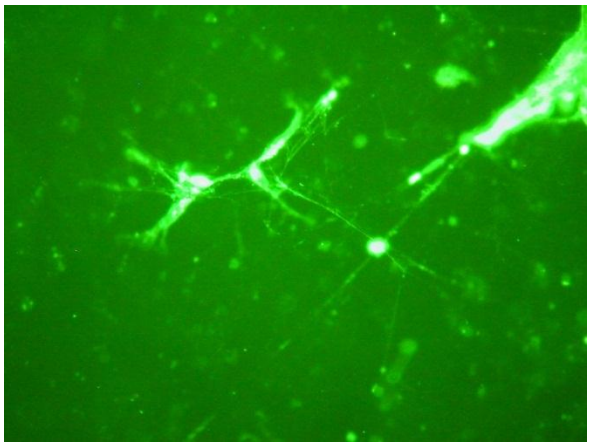
IL-6 (1.5 pg/ml) with *S. aureus*

Figure 3.19 NET visualisation photos: TNFα and IL-6 with *S. aureus*

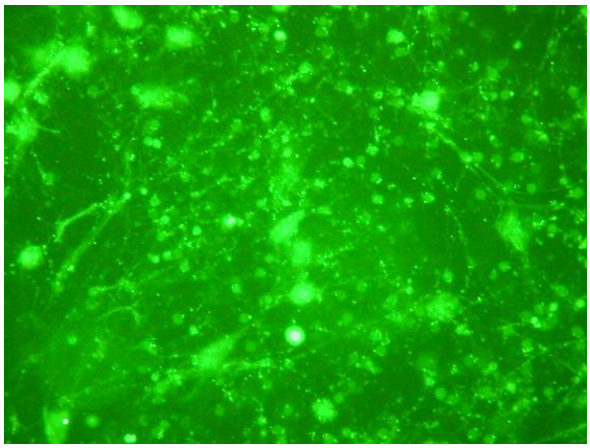
NET visualisation photos of resistin, GM-CSF and IL-8 with *S. aureus*



Resistin (33 ng/ml) and *S. aureus*



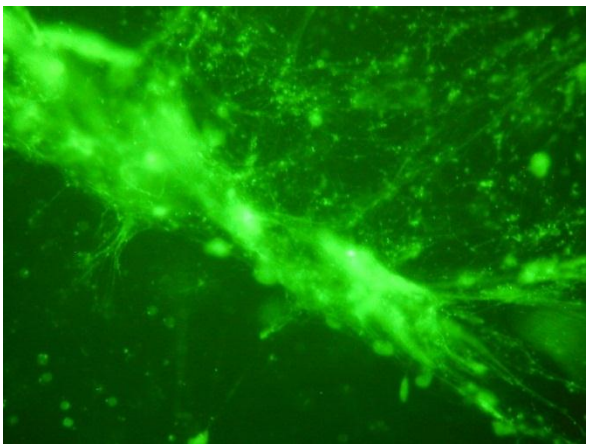
IL-8 (10 ng/ml) and *S. aureus*



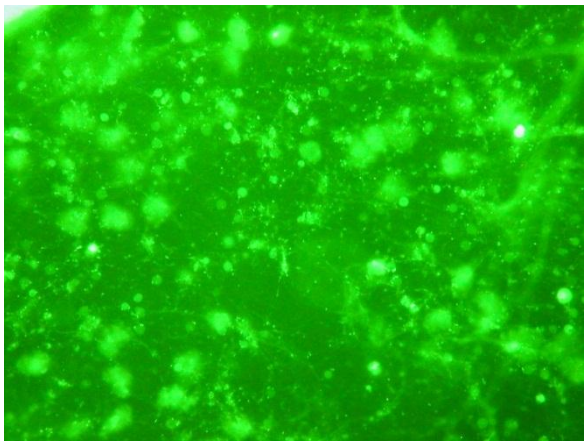
GM-CSF (100 ng/ml) and *S. aureus*

Figure 3.20 NET visualisation photos: adipokines with *S. aureus*

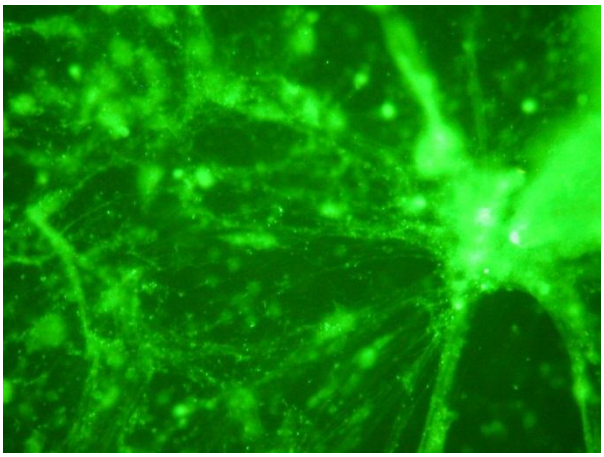
NET visualisation photos of adiponectin, leptin, GM-CSF and IL-8 with PMA



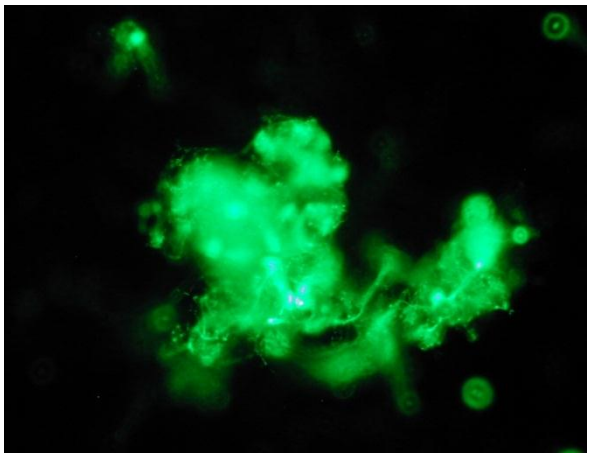
Adiponectin (8.6 µg/ml) and PMA



Leptin (23.45 ng/ml) and PMA



IL-8 (10 ng/ml) and PMA



GM-CSF (100 ng/ml) and PMA

Figure 3.21 NET visualisation photos: adipokines with PMA

4. Discussion

This project shows that adipokine concentrations reflective of obese individuals appear capable of affecting neutrophil function when challenged with an activating stimulus over lean concentrations under certain stimulation conditions. Obese concentrations of IL-6, leptin and adiponectin enhanced the ROS potential of neutrophils derived from healthy lean males compared to incubation with lean concentrations. Furthermore resistin, IL-6 and TNFα were able to increase NET production in the neutrophils over stimulus alone. These results suggest a pro-inflammatory potential of these agents in contributing to the low-grade chronic inflammatory phenotype present in obese individuals. Adiponectin, which has largely anti-inflammatory roles (as highlighted in table 1.1) is lower in obese individuals compared to lean and results of this study support the protective role of adiponectin as obese concentrations generated significantly higher ROS than in neutrophils pre-incubated with lean concentrations.

It is important to note the different effects these adipokines can have on neutrophil activity, for example, at low concentrations, IL-8 stimulates L-selectin shedding and the increased expression of β2 integrins which aid the process of neutrophil arrest during migration to the site of inflammation. At higher concentrations the oxidative burst is initiated, and higher still IL-8 induces degranulation of neutrophils (Ley 2002).

Leptin, resistin and adiponectin concentrations were taken from studies that employed ELISAs to determine the lean and obese concentration from blood plasma of volunteers, whose numbers ranged from 8 (Rosicka *et al.* 2003) to 52 people (Cohen *et al.* 2008). In contrast TNFα and IL-6 concentrations were derived from a study by Murdolo *et al.* (2008) that measured *in situ* profiling of adipokines in

subcutaneous microdialysates from 6 lean and 6 obese individuals. How truly representative these chosen concentrations are of physiological levels is not clear; the literature review (table 3.1) identified a large variation in reported levels of some of the adipokines. This could be for several reasons which are outlined below.

a) Differences in experimental technique, for example Wozniak *et al.* (1993) incubated neutrophils for under 10 minutes prior to and after the addition of priming agent and stimuli, and a longer incubation time may generate different results.
b) The sex of the volunteers can impact upon physiological adipokine concentration findings, for example, serum leptin levels have been found to be significantly higher in healthy women (29.5 ng/ml) than healthy men (4 ng/ml) (Cohen *et al.* 2011).
c) Adipokine levels can differ greatly within sexes, for example a study by Illan-Gomez *et al.* 2012 found adiponectin levels in obese women (5.82 µg/ml with a SD of 2.93) and lean women (16.68 µg/ml with a SD of 64.17), clearly within lean women there is huge variation.
d) Age differences of the volunteers can impact upon results, for example Andreasson *et al.* 2012 found leptin and adiponectin levels increased with advancing age adults. Furthermore older persons tend to have higher fat composition and therefore risk assessment by BMI alone is less accurate.
e) The health status of the volunteers had to be taken into consideration when selecting obese and lean concentrations from the literature, for example Onat *et al.* 2008 used volunteers that comprised individuals with disease such as type 2 diabetes and coronary heart disease.
f) The sample in which the adipokine levels were measured (e.g. plasma versus serum) can also give varied concentrations in lean and obese individuals.

Indeed Murdolo *et al.* 2008 acknowledge the concentrations of adipokines they found may not be truly representable of actual interstitial levels, for example, the values were not adjusted for the *in vivo* recovery factor.

g) Another challenge when addressing which concentrations to use was those found in morbidly obese (BMI > 40 kg/m^2) versus obese individuals (BMI > 27 and > 40 kg/m^2) – ultimately obese levels in otherwise healthy volunteers were selected as these studies generally used "healthy" obese – i.e. no other accompanying obesity-linked diseases such as type 2 diabetes or atherosclerosis.

Another point to consider relates to the "priming concentrations" of adipokines used in this project. Some of the studies noted effects of the adipokines on cells other than neutrophils, for example Lovren *et al.* (2010) used monocytes when studying the effects of adiponectin. Added to this is the effect of the adipokines on the cells, for example Lovren *et al.* found the effects of adiponectin to be anti-inflammatory (as expected), priming human monocytes to differentiate into anti-inflammatory M2 macrophages. Adiponectin "priming" concentrations in neutrophils would presumably lower ROS generation and NET formation; however, due to the low numbers of volunteers used in this project, it is not possible to come to any reliable conclusion. There were also differences in findings for the influence of adipokines on cellular activity; for example, a study by Iqbal *et al.* (2005) found no difference in resistin levels in severe obese individuals after a 6 month dietary intervention program.

Neutrophils are short-lived cells; however the length of time in which they can be bathed in adipokines in the body is considerably longer than the 30 minutes used in this *ex vivo* study. Added to this is the fact that these agents do not work in isolation,

but rather in complex networks involving both pro and anti-inflammatory effects (Preshaw and Taylor 2011). For example, the raised IL-6 levels in obese individuals is can be a consequence of TNF-α release, which can trigger IL-6 release and increase systemic levels of IL-6 (Peterson and Pedersen 2005). Studies in rodents have demonstrated a possible role of leptin in regulating adiponectin; that through fasting a state that lowers leptin levels can also lower adiponectin expression in adipose tissue, with re-feeding increasing levels (Zhang *et al.* 2002). The adipokines can also show divergent effects depending on their concentration, for example, Van Den Berg *et al.* (2001) showed the effects of TNFα to be dose dependent; at low doses (0.1-1 ng/ml) of TNFα neutrophil survival increased, but at higher doses (10 ng/ml) the effect was lost.

In periodontal pathogenesis cytokines have wide ranging and overlapping functions, the balance between pro- and anti-inflammatory cytokines and regulation of their receptors and signalling pathways determines the extent of periodontal tissue destruction.

Studies of ROS generation by peripheral blood neutrophils in periodontal disease have used a variety of patient groups, pathways of activation, neutrophil isolation techniques and differing methods of ROS detection. Results obtained from chemiluminescent assays for detecting ROS can show considerable day-to-day variation, thus selecting gender and age-matched individuals is important to obtain as consistent results as possible. When looking at the integration and peak results it is clear there are some outliers and this could mask the identification of potentially significant responses, for example there may be a general trend shown by the majority of volunteers, however one outlier may prevent statistical significance being achieved.

All experiments were performed in the presence of divalent cations (Mg^{2+} and Ca^{2+}) and for ROS glucose, in an attempt to reflect more accurately the *in vivo* situation. This has been used in similar studies of neutrophils (Matthews *et al.* 2007b) however some of the studies identified from the literature review on adipokines affecting neutrophil function did not enrich buffers with them, for example Nathan *et al.* (1989) and Cohen *et al.* (2008) for the studies of GM-CSF and resistin respectively. Most public work on cytokine biology and immune responses in periodontitis has involved model systems, which are far removed from the microanaomical complexity of the human periodontium (Preshaw and Taylor 2011). More meaningful *in vivo* and *ex vivo* models of periodontits will give a better understanding of how the human host responds to a polymicrobial challenge. Future work could involve the study of other adipokines, such as apelin, vaspin and omentin to provide a more in depth profile of the potential effects of adiopkines on neutrophil function in lean versus obese individuals. The stimulation of neutrophils via other receptors could also be studied, such as Nod-like receptors (NLRs), which are activated by PAMPs such as muramyl dipeptide (MDP) (Franchi *et al.* 2009), which is found on the surface of the peptidoglycan layer of nearly all Gram positive and Gram negative organisms (McDonald *et al.* 2005). NLR1, NLR2 and NLRP3 are expressed by neutrophils and have been shown to bind MDP (Ekman and Cardell 2009).

5. Conclusion

Adipokine concentrations representative of those found in obese individuals have been shown in this pilot study to enhance neutrophil ROS and NET generation after

exposure to bacterial stimulus. The effects are however stimulus dependent and require longer studies to validate any significant results.

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7. Supplementary figures

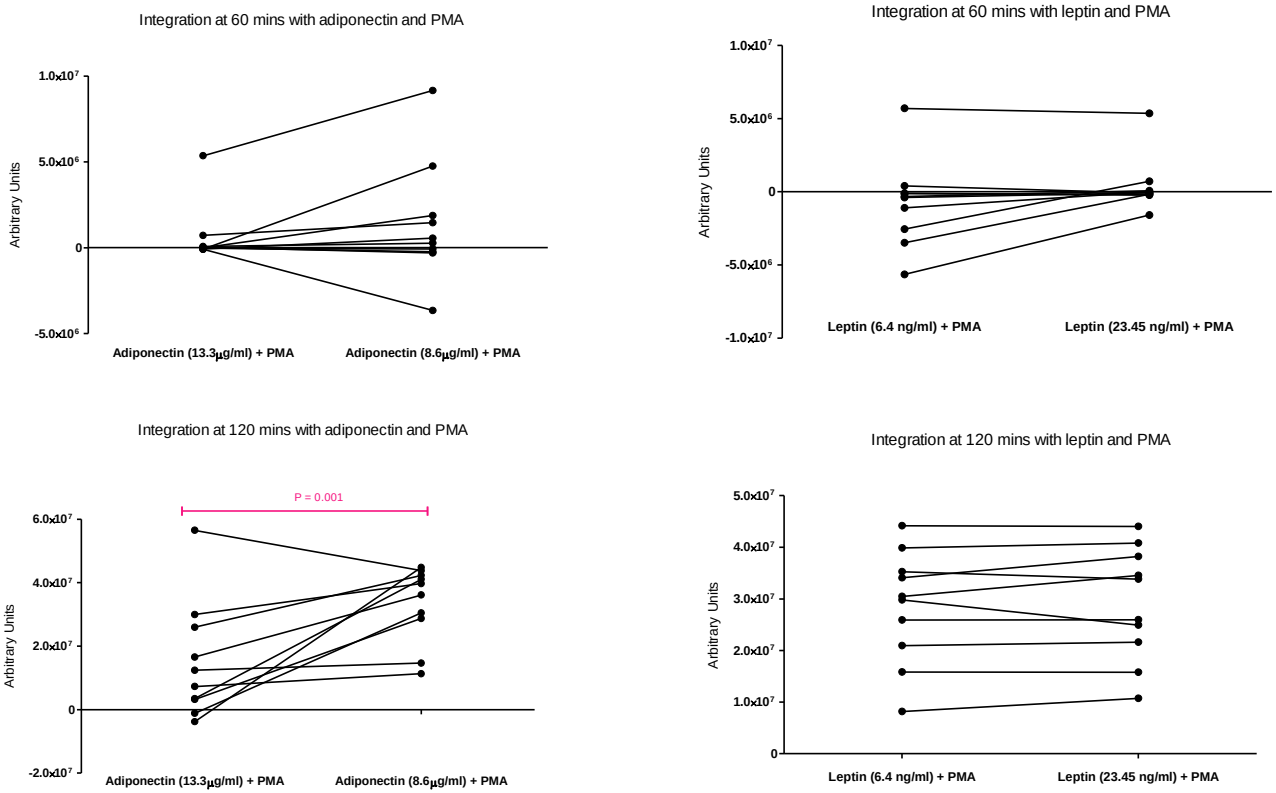


Figure S.1 Integration ROS results with adiponectin and leptin with PMA stimulus.

Integration ROS results with lean versus obese concentrations of resistin and positive control GM-CSF with PMA stimulus

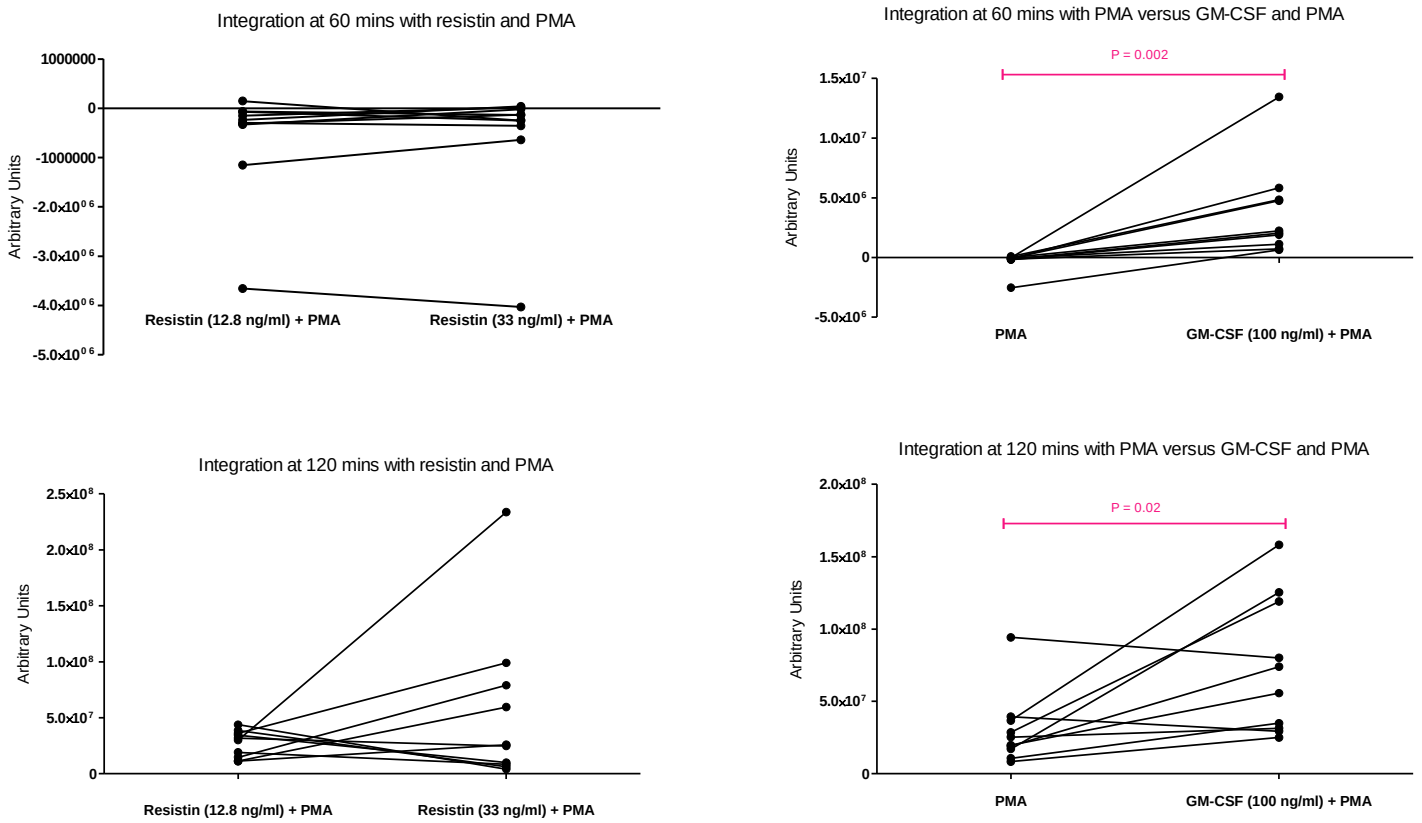


Figure S.2 Integration ROS results with resistin and GM-CSF with PMA stimulus.

Integration ROS results with positive control IL-8 with PMA stimulus

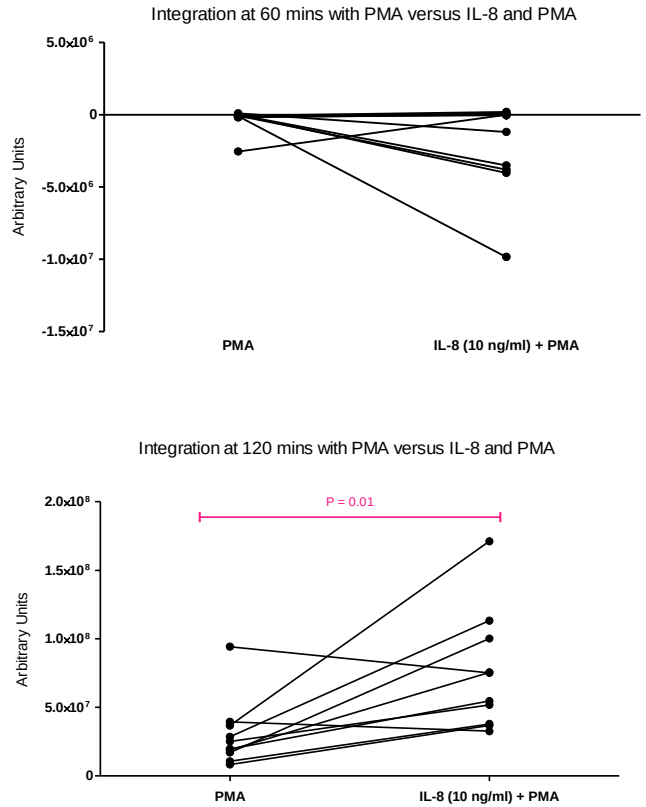


Figure S.3 Integration ROS results with IL-8 with PMA stimulus.

Peak ROS results with lean versus obese concentrations of adiponectin and leptin with PMA stimulus

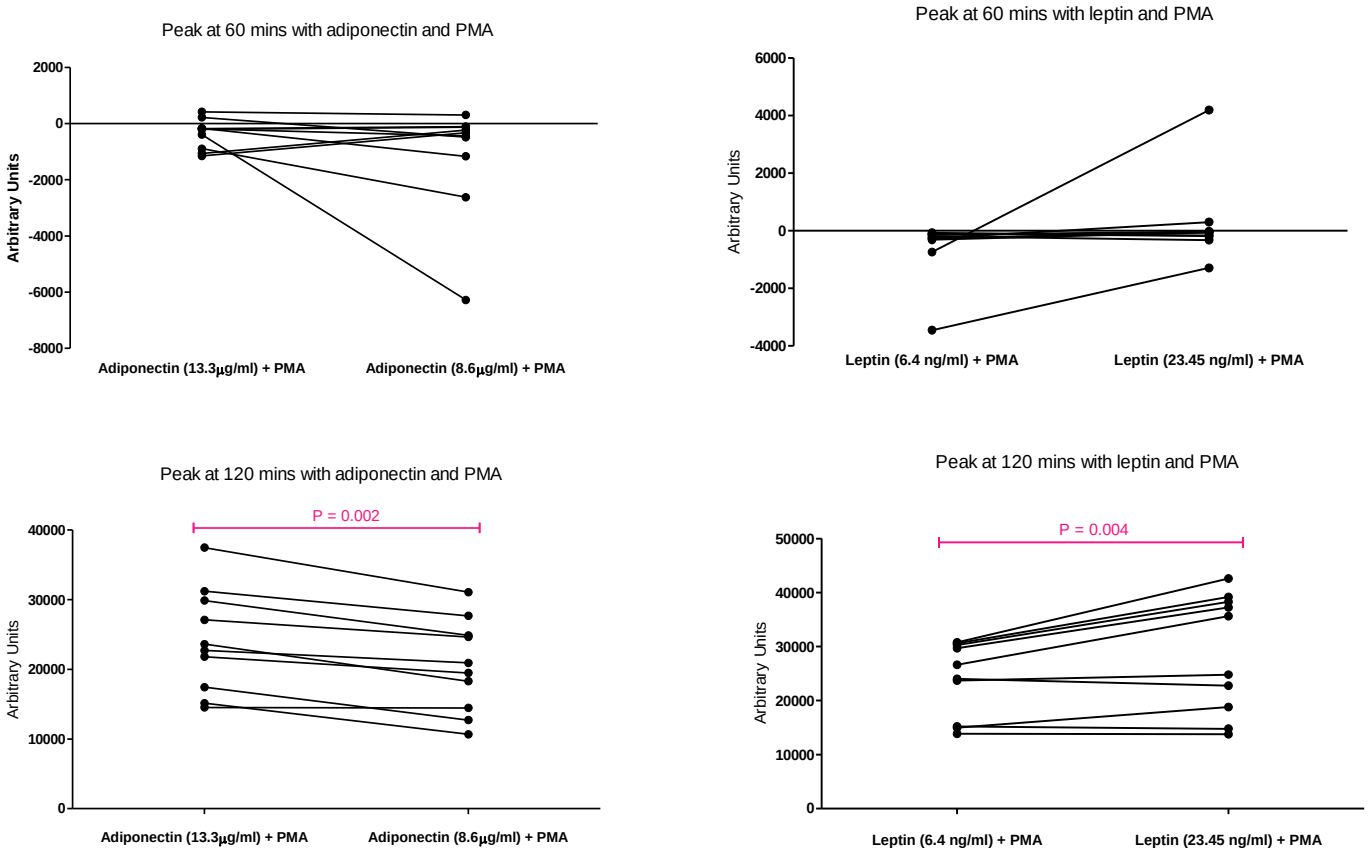


Figure S.4 Peak ROS results with adiponectin and leptin with PMA stimulus.

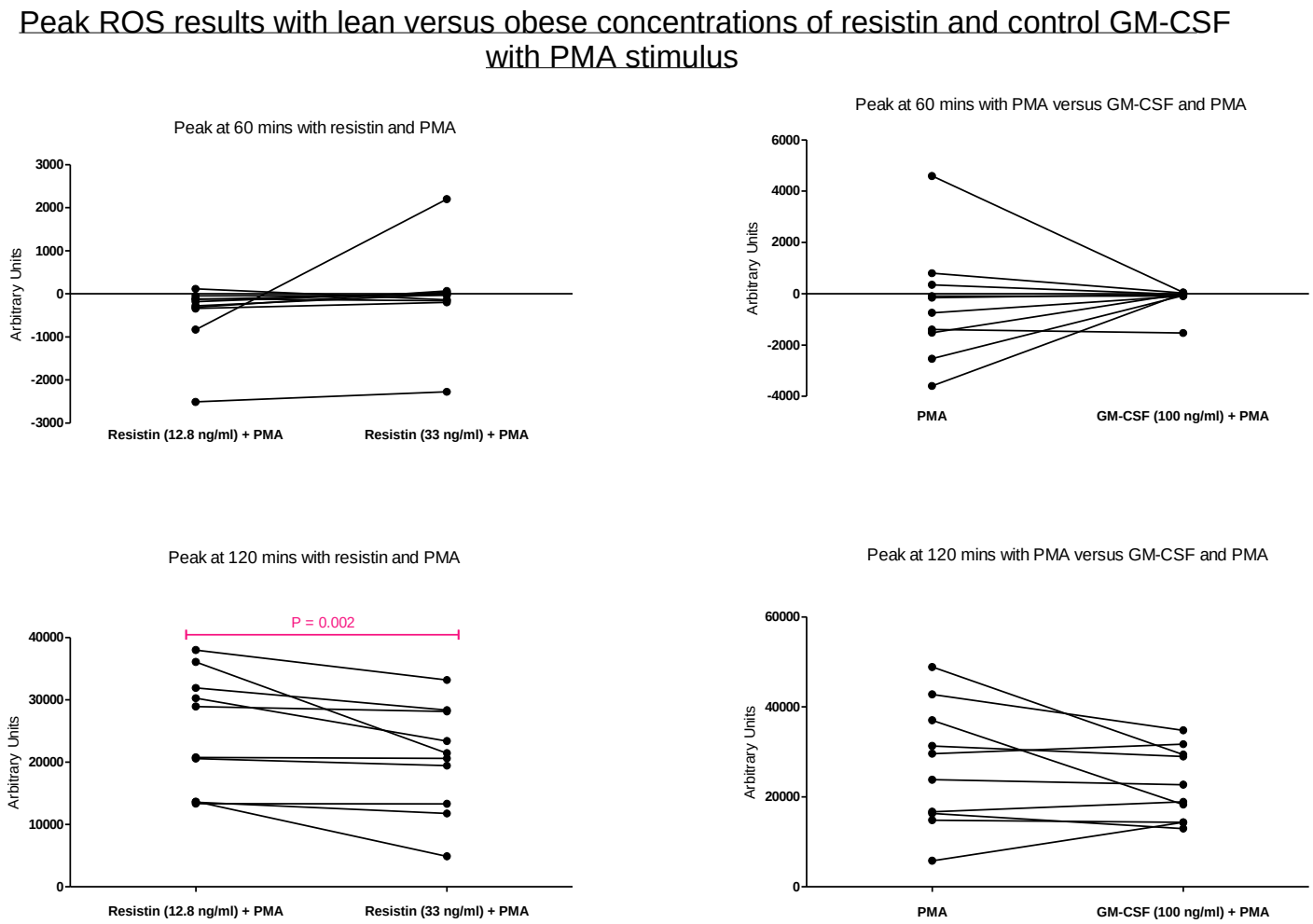


Figure S.5 Peak ROS results with resistin and GM-CSF with PMA stimulus.

Peak ROS results with control IL-8 and PMA stimulus

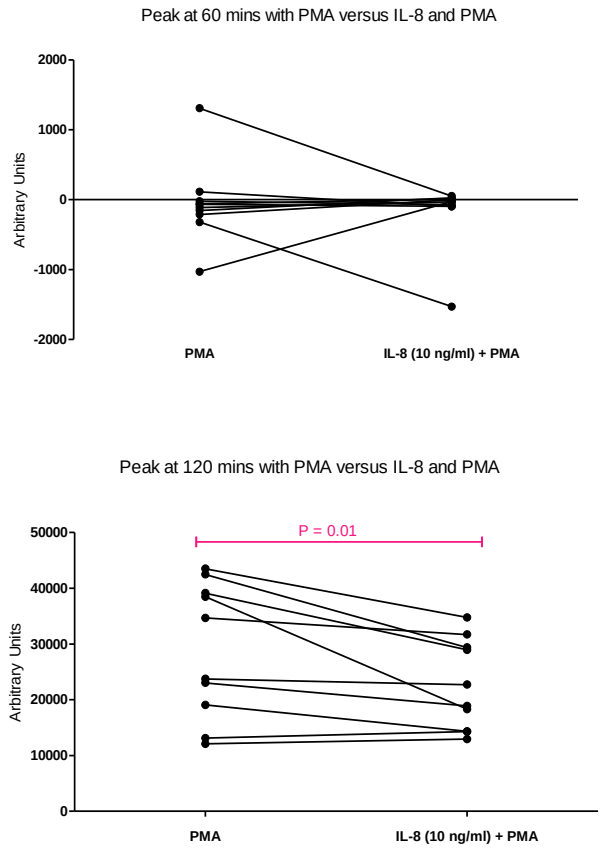
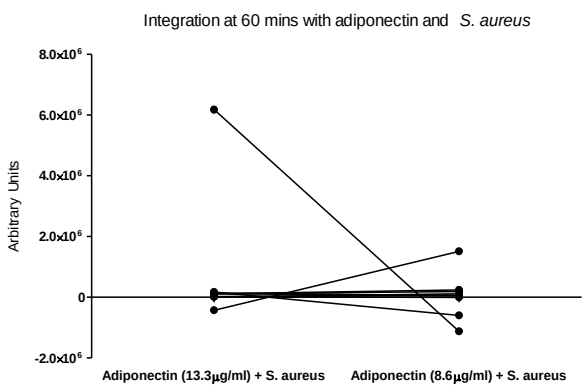
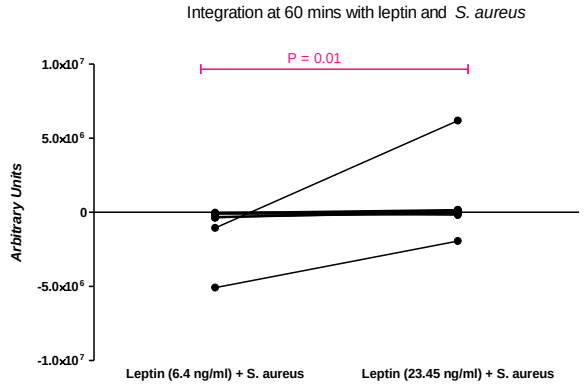
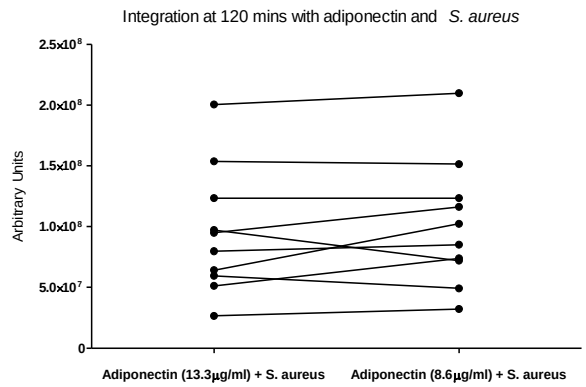


Figure S.6 Peak ROS results with IL-8 with PMA stimulus.

Integration ROS results with lean versus obese concentrations of adiponectin and leptin with *S. aureus* stimulus







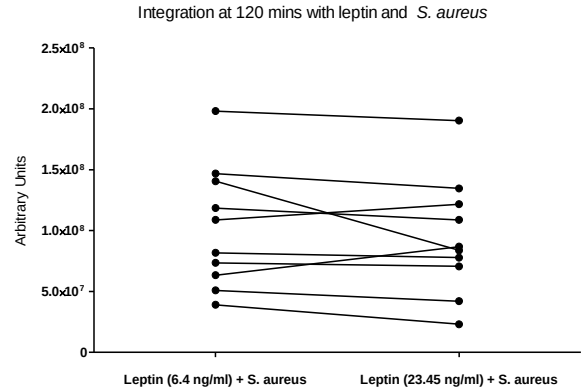


Figure S.7 Integration ROS results with adiponectin and leptin with *S. aureus* stimulus.

Integration ROS results with lean versus obese concentrations of resistin and GM-CSF control with *S. aureus* stimulus

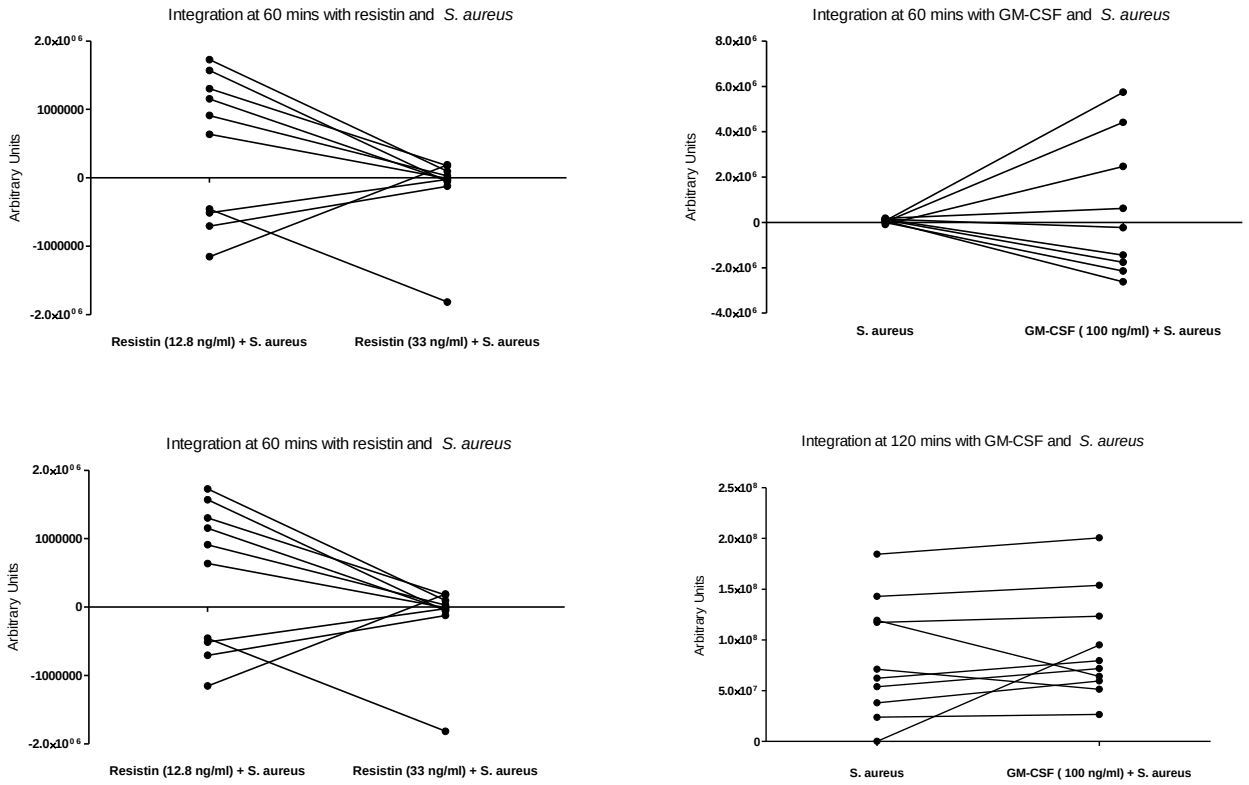


Figure S.8 Integration ROS results with resistin and GM-CSF with *S. aureus* stimulus.

Integration ROS result with control IL-8 and *S. aureus* stimulus

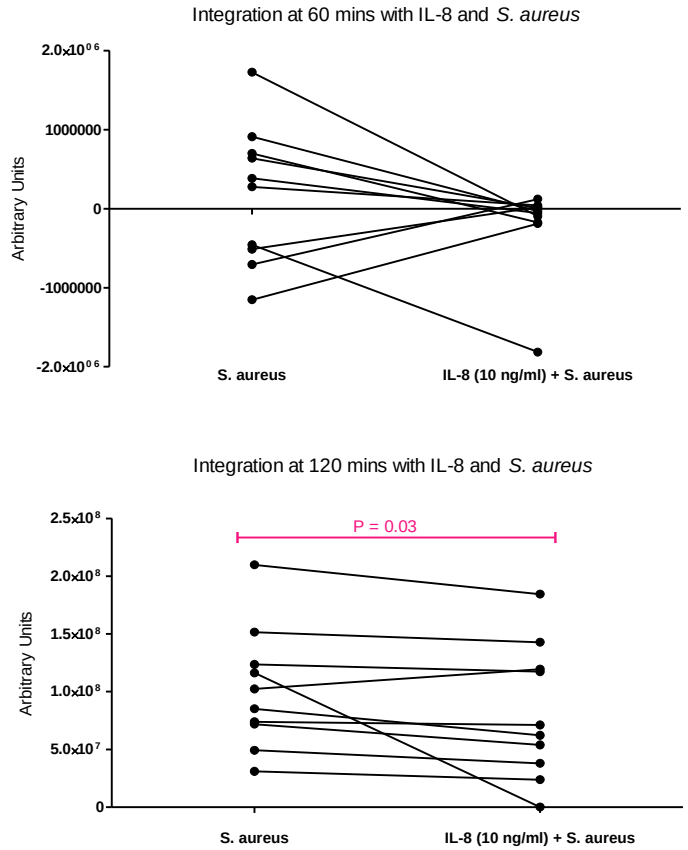


Figure S.9Integration ROS results with IL-8 with *S. aureus* stimulus.

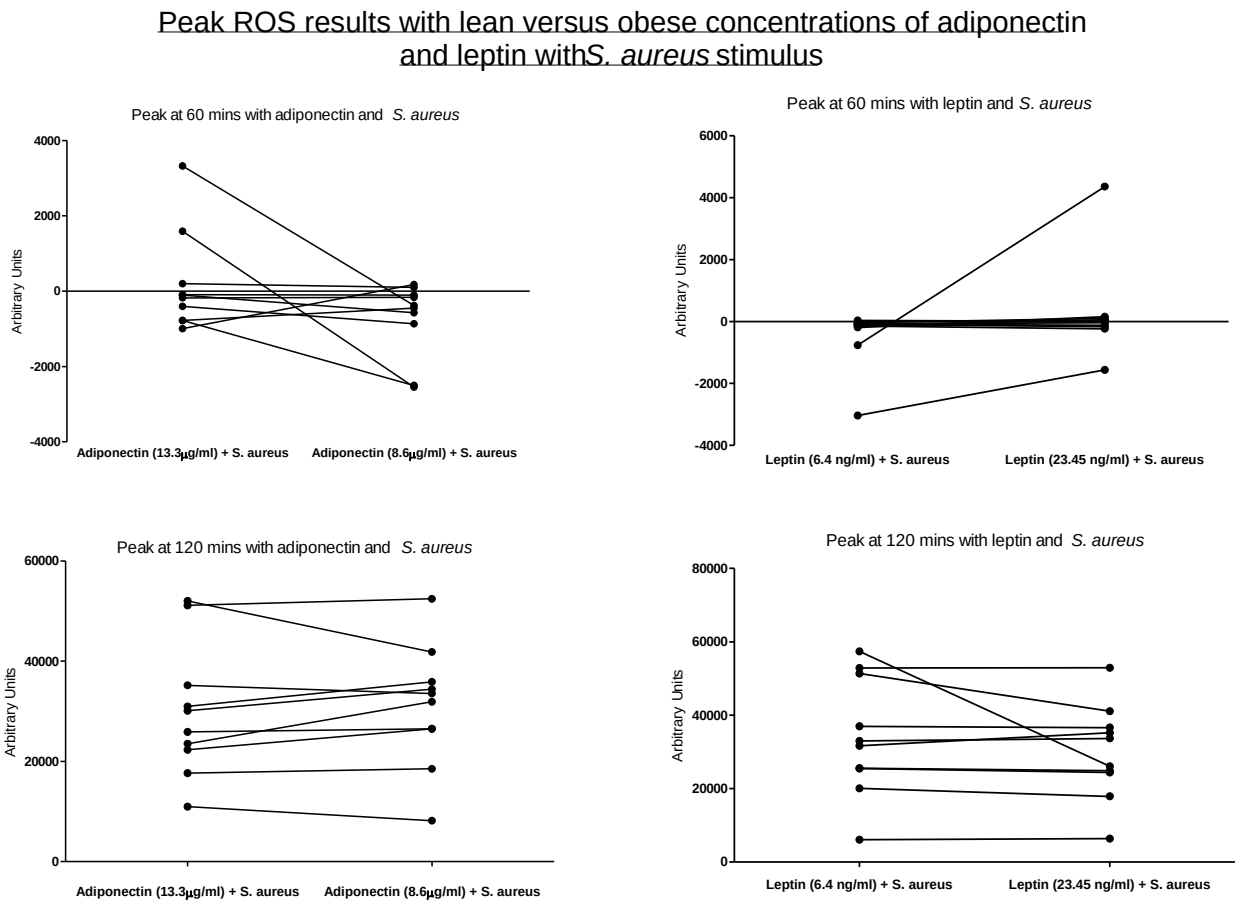


Figure S.10 Peak ROS results with adiponectin and leptin with *S. aureus* stimulus.

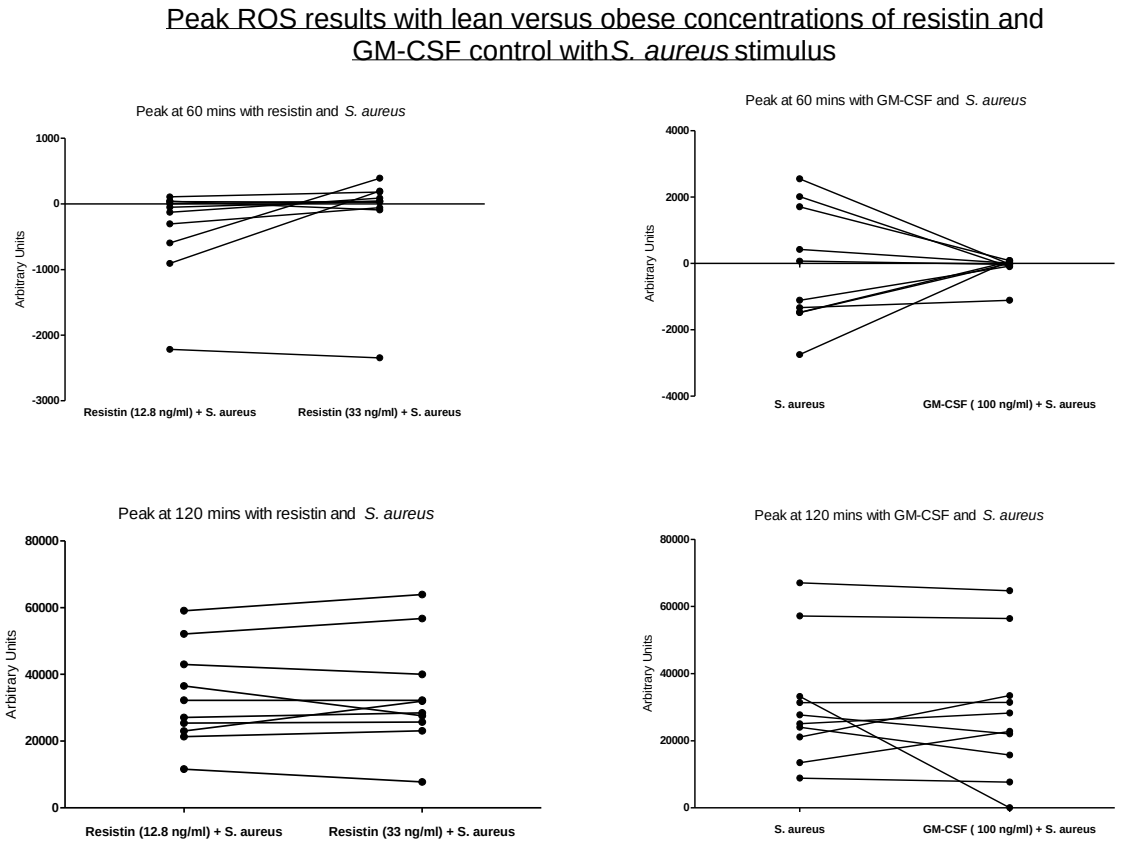


Figure S.11 Peak ROS results with resistin and GM-CSF with *S. aureu s* stimulus.

Peak ROS result with control IL-8 and *S. aureus* stimulus

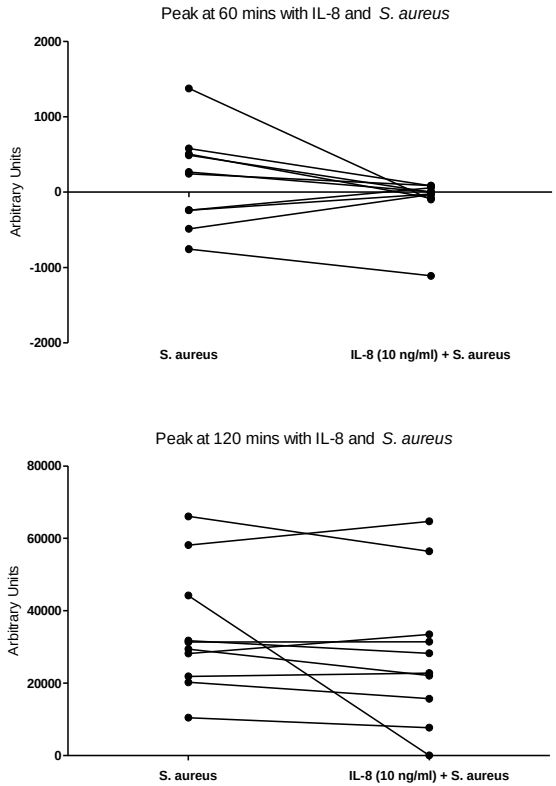


Figure S.12 Peak ROS results with IL-8 with *S. aureus* stimulus.

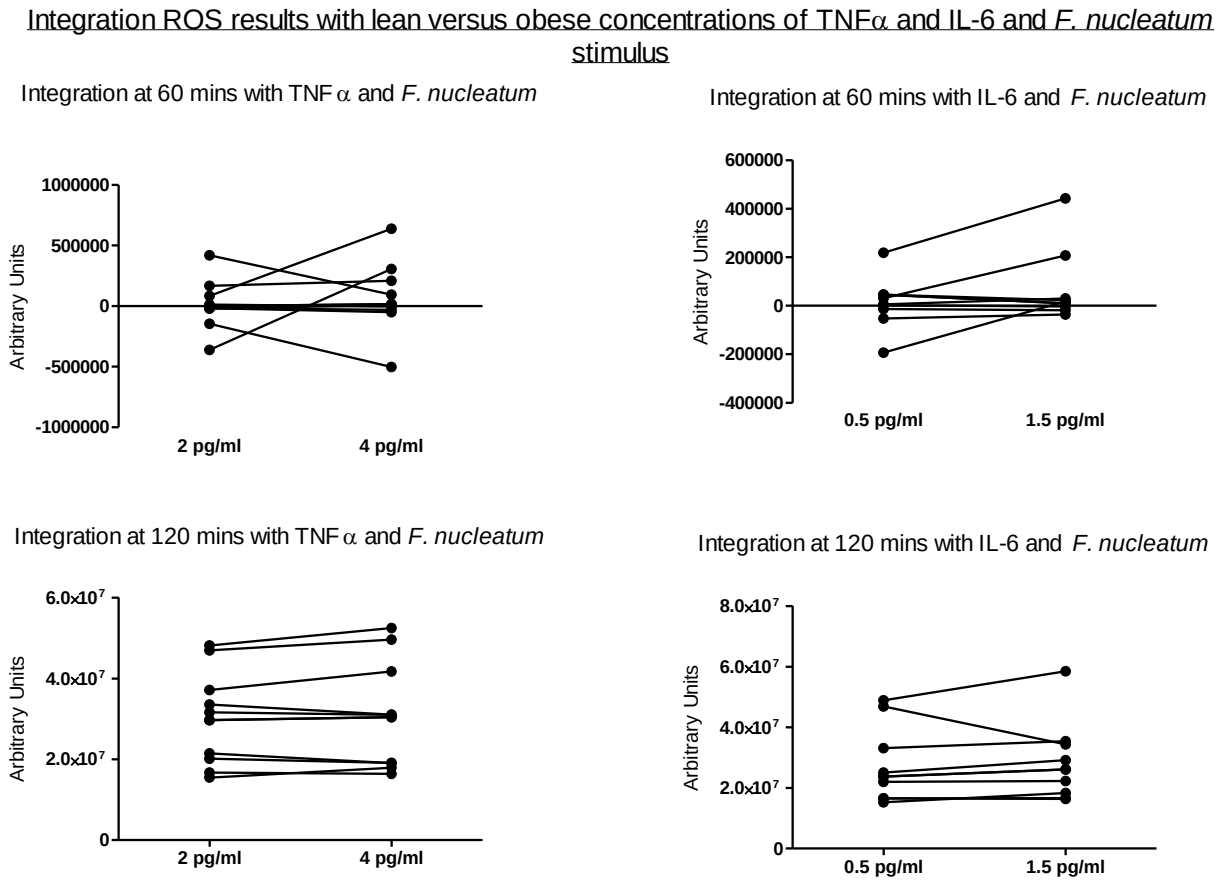


Figure S.13 Integration ROS results with TNFα and IL-6 with *F. nucleatum* stimulus.

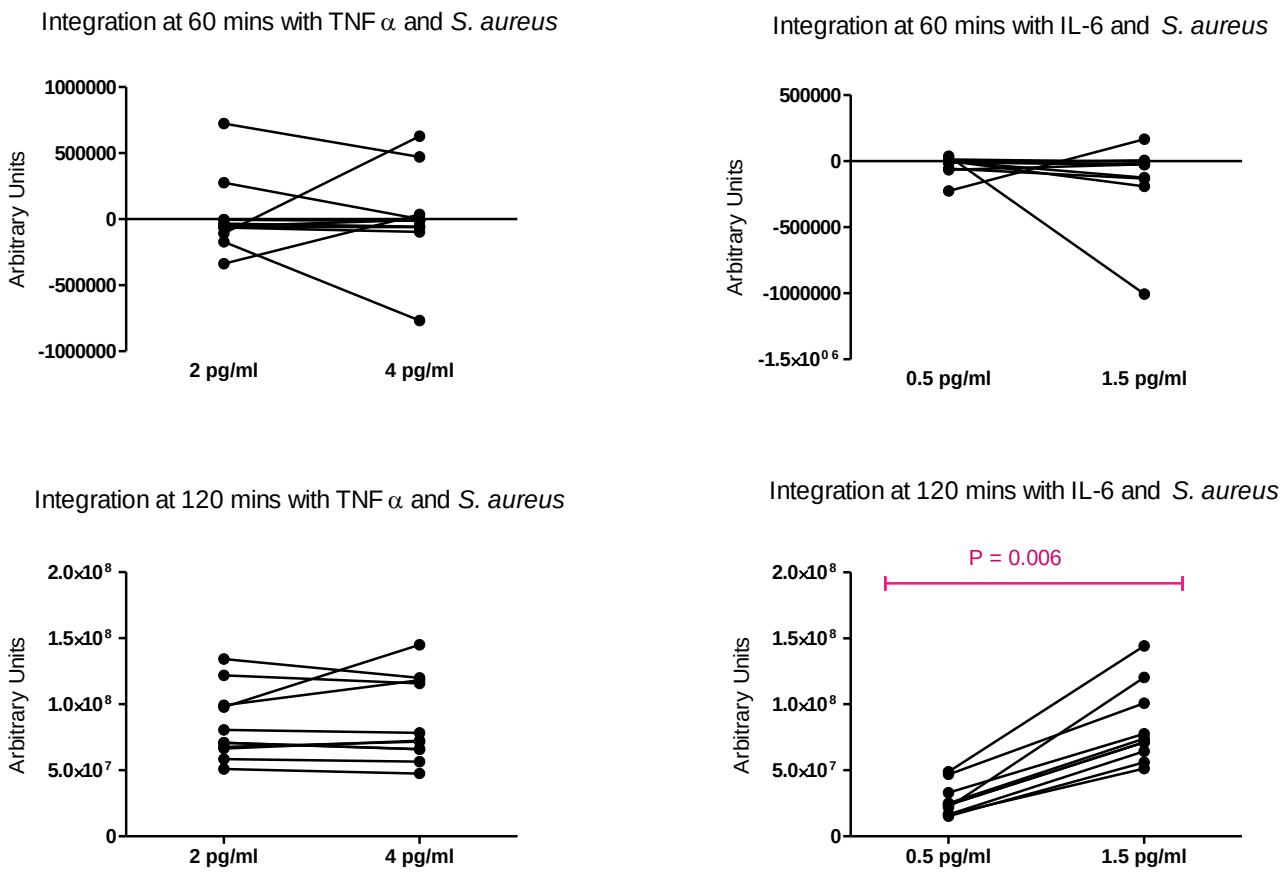


Figure S.14 Integration ROS results with TNFα and IL-6 with *S. aureu* stimulus *s.*

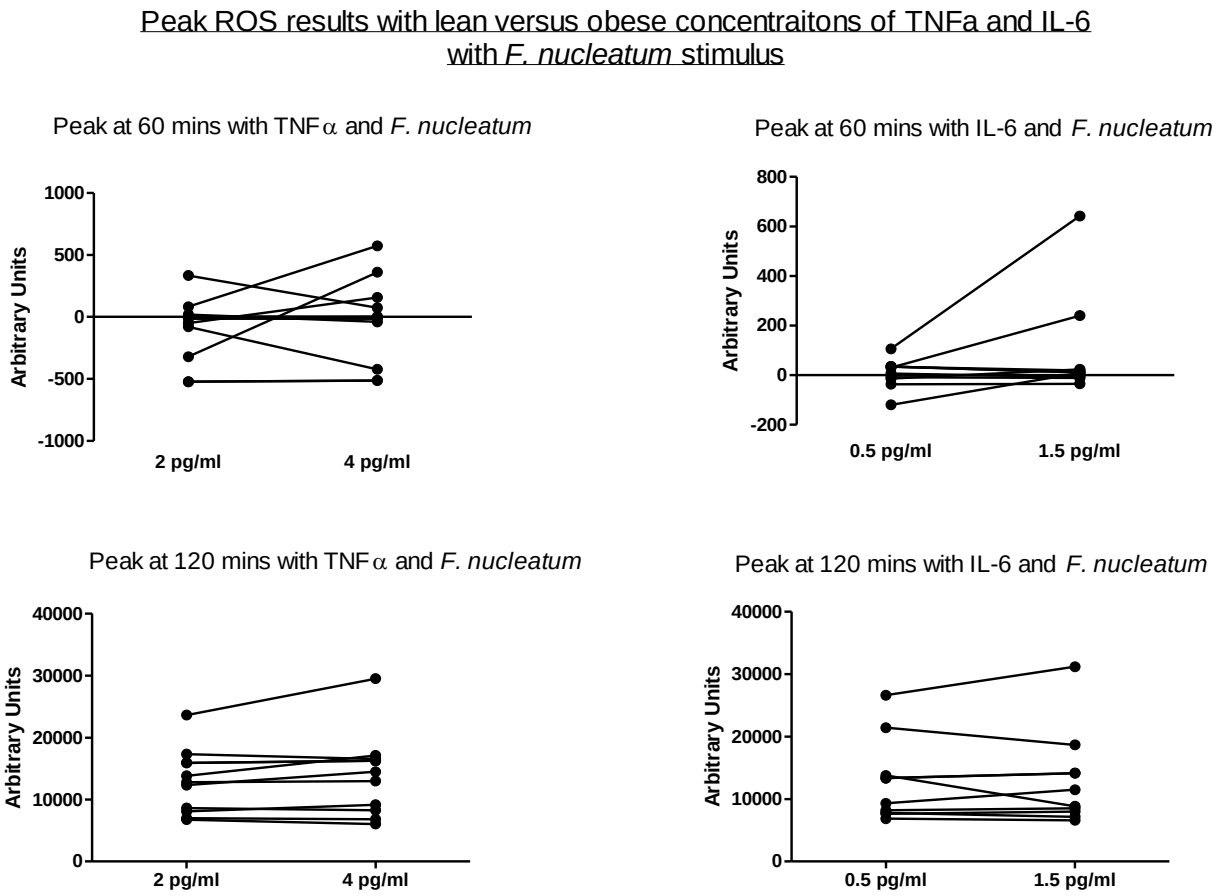


Figure S.15 PeakROS results with TNFα and IL-6 with *F. nucleatum* stimulus.

Peak ROS results of lean versus obese concentrations of TNFa and IL-6 and *S. aureus* stimulus

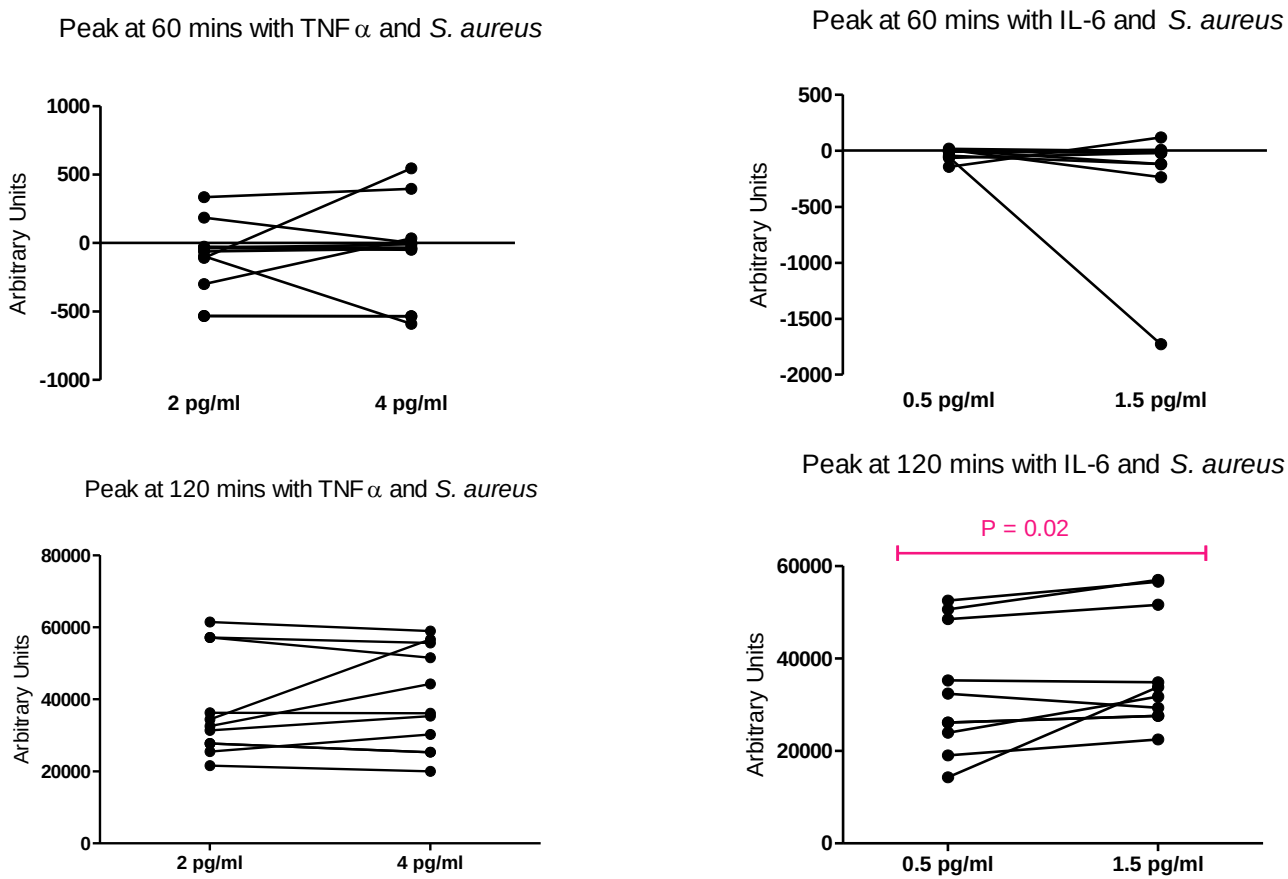


Figure S.16 Peak ROS results with TNFα and IL-6 with *S. aureus* stimulus.

Effect of different adipokine concentrations upon neutrophil directional chemotaxis

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Supervisors:
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Abstract

The obesity epidemic is a growing public health problem in all worldwide populations. It is well known that obese individuals exhibit low grade chronic inflammation which exacerbates obesity-associated diseases. The link between obesity and the occurrence and severity of periodontitis is well characterised and obesity constitutes a major risk factor for periodontal tissue destruction, however direct biological evidence is yet to be established. Adipose tissue, once overlooked as simply inert, fat storing cells of the body, has been shown to function in a similar way to endocrine organs through the secretion of cytokines (adipokines) can contribute towards dysregulated immune responses to bacterial infection. The globally secreted cytokines tumour necrosis factor (TNFα) and interleukin-6 (IL-6), as well as adipose-specific adipokines resistin, leptin and adiponectin have been previously shown to prime/affect neutrophils which are the primary cells involved in immunity to periodontal bacteria at sufficiently high concentrations. This study aimed to ascertain the effect of these adipokines on neutrophil chemotaxis at concentrations reflective of adipokine rich (obese) and adipokine normal (lean) individuals.

Blood was isolated from healthy male volunteers, with no history of diabetes and with normal body mass indices BMI's (range 20-25). Peripheral blood neutrophils were isolated by Percoll^{TM} discontinuous gradient centrifugation. The chemoattractive properties of these adipokines were investigated using an "Insall" chamber by exposing the neutrophils to lean/obese concentrations. Neutrophil movement was captured by microscopic imaging.

The results revealed significant increase in chemotactic index, speed and velocity when the neutrophils were pre-incubated with concentrations of adipokines found in obese patients relative to non-obese levels.

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1. Introduction

1.1 Periodontitis

Chronic periodontitis is a major cause of tooth loss in adults and is associated with other inflammatory diseases including cardiovascular diseases and type II diabetes (Pihilstrom 2005). Chronic periodontitis is thought to arise in pre-disposed individuals whose immune systems respond abnormally to the microorganisms that contribute to the plaque biofilm found at the dento-gingival junction complex. The periodontal tissue comprises gingival epithelium, a highly porous tissue composed of junctional epithelial cells alongside gingival connective tissues, periodontal ligaments and alveolar bone (Bosshardt and Lang 2005). To cope with constant microbial exposure, the gingival cells express numerous host mediators and the tissues are infiltrated by innate immune cells, including neutrophils. Approximately 30,000 neutrophils pass through the periodontal tissues every minute (Schiott and Loe 1970). Bacterial challenge at periodontally healthy sites aids immune resistance to colonisation of potentially pathogenic microbial species, however at diseased sites there is an alteration in these normal defence mechanisms (Darveau 2010).

The precursor to periodontitis is gingivitis, which requires the presence of plaque bacteria (Loe et al. 1965; Theilade et al. 1966) that induce pathological changes in the periodontal tissues resulting in the formation of a "lesion". However not all sufferers of gingivitis develop periodontitis.

Periodontitis is characterised by a microbial-shift in which Gram positive organisms that initially colonise are replaced by Gram negative (Marsh 1994). The presence and persistence of pathogenic bacteria, such as subsets of *Porphryromonas gingivalis,* in high enough numbers can illicit chronic inflammatory responses. The abnormal host inflammatory response against the bacteria includes the excessive

generation of reactive oxygen species (ROS) and results in connective tissue destruction and tooth loss (Chapple and Matthews 2007; The American Academy of Periodontology 1999). An aberrant host immune response to this colonisation infection drives tissues damage and is responsible for the clinical manifestations of the disease which is a result of imbalance or disruption of the inflammatory mediators generated in response to infection (Champagne et al. 2003; Van Dyke 2008).

Studies of aggressive periodontitis sufferers, who have a distinctly different pathogenesis to chronic periodontitis, have been found to have impaired neutrophil function characterised by impaired neutrophil chemotactic activity in most cases (Clark et al. 1977; Van Dyke et al. 1980; Daniel et al. 1993). The patients suffer increased susceptibility to infections and severe periodontal disease. It should be noted that these studies used a Boyden chamber, an indirect method that involves counting the number of cells that migrate towards a chemoattractant source, and results are more likely a reflection of chemokinesis, which is the movement of cells in a non-directional fashion.

1.2 The host response to bacterial infection: the neutrophil

Neutrophils are a member of the granulocyte family of white blood cells. They are formed in the bone marrow and are terminally differentiated from myeloblasts (Amulic et al. 2012). In non-infected individuals the release of neutrophils from the bone marrow is tightly regulated and those in circulation are confined to the vascular capillaries, however challenge with infectious agents such as bacteria results in a shift of the neutrophil into an adherent state involving the migration of cells to and subsequent activation within the site of inflammation. The host immune response to

bacterial infection is characterised by infiltration of neutrophils, macrophages, lymphocytes and other immune cells into the infected tissues as well as the secretion of inflammatory mediators including cytokines and matrix metalloproteinases that actively recruit these cells and drive inflammation.

Cytokines are a diverse group of molecules with potent biological activity whose primary function is to regulate immune responses. Neutrophils are the first line of innate immune defence against non-specific infectious or chemical agents and are recruited to sites of inflammation by chemoattractants secreted by affected tissue (Detmers et al. 1996). Products generated from bacteria and other infectious agents can also act as chemoattractants such as fMLP (N-formyl-Met-Leu-Phe) and lipopolysacharride (LPS) (Chen and Pan 2009). Cell migration from blood vessels to the site of infection is the first step in phagocytosis and involves several steps including capture, rolling, activation and transcellular movement. The signalling molecules phosphatidylinositol 3-kinase (PI3K) and Akt/protein kinase B regulate neutrophil migration (Sasaki et al. 2000). Figure 1.1 outlines the process of leukocyte transendothelial migration, which occurs in the tight junctions between the vascular endothelial cells.

Chemokines such as interleukin-8 (IL-8) are released in response to inflammatory stimuli and attract and activate neutrophils to adhere to and cross the the endothelial walls of blood vessels (Huber et al. 1991). Neutrophil chemoattractant receptors are members of the transmembrane G-protein coupled receptor family, activation of which triggers various signaling cascades that enable the cell to move in a direction-specific manner to towards the source of the chemoattractant. Receptor-ligand binding of these agents results in the activation of protein kinase C, tyrosine kinases and the GTP binding protein Ras (Worthen et al. 1994). Activation of further proteins within the cell results in the actin-dependent movement of the neutrophil in

a gradient-driven manner. The activation of chemoattractant receptors also serves to activate phosphoinositol-3-kinase which is involved in NAPDH oxidase activation (Klippel et al. 1996).

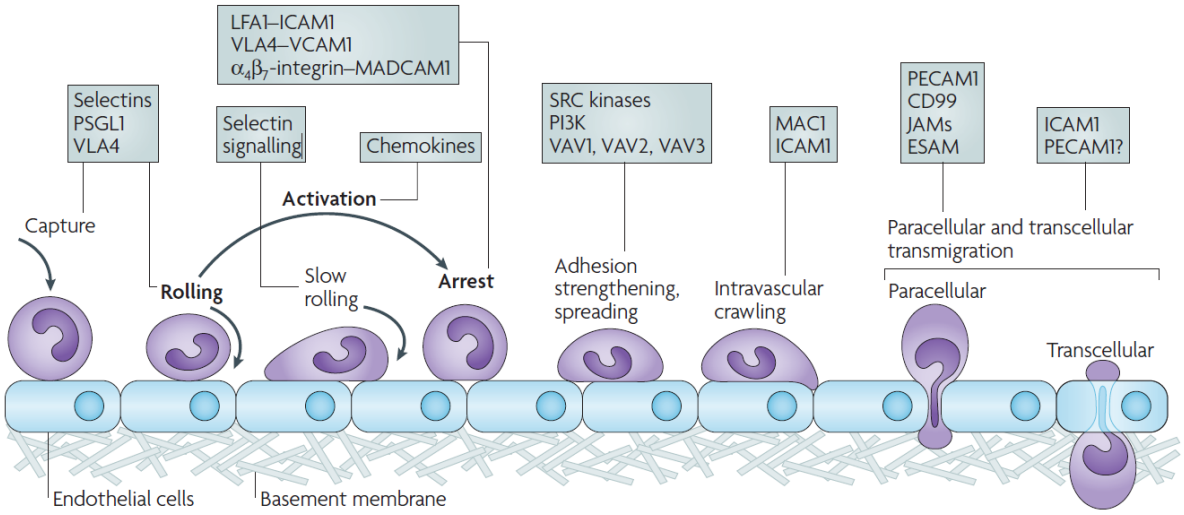


Figure 1.1 Adhesion and migration of leukocytes. Rolling is mediated by selectins. Activaiton is mediated by chemokines and arrest via integrins. All three of the major steps result in the recruitment of the cell to the endothelial wall and subsequent passage out of the vessel (Ley et al. 2007).

1.3 Cell migration: chemotaxis

Molecular gradients are an inherent feature of mammalian physiology required to activate intracellular signalling events and establishing directional cues for migrating cells. Chemotaxis is the process by which the direction of motile cells is biased along a concentration gradient of soluble factors/extracellular signals known as chemoattractants (Insall 2010). Chemotaxis exists in a large and diverse number of organisms from amoebas to eukaryotes (Van Haarstert 2004). Neutrophils migrate to the site of infection along a chemoattractive gradient in order to kill invading microorganisms. This process involves a number of interacting processes including recognition of the chemoattractant, detection of a possible gradient and transmission of the information to the cell's motility machinery. Neutrophils move by producing actin-rich pseudopods at the leading edge of the cell while retracting other regions The orientation of these protrusions towards a chemoattractant determines the direction of migration (Andrew and Insall 2007). Chemokines are divided into four structurally similar sub-families (CXC, CX3C, CC and C) (Bonecchi et al. 2009). Neutrophils express the chemokine receptors CXCR1 and CXCR2 which bind IL-8 (CXCL8), GCP2 (CXCL6) and GROα (CXCL1) (Viola and Luster 2008). Chemokine receptor-ligand binding activates pathways including MAPKs and nuclear factor (NF)-κB. Disturbances in the ability of cells to migrate up a chemotactic gradient can increase the risk for bacterial infections.

1.4 The resolution of inflammation

The resolution of inflammation is a vital process that limits further infiltration of activated cells and removes apoptotic cells from sites of inflammation. Ingestion of apoptic cells is driven by anti-inflammatory cytokines such as transforming growth factor (TGF)-β and IL-10 (Kennedy and DeLeo 2009). Late in the inflammatory response neutrophils switch from producing pro-inflammatory lipid mediators such as prostaglandins, to anti-inflammatory lipids which include resolvins and lipoxins. The production of these lipids is a result of the interaction of neutrophils with other cell types including epithelial cells, fibroblasts, platelets and leukocytes. The anti-inflammatory lipid mediators promote macrophage infiltration to the site and also inhibit neutrophil recruitment and function. Figure 1.2 shows a diagrammatic representation of the sequential process of inflammation resolution.

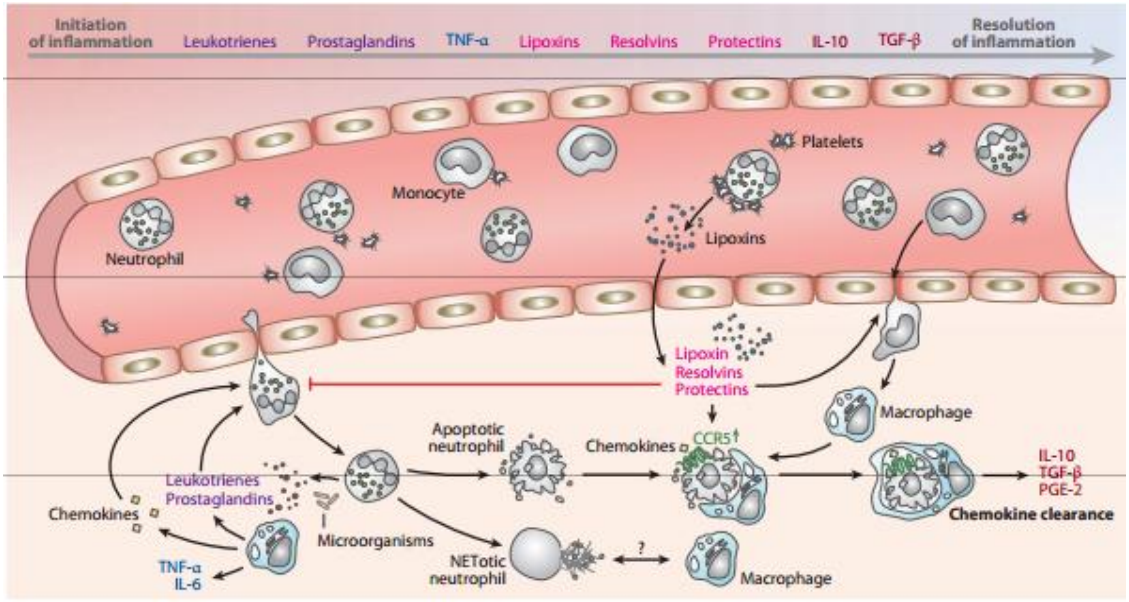


Figure 1.2. Proinflammatory mediators and chemoattractants secreted by monocytes to recruit other immune cells, particularly neutrophils, to the site of infection (Amulic et al. 2012). When the pathogen is no longer detected, a range of other anti-inflammatory mediators are secreted including resolvins and lipoxins that aid the restoration of tissue homeostasis. Neutrophil exposure to anti-inflammatory lipids results in the expression of CCR5 on the surface of apoptotic neutrophils, effectively "mopping up" free chemoattracitants such as CCL3 and CCL5. This prevents further recruitment of neutrophils to the site (Ariel et al. 2006).

1.5 Obesity and inflammation

Obesity is a major worldwide medical threat. The number of people "overweight" or "obese", characterised by excessive fat accumulation and defined as a body mass index (BMI) of over 25 and 30 respectively, is predicted to be 1.5 billion by 2015 according to the WHO. Obesity is strongly associated with a number of diseases including type II diabetes and coronary heart disease.

Adipose tissue, which contains the fat-storing cells known as adipocytes, produces a number of bioactive molecules that influence and communicate with other organ systems and can contribute to systemic inflammation. These so-called adipokines have been found to make a significant contribution to chronic low-grade systemic inflammation. They include chemokines, complement components, pro-inflammatory and anti-inflammatory cytokines (Li et al. 2011).

Adipose tissue comprises adipocytes and cells of the immune system such as macrophages, which typically populate 5-10% of the tissue. Roles of macrophages in adipose tissue include removing necrotic adipocyte cells (Cinti et al. 2005) and forming new blood vessels (Cursiefen et al. 2004). Adipose tissue in obese individuals is found to have a significantly higher proportion (up to 60%) of total cells within the tissue (Weisberg et al. 2003). The increase in the number of macrophages in adipose tissue of obese individuals may contribute to the systemic increase in cytokines that are also secreted as adipokines such as TNFα and IL-6 (Lehrke et al. 2004). The type of macrophages found in adipose tissue of obese individuals is different to that of lean people. A study by Lumeng et al. (2007) in obese mice identified more of the pro-inflammatory "classically activated" M1 subsets of macrophages within adipose tissue compared to their lean counterparts who had a greater proportion of anti-inflammatory "alternatively activated" M2 macrophages.

Obese individuals have also been found to have elevated systemic concentrations of pro-inflammatory cytokines compared to lean (Esposito et al. 2003; Wellen and Hotamisligil 2003). Weight loss intervention studies have shown that a reduction in body weight, such as through exercise and healthy eating, correlates with a decrease in pro-inflammatory and an increase in anti-inflammatory adipokines (Bastard et al. 2000; Nicoletti et al. 2003; Ziccardi et al. 2002).

1.6 The role of adipokines

The adipokines investigated in this project include adiponectin, leptin, resistin, TNFα and IL-6. Each of these are briefly described below:

Adiponectin is a 244-residue protein that circulates in blood in different molecular forms. It is the most abundant adipokine secreted by adipocytes accounting for 0.01% of total plasma proteins (Maeda et al. 1996). It exists as three major oligomeric complexes in the blood stream: trimers, hexamers and a high-molecular weight form (Arita et al. 1999). Obese subjects have low plasma levels of adiponectin compared to lean (Arita et all. 1999) as large adipocytes in obese individuals produce lower levels of adiponectin (Berg and Scherer 2005). Adiponectin has been shown to have protective anti-inflammatory properties, for example by stimulating the production of the anti-inflammatory cytokine IL-10 in macrophages (Wulster-Radcliffe et al. 2004) and the ability to switch macrophages from the M1 to M2 phenotype (Ohashi et al. 2010). Studies have also shown adiponectin reduces the expression of pro-inflammatory TNFα in adipose tissue (Maeda et al. 2002), inhibits macrophage phagocytic activity and attenuates pro-inflammatory signalling pathways such as nuclear factor (NF)-κB (Ouchi et al. 2000;

Wulster-Radcliffe et al. 2004). Other roles of adiponectin include increasing fatty acid oxidation and decreasing gluconeogenesis in the liver (Lago et al. 2007). The administration of adiponectin in diabetic mice enhances insulin sensitivity through the reduction of hyperglycaemia, indicating the proteins role in protecting against obesity-linked metabolic dysfunction (Berg and Scherer 2005). The mechanism by which adiponectin protects against insulin sensitivity is through activation of antimicrobial peptide (AMP)-activated protein kinase in the liver and skeletal muscle, which leads to an increase in glucose uptake and fatty acid oxidation (Yamauchi et al. 2002). Plasma adiponectin levels are also negatively associated with C-reactive protein, a marker of low-grade inflammation, in obese individuals, whereas the reverse is true for healthy lean individuals (Ouchi et al. 2003).

Leptin is a 16-kDa non-glycosylated peptide member of the class I cytokine superfamily. It is expressed by inflammatory cells exposed to stimuli such as IL-6 and LPS (Faggioni et al. 2001) and it functions in regulating appetite, energy homeostasis and immune and inflammatory processes in macrophages, neutrophils and other immune cells (Otero et al. 2006; Matarese et al. 2005). Upon binding to its receptors, of which there are two isoforms (Ob-Ra and Ob-Rb) intracellular pathways including JAKs, STAT1 and NF-κB are activated (Eder 2009). Ob-Ra, the short form of the leptin receptor is expressed on neutrophils, whereas the long isoform Ob-Rb, is expressed by monocytes (Zarkesh-Esfahani et al. 2004). Leptin has been shown to increase the production of TNF and IL-6 in monocytes and stimulates chemokine ligand production in macrophages (Santos-Alvarez et al. 1999; Kiguchi et al. 2009). A diet deficient in essential nutrients correlates with low leptin levels and this is associated with immune impairment and increased susceptibility to infection.

Resistin is a 12.5-kDa dimeric protein mainly expressed in neutrophils and macrophages (Patel et al. 2003). LPS has been shown to induce the expression of resistin in macrophages. IL-6 and TNFα can induce and be induced by resistin secretion (Bokarewa et al. 2005) indicating its role as a pro-inflammatory cytokine.

Tumour necrosis factor (TNFα) is produced by a number of cell types, particularly macrophages and T lymphocytes, but also neutrophils and osteoclasts (Ware 2008). TNFα is a pro-inflammatory cytokine and upon binding to either of its two receptors (TNFR1 and TNFR2) activates the MAPKs and NF-κB (Locksley et al. 2001). TNFα increases expression of cell adhesion molecules such as E-selectin and ICAM-1 on the surface of vascular endothelial cells (Bradley 2008), which enable neutrophils to bind to these integrins expressed on activated endothelial cells via complementary neutrophil receptors such as LFA-1.

IL-6 is a pro-inflammatory cytokine produced by many immune cells including adipocytes. IL-6 promotes bone resorption, stimulates angiogenesis and the release of acute phase proteins (Blanchard et al. 2009).

1.7 Obesity and periodontitis

Obesity has emerged as one of the major risk factors for periodontal disease and many studies have demonstrated this association in both young, middle aged and older people (Al-Zahrani et al. 2003, Dalla Vecchia et al. 2005, Ekuni et al. 2008, Khader et al, 2009, Kongstadt et al. 2009, Saito et al. 2005). A recent study by

Dickie de Castilhos et al. (2012) identified an association between young obese individuals and an increased incidence of gingivitis, which is a risk factor for periodontitis. It appears the distribution of fat is associated with the risk of periodontitis, in particular visceral fat accumulation (Saito et al. 2001).

The mechanism linking obesity with chronic inflammatory diseases such as periodontitis is thought to be mediated by adipokines secreted form adipose tissue.

Adipose dysfunction as a consequence of adipose tissue expansion can have local and systemic effects on inflammatory responses, which can contribute to the initiation and progression of inflammatory-linked diseases. One such consequence is the resorption of alveolar bone, which is a hallmark of periodontitis. This is caused by the increased expression of RANKL (receptor-activator of nuclear factor-κB ligand), which binds osteoclast pre-cursors and causes them to differentiate into bone resorbing cells (Cochran 2008). RANKL can be induced by TNFα and IL-1β (Nagasawa et al. 2007).

1.8 The effect of adipokines on chemotaxis

Numerous studies have shown that adipokines affect cellular behaviour, for example pro-inflammatory adipokines can activate neutrophils or "prime" them for the enhanced generation of reactive oxygen species (ROS) as described in project 1 section 1.7. Below are a few examples of studies that have demonstrated the effects of non-physiological concentrations of the adipokines used in this study specifically on neutrophil chemotaxis.

A study by Okamoto et al. 2008 showed the ability of anti-inflammatory adiponectin to inhibit CXCR3 chemokine ligands in monocyte-derived macrophages at concentrations ranging from 0.01- 10 μg/ml after stimulation with LPS.

Leptin has been shown to act as a neutrophil chemoattractant showing maximal response at 50 ng/ml in a study by Montecucco et al. (2006) showed neutrophils exposed to leptin induced chemotaxis but did not lead to cell activation because of a lack of intracellular calcium mobilisation and oxidant production. The study also showed intracellular pathways in leptin-mediated chemotaxis involve the activation of p38 MAPK. Another study showed leptin induced neutrophil chemotaxis towards fMLP and also acted as a chemotactic agent at a much higher concentration (250 ng/ml) (Caldelfie-chezet et al. 2003).

Resistin at a concentration of 30 nM has been shown to induce neutrophil arrest and the expression of cell adhesion molecules including VCAM-1 and ICAM-1 (Kawanami et al. 2003), which occurs during the leucocyte migration cascade.

GM-CSF is a cytokine released by different cell types including activated T cells and macrophages (Arai et al. 1990). As well as its other pro-stimulatory effects on neutrophils, GM-CSF can induce the expression of neutrophil receptors for CC chemokines (Cheng et al. 2001). GM-CSF has been shown to induce neutrophil chemotaxis and chemokinesis of neutrophils at a maximally effective concentration of 60 ng/ml. This response was similar to that of the chemokine IL-8 (also at 60 ng/ml) however both IL-8 and GM-CSF were less potent than fMLP (Gomez-Cambronero et al. 2003).

TNFα at high concentrations has been shown to act as a chemoattractant for neutrophils (Shalaby et al. 1985). In particular, studies Gamble et al. (1985) and Ming et al. (1987) showed the ability of neutrophils to adhere to the endothelium of umbilical cord veins when pre-incubated with TNFα at concentrations up to 10,000 Units/ml.

If chemokines are not removed from the site of inflammation or sufficient quantities of anti-inflammatory chemokines are not produced, resolution of inflammation can

be compromised. Obese individuals have higher circulating adipokines than lean, and these adipokines at higher concentrations than those found phsiologically have chemotactic properties. If physiological concentrations show also to have an effect on chemotaxis, this could be one of the factors that prevent the efficient end of inflammatory resolution and could therefore contribute to the chronic low-grade inflammatory state present in obese individuals..

1.9 Methods of studying neutrophil chemotaxis

The purpose of studying chemotaxis is to allow the behaviour of cells subjected to a linear gradient of chemoattractant to be observed directly using a light microscope. Most assays use the two-well design whereby cells are seeded between the wells, one containing a control or buffer substance and the other with the chemoattractant and cells lying within the gradient are able to migrate between them. This enables the study of chemokinesis. However direct visualisation chambers allow cells to be observed migrating using time-lapse microscopy in real-time and are considered the gold standard for investigating true "chemotaxis" (Wells 2000). Bridge chambers provide a visualisation platform for observing the behaviour of cells between the two wells; the cells are plated onto coverslips which are inverted leaving a gap between the chamber and coverslip for the addition of desired chemoattractant. They include the Zigmond, Dunn and Insall chambers, which are briefly described below and illustrated in figure 1.3.

- The Zigmond chamber was first described in 1977 for the study of polymorphonuclear cells (Zigmond 1977). The chamber permits the generation of shallow gradients at a near steady state linear gradient. The Zigmond chamber has a variable gap between the bridge and coverslip which

can result in unpredictable variations in the chemotactic gradient (Zicha et al. 1991).

- The Dunn chamber is derived from the original Zigmond chamber and achieves stability by being a sealed, rigid, glass enclosure entirely filled with incompressible medium using thick (0.25-0.35 mm) coverslips. The circular bridge design can result in variable chemoattractant gradient orientation and changing the gradient in Dunn chambers is inefficient (Muionen-Martin 2010).
- The Insall chamber, derived from the Dunn chamber (Muinonen-Martin et al. 2010), is designed to ensure better stability between the gap between the bridge and coverslip. The bridge structure is a square arrangement with two linear bridges of differing widths on opposite sides and the other two sides are large coverslip supports. The ease of handling, gradients with defined directions and the ability to use thin coverslips are some of the advantages of this type of bridge chamber. The Insall chamber was used in this project.

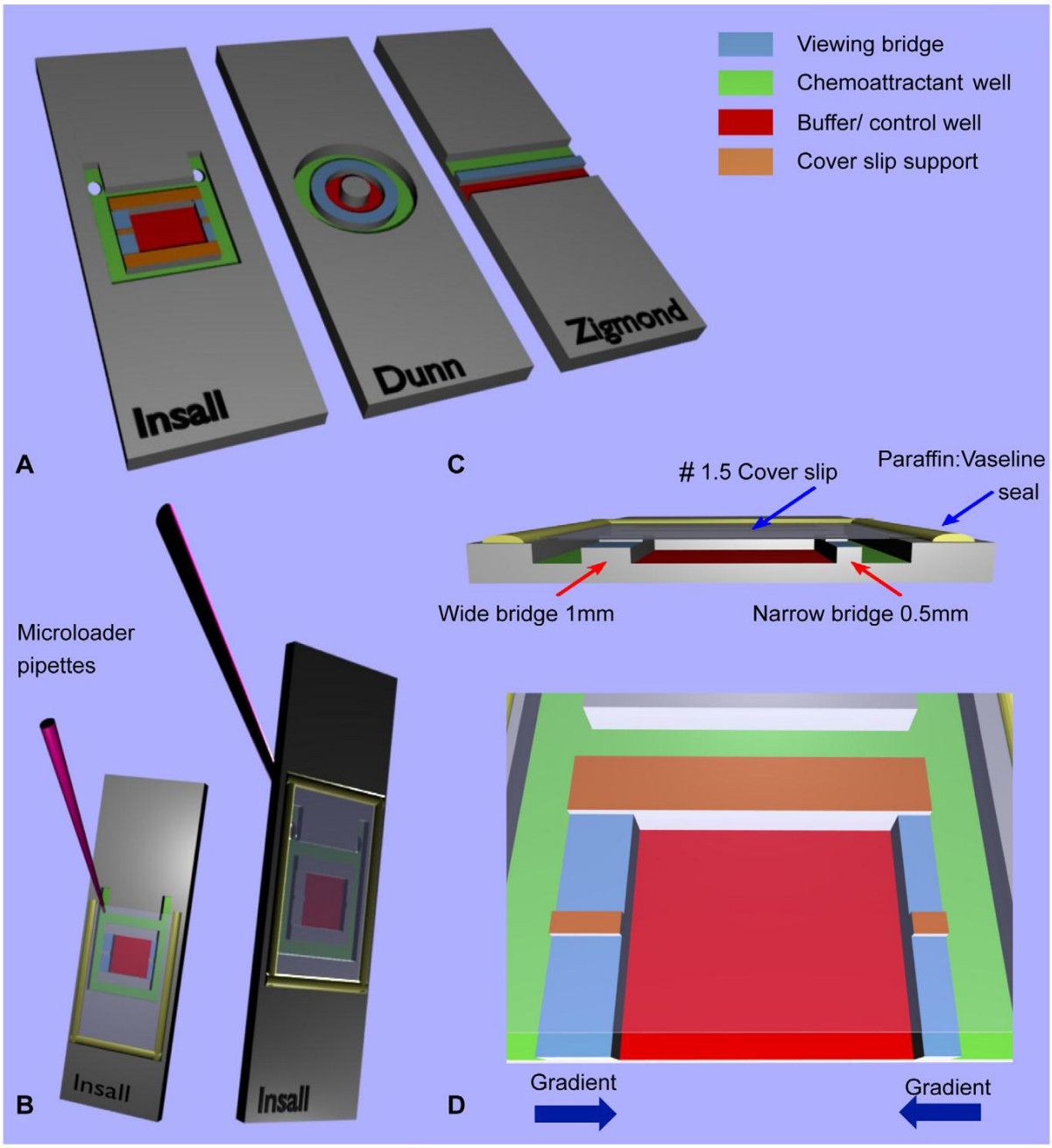


Figure 1.3. The major bridge chambers commercially available. The Insall chamber is the most recently developed chamber and offers advantages over the Dunn and Zigmond. A) shows the schematics of all three chambers – both Zigmond and Dunn lack a support structure for the coverslip. B) The desired chemoattractant can be loaded into the Insall chamber once the coverslip is attached. C) The differences in bridge widths provide different gradient steepnesses. D) The gradients are unidirectional across each bridge (Muinonen-Martin et al. 2010).

1.10 Project aims

The aim of this project was:

- To investigate the chemotactic potential of the adipokines, resistin, adiponectin, leptin, IL-6 and TNFα at concentrations found in obese and lean individuals using a novel directional chemotaxis assay – the "Insall Chamber".

2. Methods

2.1 Blood collection

Blood was taken from staff and student volunteers of the Birmingham Dental School. Volunteers had a BMI and waist-hip ration within the normal range (22.06±0.22 and 0.86±0.005 respectively). All were under 30, non-smokers, non-diabetic males and not taking any anti-inflammatory medication. Approximately 25 ml of blood was collected from each volunteer into Vacutainer^{TM} (Greiner Bio-One Ltd, Stonehouse, UK) lithium heparin (17 IU/ml) tubes. The tubes were inverted several times and after a time lapse of approximately 20 minutes the blood was removed from the tubes for neutrophil isolation. Donors gave their consent and ethical approval was provided by the University of Birmingham (ERN_10-0518).

2.2 Neutrophil isolation

Neutrophils were isolated from blood samples using percoll (GE Healthcare) and a full description is given in project 1 (section 2.3).

2.3 Adipokines

A literature review to identify the lean and obese concentrations of adipokines used in this project was performed (the details of which are shown in table 3.1 of project 1). The chosen concentrations and references are shown in table 2.1. GM-CSF and IL-8 were used as positive controls and therefore concentrations were chosen that have been shown in previous studies to affect neutrophil behaviour.

Adipokine	Lean concentration	Reference for lean concentration	Obese concentration	Reference for obese concentration	Priming concentration	Reference for priming concentration
TNFα	2 pg/ml	Murdolo et al. 2008	4 pg/ml	Murdolo et al. 2008		
IL-6	0.5 pg/ml	Murdolo et al. 2008	1.5 pg/ml	Murdolo et al. 2008		
Adiponectin	13.3 µg/ml	Silha et al. 2003	8.6 µg/ml	Silha et al. 2003		
Leptin	6.4 ng/ml	Rosicka et al. 2003	23.45 ng/ml	Rosicka et al. 2003		
Resistin	12.8 ng/ml	Cohen et al. 2008	33 ng/ml	Cohen et al. 2008		
IL-8 (positive control)					10 ng/ml	Wozniak et al. 1993
GM-CSF (positive control)					100 ng/ml	Nathan 1989

Table 2.1 Lean and obese concentrations of adipokines used in this project.

2.4 Chemotaxis assay

For the chemotaxis assay, the isolated neutrophils were required at a concentration of 2 x 10^6 per ml suspended in RPMI. 1.5 µl of 7.5% Albumin Bovine Fraction (Sigma: A8412) was also added per ml of cell suspension. Coverslips were washed in 0.2 M HCl followed by sterile water and placed on filter paper. The coverslips were coated in 500 µl of 7.5% serum Albumin Fraction and subsequently loaded with 400 µl of the neutrophil suspension and incubated for 30 minutes at room temperature.

The Insall chamber (Muinonen-Martin et al. 2010) was used to study chemotaxis. The chamber was washed 4 times with approximately 400 µl of RPMI before a large droplet of RPMI was left on the chamber. After the 30 minute incubation, the coverslip was inverted and placed at the top of the chemotaxis chamber ensuring that the pipette loading bays were exposed. Using filter paper, excess fluid surrounding the coverslip was absorbed and the fluid within the chemoattractant wells was removed. Finally 80 µl of desired chemoattractant (adipokine or control) were injected into the chemoattractant well and analysed by microscopy. The chamber was viewed using a Zeiss Primovert microscope at x 40 magnification and images were captured using a Q Imaging Retiga 2000R camera.

2.5 Image analysis

The images were processed using Q proimaging software. Images were generated every 12 seconds for a total of 36 frames per condition and each stack of images were generated within an 8 minute time frame. For each adipokine concentration 5 image stacks were captured.

The images were analysed further using Image J 1.45SR software. The manual tracking plug-in was used and for each set of images 10 cells were chosen and

tracked through the 36 frames. The numerical data generated was then exported into excel and averaged to calculate:

a) Cell speed: which measures the movement of each cell between frames in any direction (chemokinesis).
b) Cell velocity: a measure of the movement of a cell in a focused direction (chemotaxis).
c) Cell persistence: a measure of the commitment of the cell in a particular direction (as illustrated in figure 2.1).
d) Chemotactic index: a measure of the accuracy of orientation towards a chemoattractant.

These measurements have been calculated as derived from those of Sapey et al. (2011).

Statistical analysis of all four parameters of lean versus obese adipokine concentrations was done using Prism 5.0 software and any significance difference was detected by the non-parametric Wilcoxon rank-sum test.

It should be noted that calculation of the persistence and chemotactic index results are not fully understood, and the results shown for them in the next section does not fully complement the other results (chemotactic speed or velocity) or the qualitative results (images).

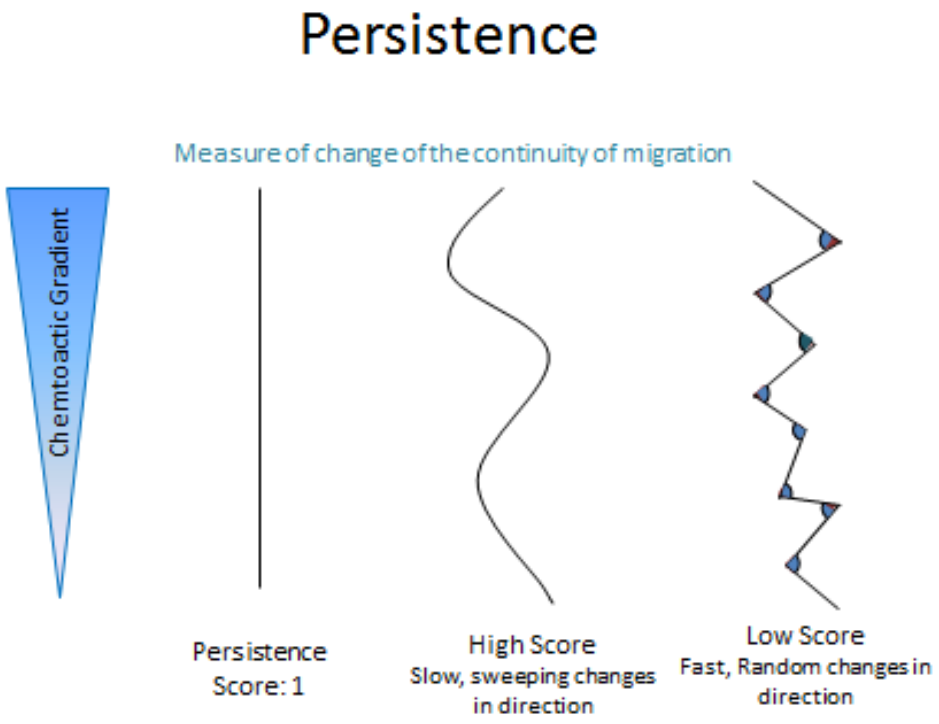


Figure 2.1. The parameter persistence as a measure of chemotaxis. The more linear the cell track, the greater the persistence.

3. Results

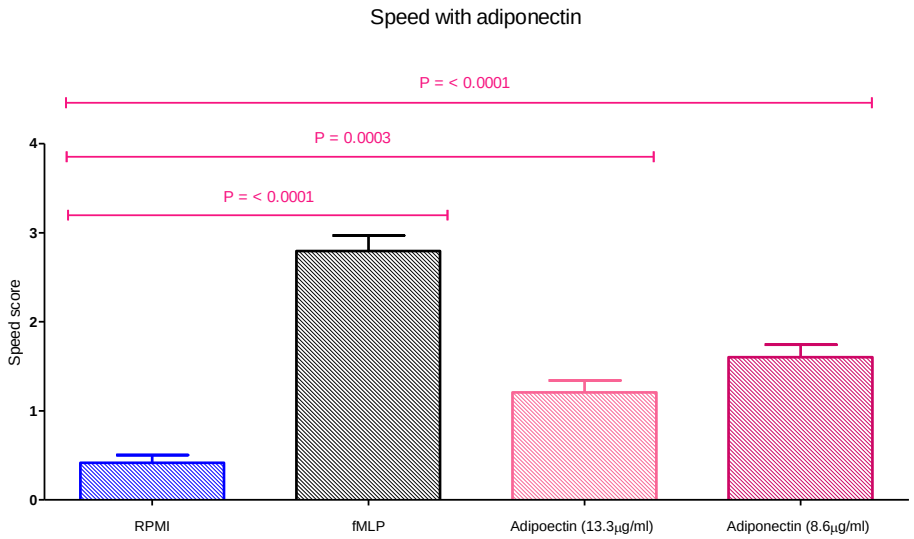
3.1 Chemotactic speed

Chemotactic speed is a measure of how far a cell has moved from the beginning image to the end frame of an experiment. Table 3.1 shows a summary of the significant differences in the speed of the cells between two different agents (adipokine or other). Figures 3.1a-f show the raw data of chemotactic speed using lean and obese concentrations of adipokines, the positive stimuli GM-CSF and IL-8 and control (RPMI).

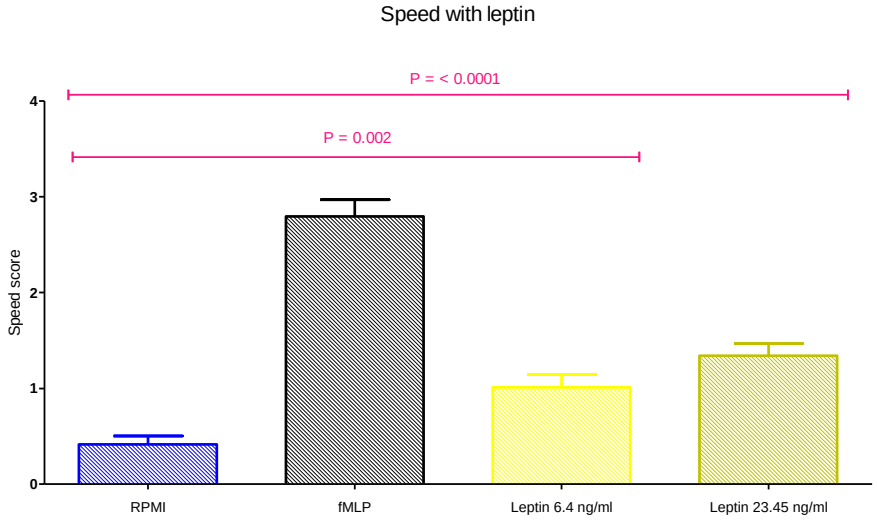
As expected there was a significant difference between the negative control and the positive control chemoattractant fMLP ($P = <0.0001$ shown in figure 3.1a). The same was true of the positive controls GM-CSF and IL-8 ($P = 0.0007$ and $P = 0.0002$ respectively) shown in table 3.1 and figure 3.1f. Adiponectin in both lean and obese conditions increases neutrophil speed compared to RPMI ($P= 0.003$ and < 0.0001 respectively) however there was no significant difference in neutrophil speed between the two concentrations (table 3.1 and figure 3.1a). Leptin showed a similar effect ($P = 0.001$ and < 0.0001) as did IL-6 ($P = 0.04$ and 0.0003) for lean and obese levels respectively (table 3.1 and figures 3.1b and 3.1c). Resistin showed a significant increase for lean and obese concentrations compared to RPMI ($P = <0.001$ and 0.0001 respectively) however resistin lean caused a significant increase in neutrophil speed compared to obese ($P = 0.005$) as illustrated in figure 3.1c. TNFα lean and obese levels increased cell speed over RPMI controls ($P = 0.0001$ for both) and obese was significantly increased over lean (table 3.1 and figure 3.1d)

Column 1 vs. Column 2		
Adipokine/RPMI	**Adipokine/stimulus**	**P value**
RPMI	fMLP	< 0.001
RPMI	Adiponectin lean (13.3 µg/ml)	0.003
RPMI	Adiponectin obese (8.6 µg/ml)	< 0.0001
RPMI	Leptin lean (6.4 ng/ml)	0.001
RPMI	Leptin obese (23.45 ng/ml)	< 0.0001
RPMI	Resistin lean (12.8 ng/ml)	< 0.001
RPMI	Resistin obese (33 ng/ml)	0.0001
Resistin lean (12.8 ng/ml)	Resistin obese (33 ng/ml)	0.005*
RPMI	TNFα lean (2 pg/ml)	0.0001
RPMI	TNFa obese (4 pg/ml)	0.0001
TNFα lean (2 pg/ml)	TNFa obese (4 pg/ml)	0.6
RPMI	IL-6 lean (0.5 pg/ml)	0.04
RPMI	IL-6 obese (1.5 pg/ml)	0.0003
RPMI	GM-CSF (100 ng/ml)	0.0007
RPMI	IL-8 (10 ng/ml)	0.0002

Table 3.1 Significant results from the measurement of chemotactic speed. Increases arise from statistical comparison of column 2 compared with column 1. The exception is highlighted (*) which represents a significant decrease in speed in column 2 compared to column 1.

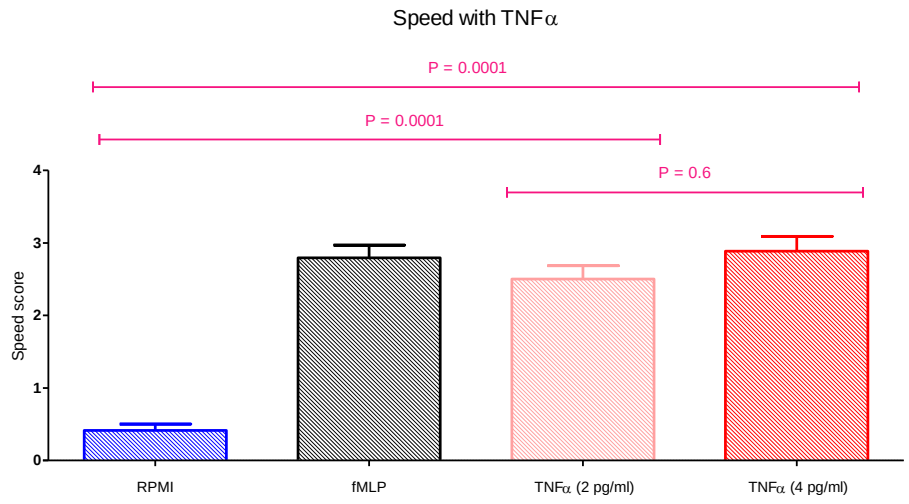


3.1 a) Speed with adiponectin

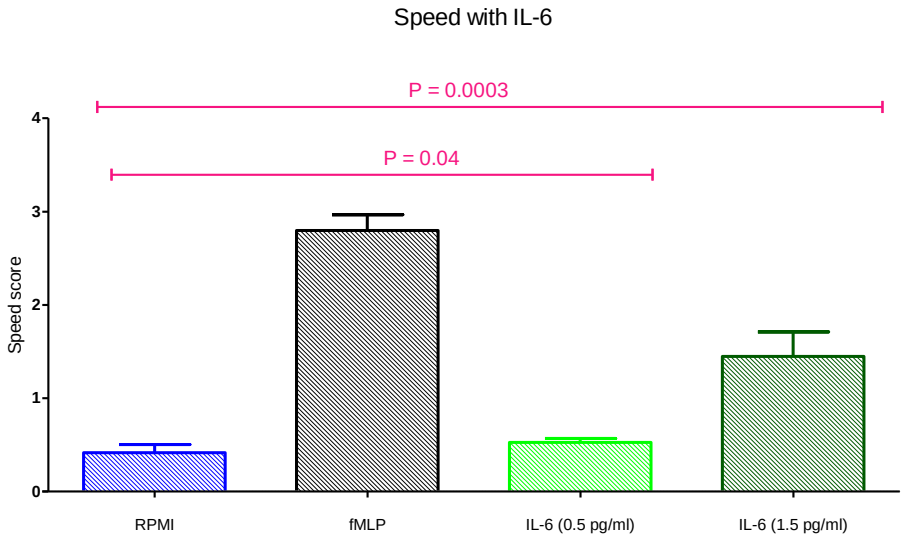


3.1 b) Speed with leptin

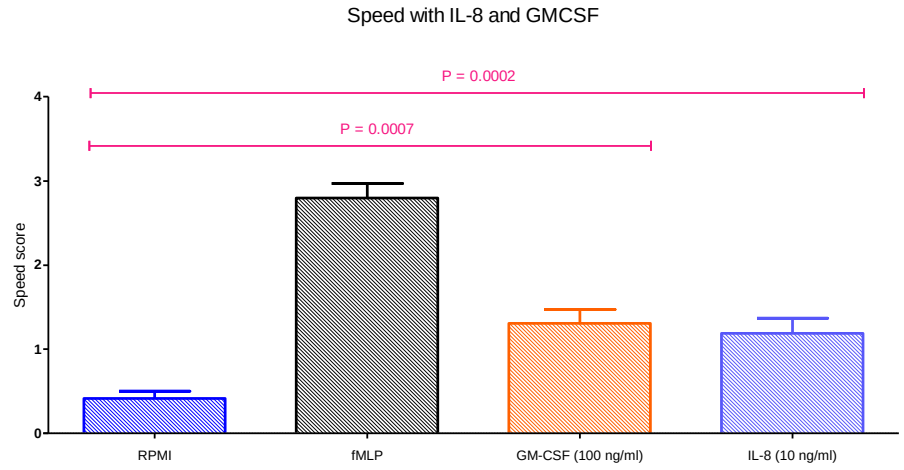
3.1 c) Speed with resistin



3.1 d) speed with TNFα



3.1 e) Speed with IL-6



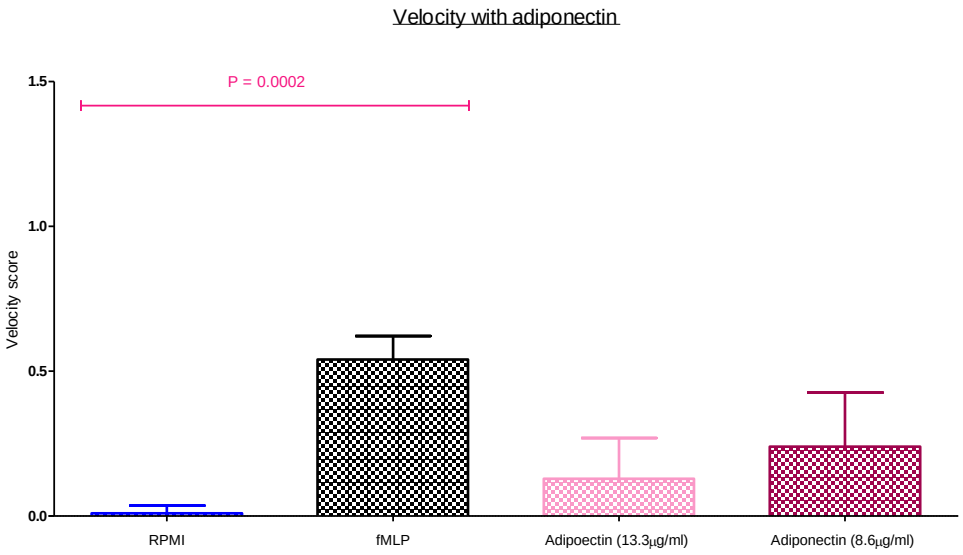
3.1 f) Speed with IL-8 and GM-CSF

3.2 Chemotactic velocity

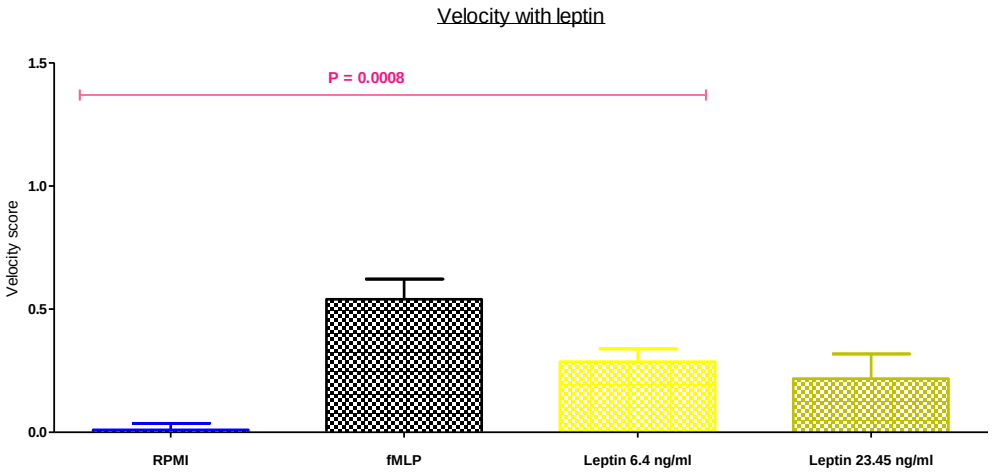
Chemotactic velocity is a measure of a cell's movement in a particular direction, i.e. towards a chemoattractant. Table 3.2 displays the statistically significant data from analysis of velocity results. Figures 3.2a-f illustrates all velocity results of the different adipokine concentrations. All three positive controls (fMLP, GM-CSF and IL-8) showed increased velocity compared to RPMI conditions (P = 0.002, 0.03 and 0.005 respectively) as shown by table 3.2 and figures 3.2a and 3.2f. There were no differences between lean and obese conditons, whilst TNFα at obese conncentrations appeared to generate a higher velocity relative to lean levels, the variation in neutrophil responses in the leaner group negated statistical significance being reached. Interestingly, IL-6 obese appeared to enhance neutrophil chemotactic velocity over that of fMLP (figure 3.2e).

Column 1 vs. Column 2		
Adipokine/RPMI	**Adipokine/stimulus**	**P value**
RPMI	fMLP	0.0002
RPMI	Leptin lean (6.4 ng/ml)	0.0008
RPMI	Resistin lean (12.8 ng/ml)	0.001
RPMI	TNFa obese (4 pg/ml)	0.0002
RPMI	IL-6 lean (0.5 pg/ml)	0.0003
RPMI	IL-6 obese (1.5 pg/ml)	0.0002
RPMI	GM-CSF (100 ng/ml)	0.03
RPMI	IL-8 (10 ng/ml)	0.005

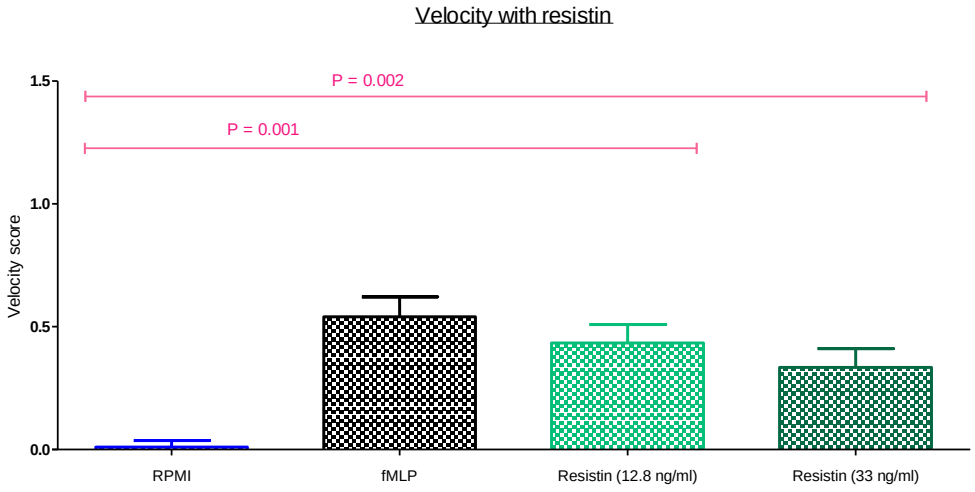
Table 3.2 Significant results from the measurement of chemotactic velocity. Increases arise from statistical comparison of column 2 compared with column 1.



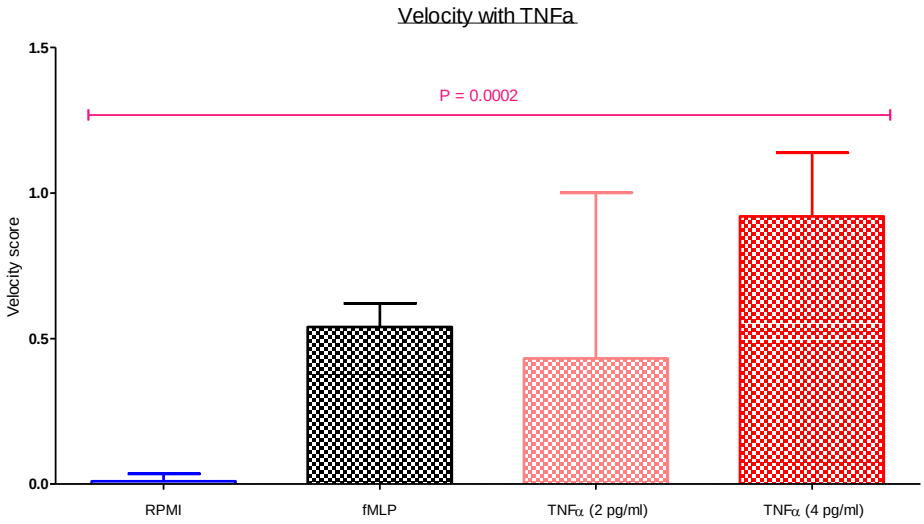
3.2 a) Velocity with adiponectin



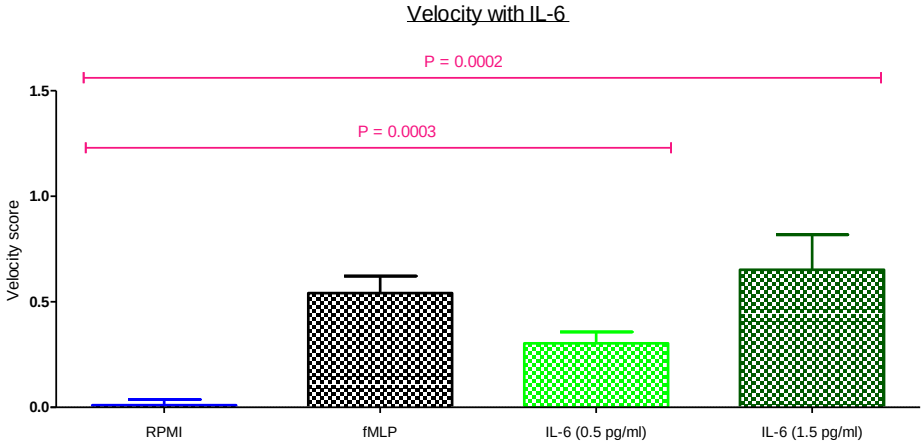
3.2 b) Velocity with leptin



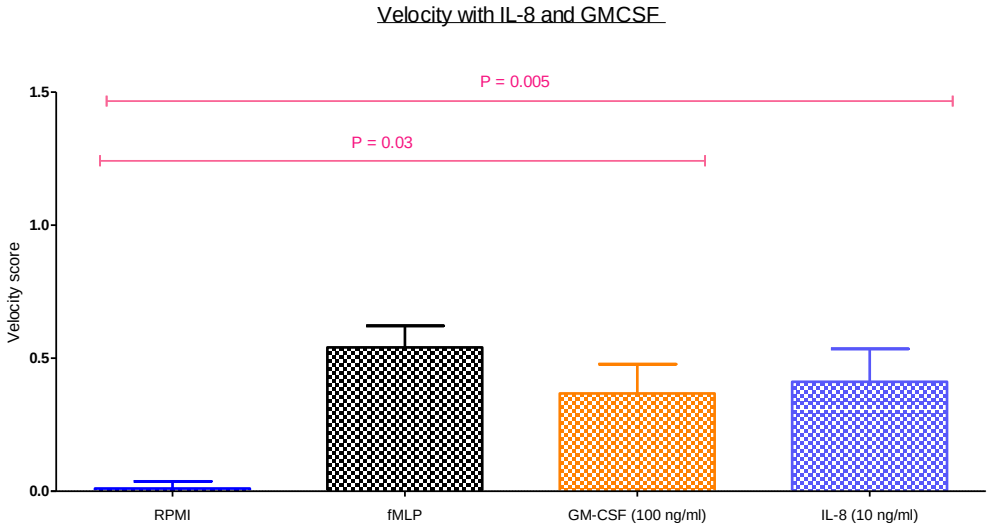
3.2 c) Velocity with resistin



3.2 d) Velocity with TNFα



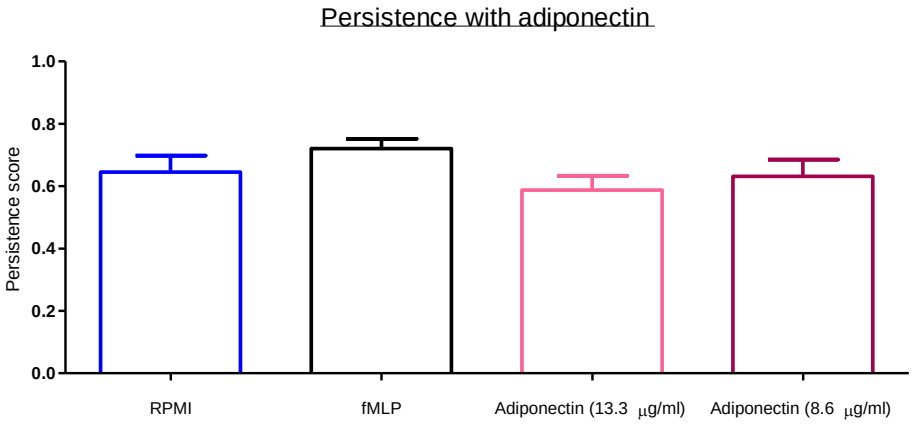
3.2 e) Velocity with IL-6



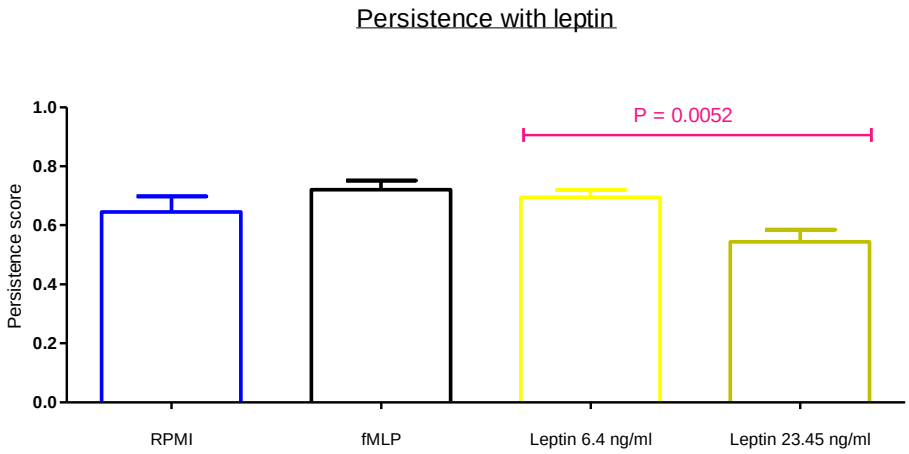
3.2 f) Velocity with IL-8 and GM-CSF

3.3 Chemotactic persistence

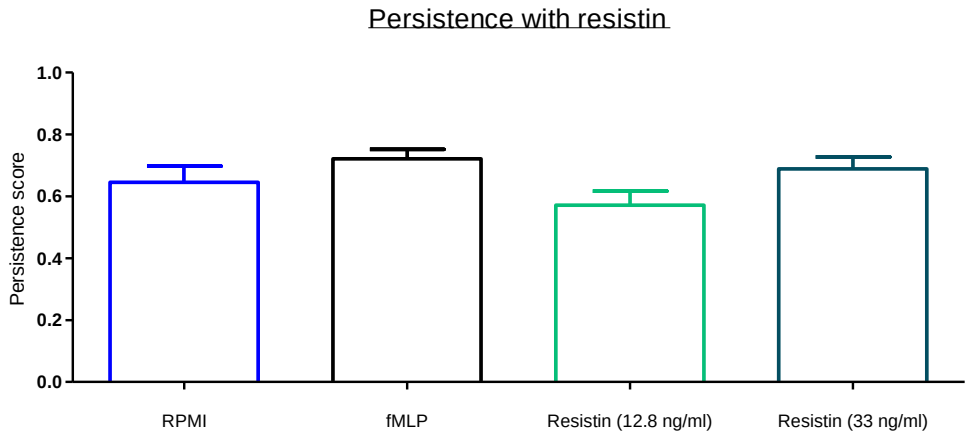
Chemotactic persistence measures how committed the cells are to a particular direction. Figures 3.3a-f illustrates the persistence results. The only significant results was a decrease between adipokine lean and obese concentrations in leptin, when the lean condition increased neutrophil persistence ($P = 0.005$) as shown in figure 3.3b.



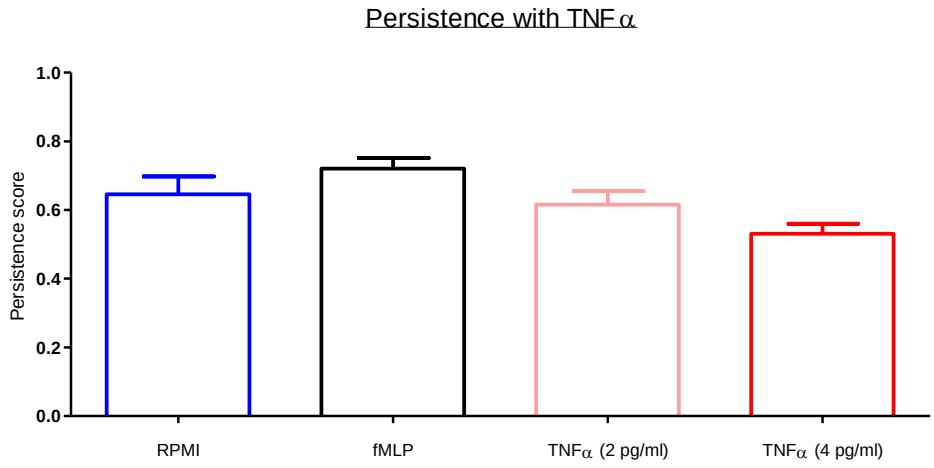
3.3 a) Persistence with adiponectin



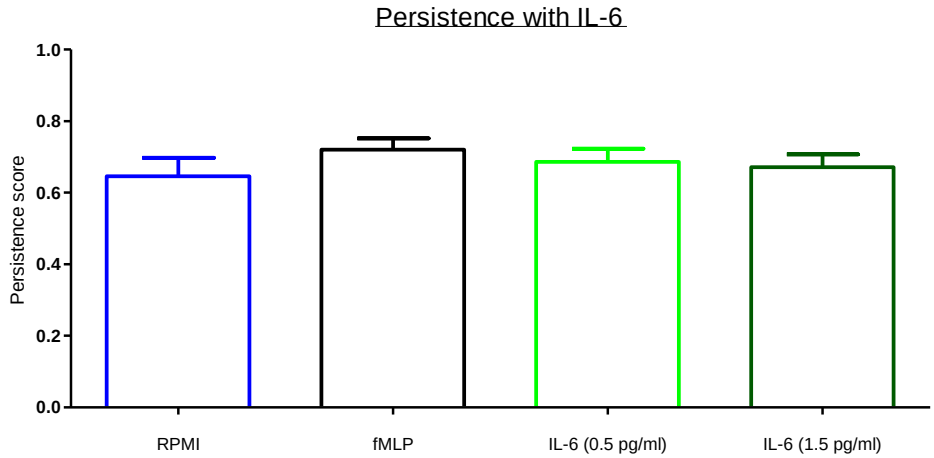
3.3 b) Persistence with leptin



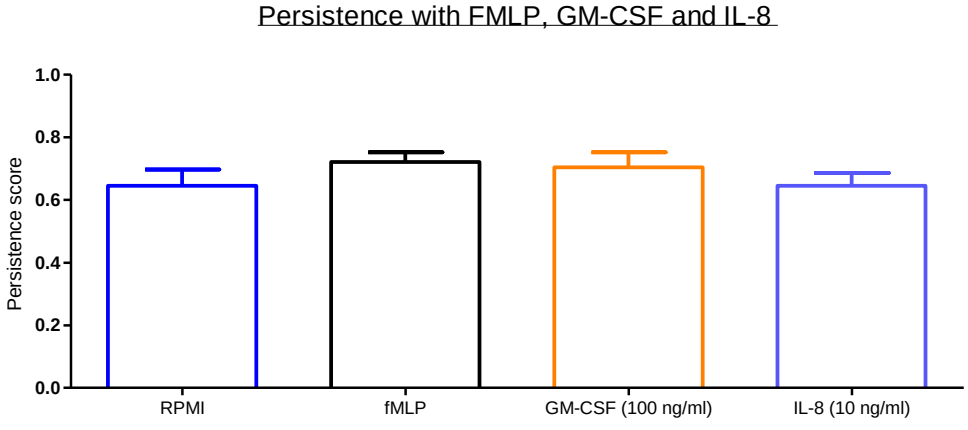
3.3 c) Persistence with resistin



3.3 d) Persistence with TNFα



3.3 e) Persistence with IL-6



3.3 f) Persistence with GM-CSF and IL-8

3.4 Chemotactic index

Chemotactic index measures how accurate the orientation of a cell is towards a chemoattractant. Table 3.3 shows the statistical results and figures 3.4a-f illustrates all results; the lower the score, the greater the index. fMLP, GM-CSF and IL-8 again show significant differences demonstrating fMLP having a higher chemotactic score than the neutrophils exposed to RPMI (P = 0.002, 0.04 and 0.03 respectively) (table 3.3 and figures 3.4a and f). Lean and obese concentrations of adiponectin, leptin and TNFα give a greater chemotactic index than control (table 3.3). Both leptin and IL-6 lean concentrations gave a lower index compared to lean (P= 0.005 and P = 0.04 respectively) (table 3.3 and figure 3.4e).

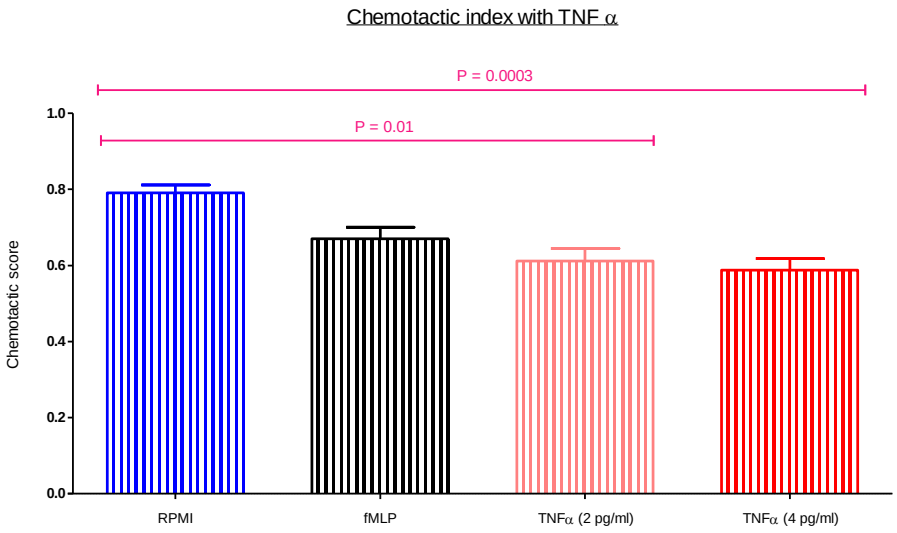
Column 1 vs. Column 2		
Adipokine/RPMI	**Adipokine/stimulus**	**P value**
RPMI	fMLP	0.002
RPMI	Adiponectin lean (13.3 µg/ml)	0.04
RPMI	Adiponectin obese (8.6 µg/ml)	0.05
RPMI	Leptin lean (6.4 ng/ml)	0.0005
Leptin lean (6.4 ng/ml)	Leptin obese (23.45 ng/ml)	0.005*
RPMI	TNFα lean (2 pg/ml)	0.01
RPMI	TNFa obese (4 pg/ml)	0.0003
IL-6 lean (0.5 pg/ml)	IL-6 obese (1.5 pg/ml)	0.04*
RPMI	GM-CSF (100 ng/ml)	0.04
RPMI	IL-8 (10 ng/ml)	0.03

Table 3.3 Chemotactic index. Significant results from the measurement of chemotactic index. Statistical comparison of column 2 compared with column 1 were made. The exception is highlighted (*) which represents a significant decrease in chemotactic index in column 2 compared to column 1.

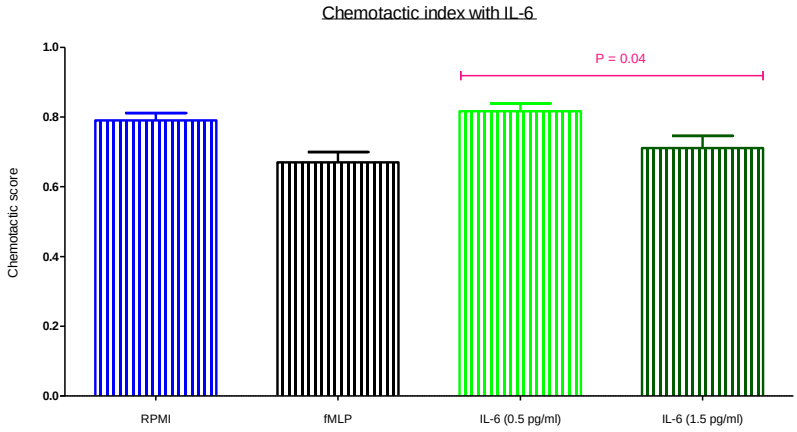
3.4 a) Chemotactic index with adiponectin

3.4 b) Chemotactic index with leptin

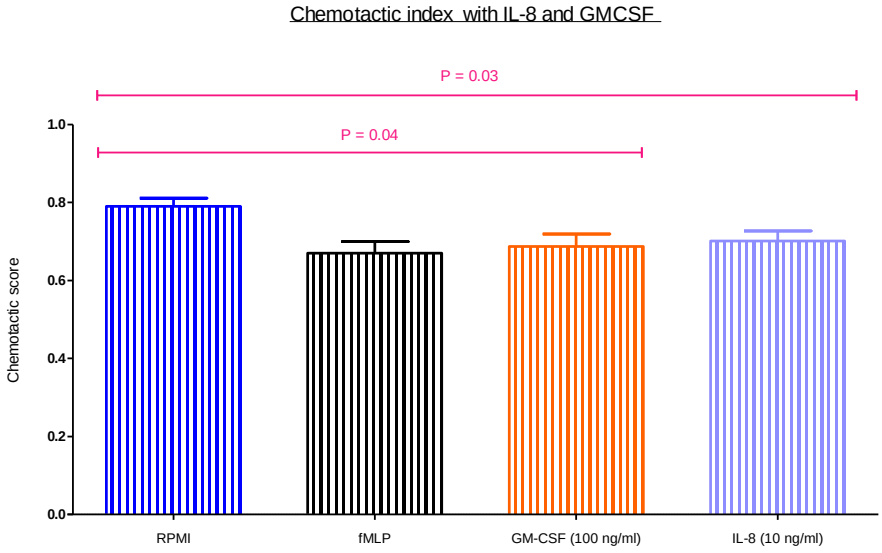
3.4 c) Chemotactic index with resistin



3.4 d) Chemotactic index with TNFα



3.4 e) Chemotactic index with IL-6



3.4 a) Chemotactic index with GM-CSF and IL-8

3.5 Chemotaxis cell tracking images

Figures 3.4a-3.7a shows the end time-point images of the neutrophils with the coloured lines mapping the cell tracks from their origin. White arrows indicate the general direction the cell. Figures 3.5b-3.7b show the cell tracks from (derived from figures 3.4b-3.7a) all of which have the same point of origin. The images provide a qualitative visual as a backdrop to the quantitative chemotaxis parameters (velocity. Speed and chemotactic index).

RPMI, positive control fMLP, TNFα lean and TNFα obese figures are shown. There are clear differences in the movement of the cells exposed to RPMI and fMLP, with fMLP neutrophils showing a directional movement upwards (towards the source of the chemoattractant) and covering a greater distance. TNFα was included because for speed, velocity and chemotactic index there were significant differences between lean or obese and RPMI control concentrations (figures 3.1d. 3.2d and 3.4d) and also obese concentrations enhanced the overall speed of the neutrophils compared to lean (3.1d). Apart from a few outliers, the majority of the cells exposed to TNFα obese concentrations are orientated in an upward direction compared to lean, which shows a speed similar to obese but in a more random direction. Supplementary figures (S.1-5) show images of neutrophils treated with other adipokines and their movement from a common point of origin.

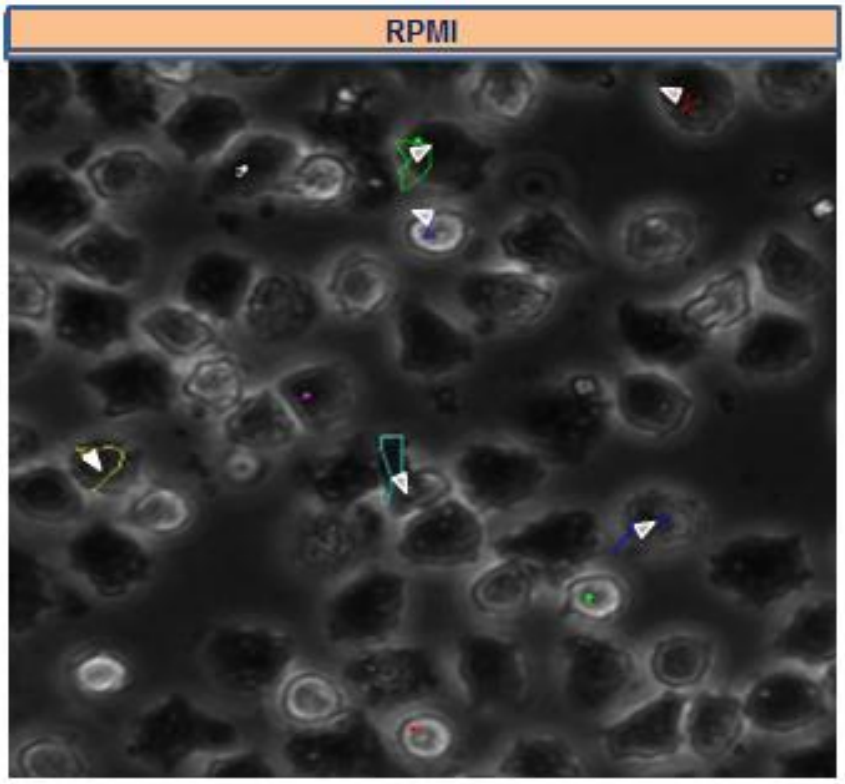


Figure 3.5 a)

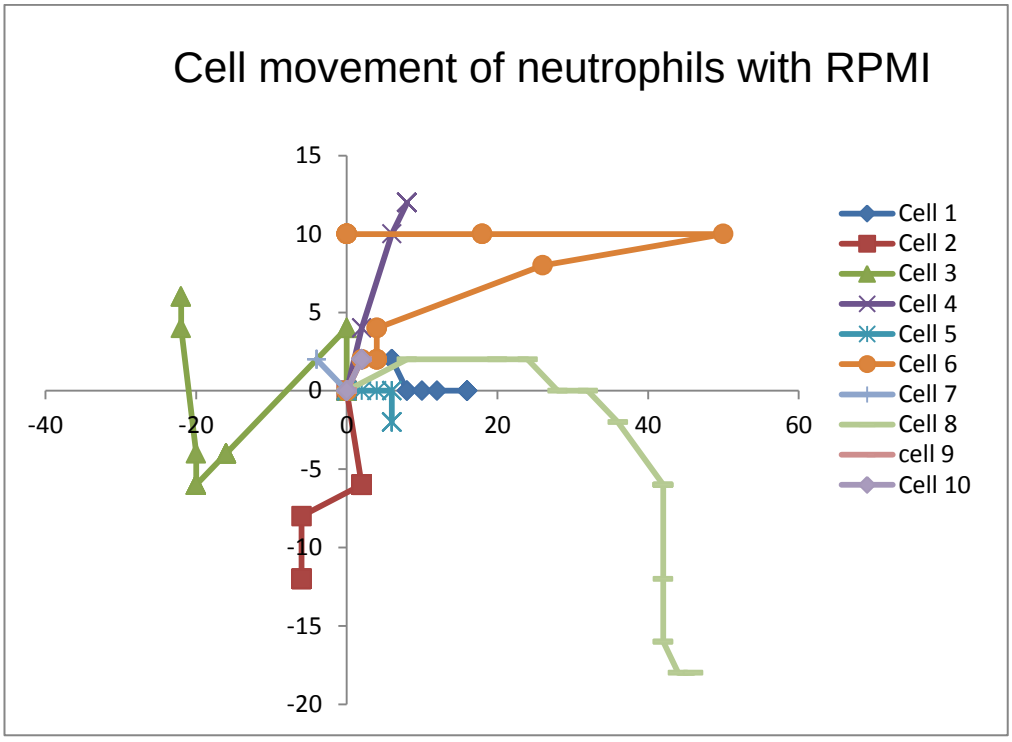


Figure 3.5 b)

Figure 3.5 a) Final image of neutrophils with RPMI control. RPMI was injected into the Insall chamber channels, of which the closest point to the channel edge is indicated by the orange bar. b) The movement of the cells arranged from a single point of origin. The graph has been drawn with RPMI source at the top.

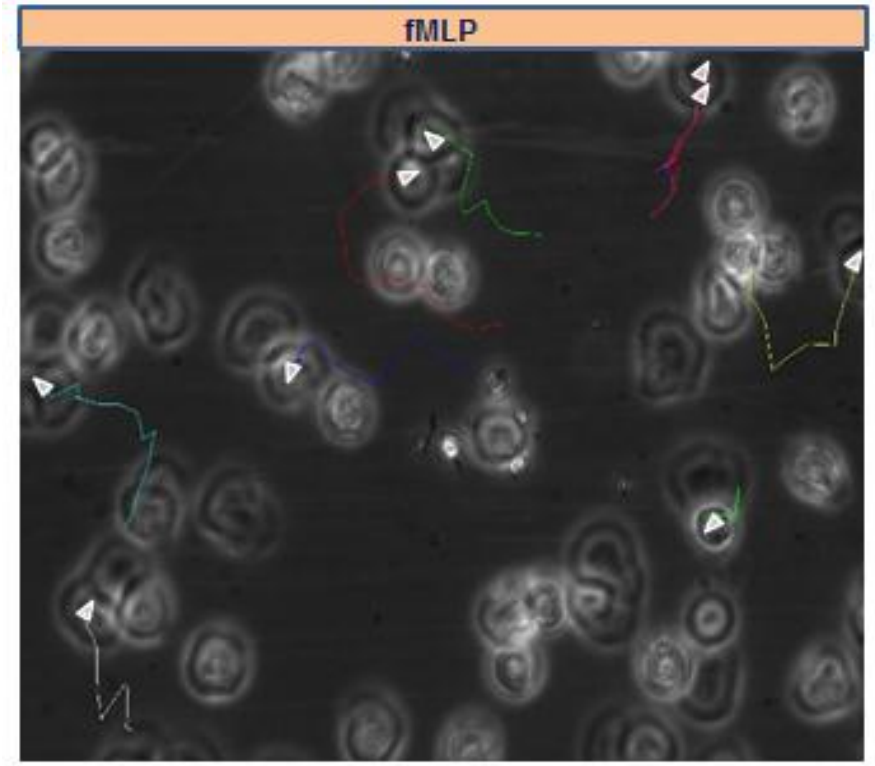


Figure 3.6 a)

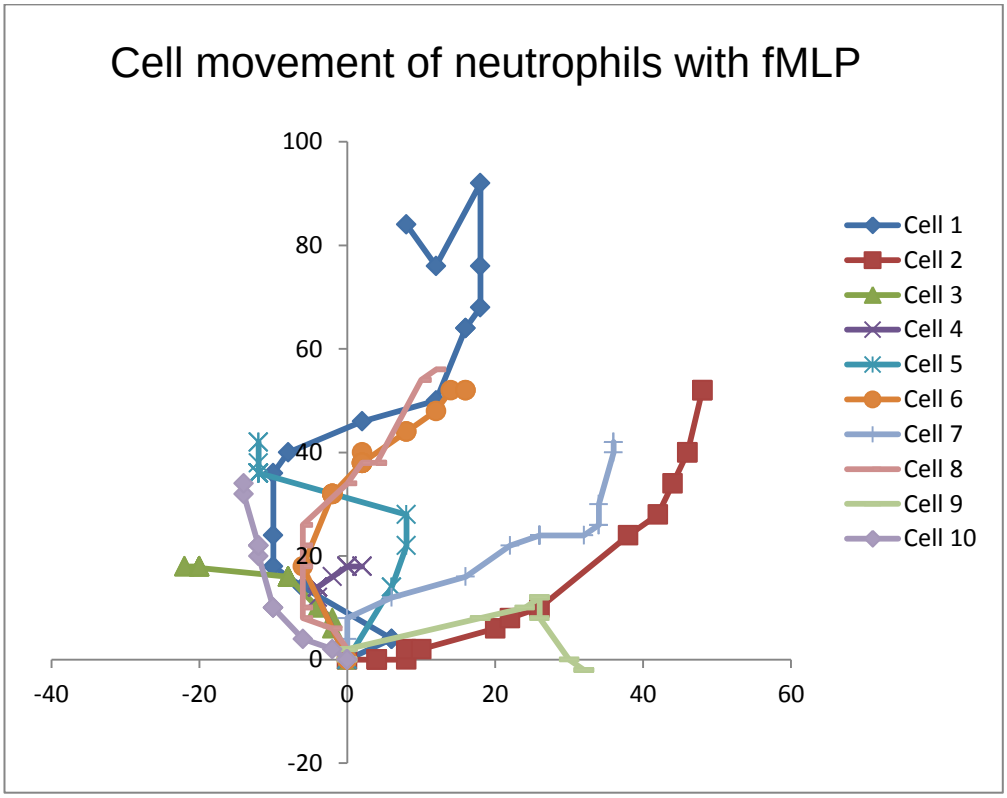


Figure 3.6 b)

Figure 3.6 a) Final image of neutrophils with fMLP postive control. The source of the fMLP was towards the top of the image (indicated by the orange bar). b) The movement of the cells arranged from a single point of origin. The graph has been drawn with fMLP source at the top.

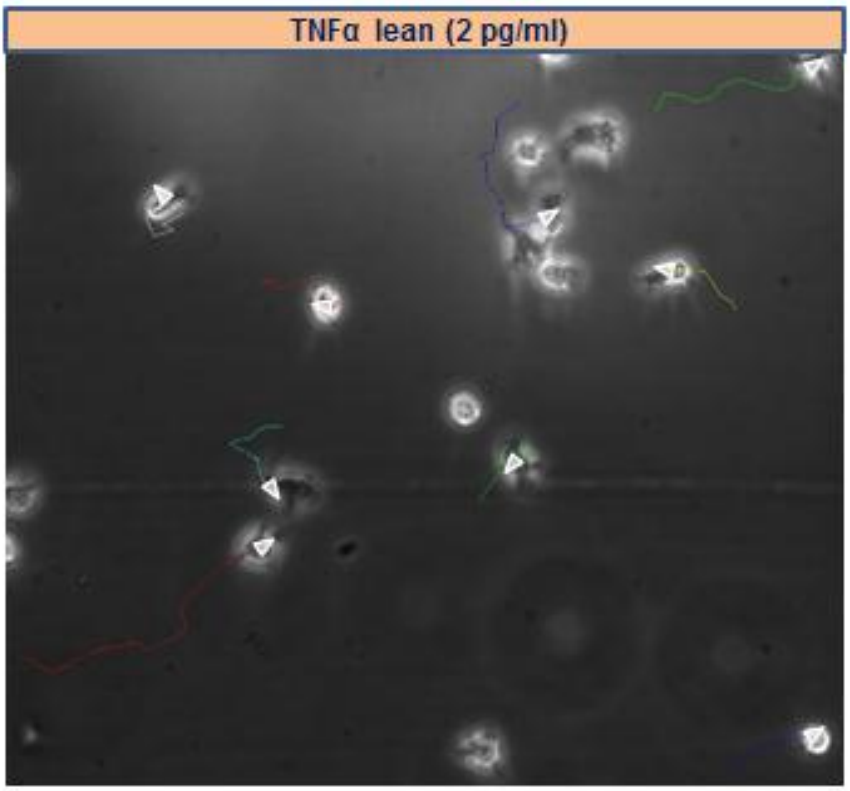


Figure 3.7 a)

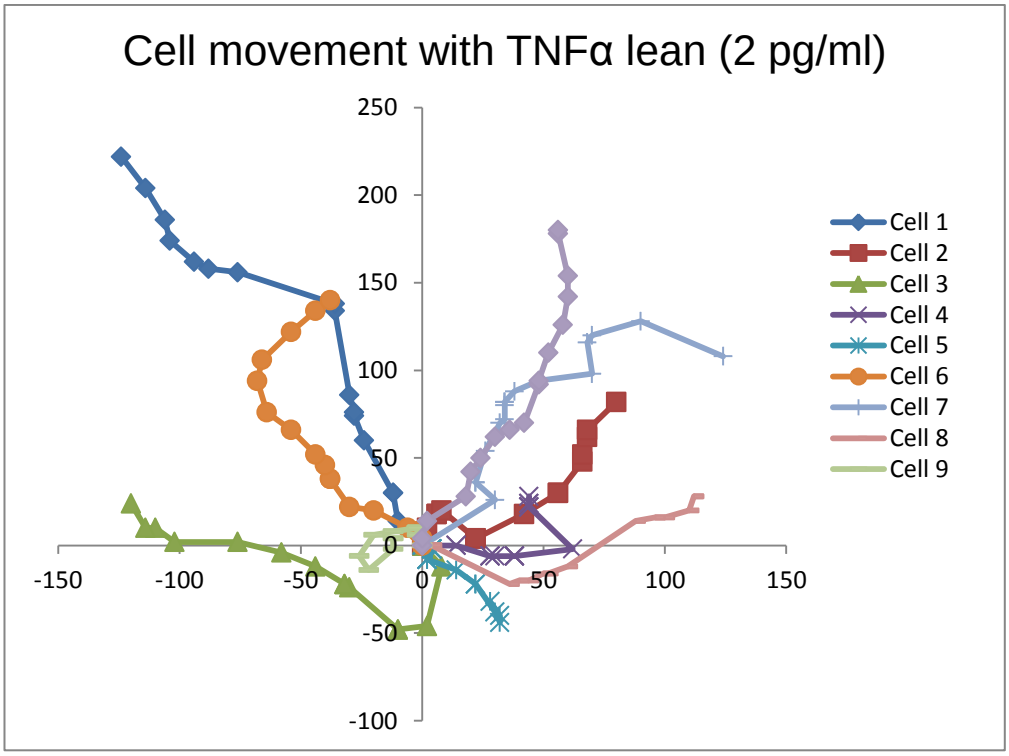


Figure 3.7 b

Figure 3.7 a) Final image of neutrophils with TNFα lean (2 pg/ml). The source of the TNFα was towards the top of the image (indicated by the orange bar). b) The movement of the cells arranged from a single point of origin. The graph has been drawn with the TNFα source at the top.

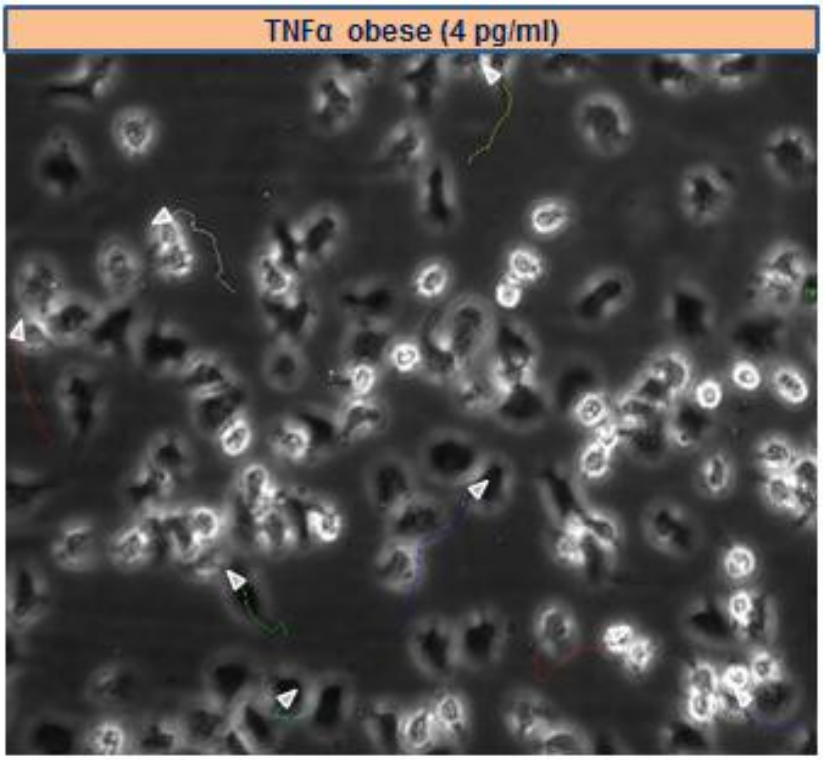


Figure 3.8 a)

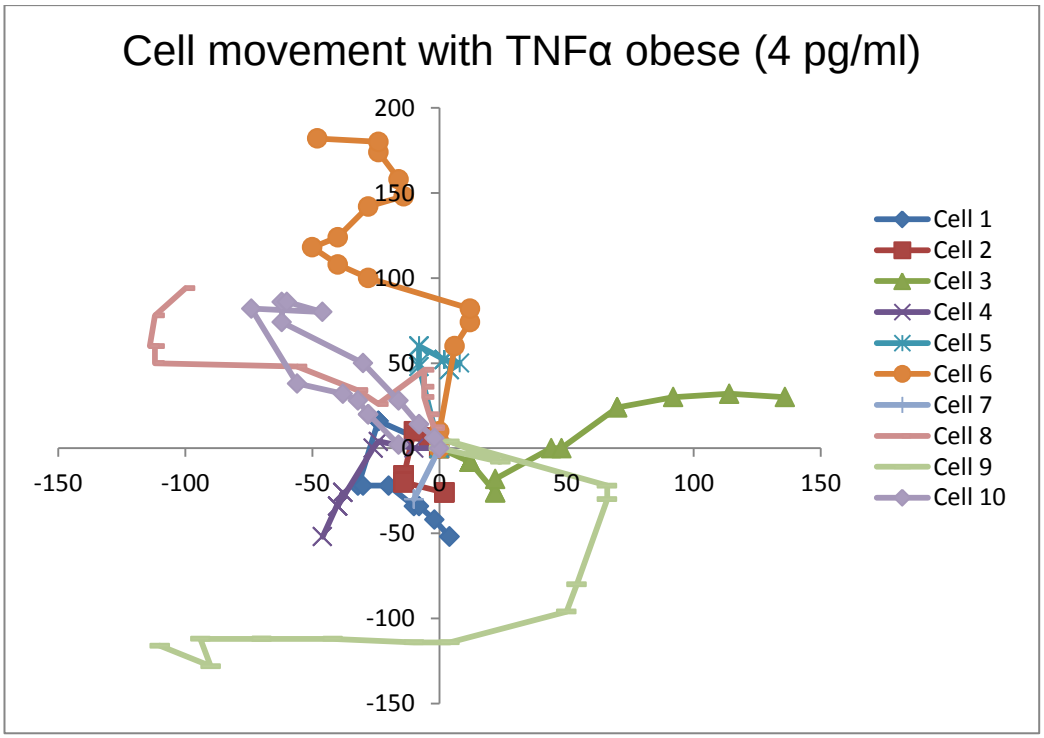


Figure 3.8 b)

Figure 3.8 a) Final image of neutrophils with TNFα obese concentration (4 pg/ml). The source of the TNFα was towards the top of the image (indicated by the orange bar). b) The movement of the cells arranged from a single point of origin. The graph has been drawn with TNFα source at the top.

4. Discussion

This project shows that adipokines reflective in lean and obese individuals can affect chemotaxis in neutrophils. Within all four of the chemotactic parameters studies, speed and velocity, which show a similar pattern for the different adipokines, give a significant result for the majority of concentrations versus control (RPMI). fMLP was shown to significantly increase neutrophil speed, velocity and have a greate chemotactic index compared to control and therefore serves to validate the rest of the results. GM-CSF and IL-8, the concentrations of which were that of "priming" (and therefore non-physiological) also showed significant increases in speed, velocity and chemotactic index, but not to the extent of fMLP (which is also supportive of the study by Gomez-Cambronero et al. 2003). The two adipokines that showed significant differences between lean and obese concentrations were resistin and TNFα. For resistin, the results in this study show cell speed to be higher with exposure to lean versus obese. This supports the study by Kawanami et al. (2003), in which resistin induced cell cycle arrest and the up-regulated expression of adhesion factors (which bind to the endothelial cell walls). Conversely TNFα obese concentrations were found to significantly increase neutrophil movement compared to lean and for obese were comparable to the effects of fMLP, this result also supports those within the literature, which show TNFα to act as a positive chemoattractant at high concentrations (Shalalby et al. 1985). Justification for the concentrations of all adipokines used in this study is described in project 1 (section 4).

The results show that these adipokines can affect neutrophil movement. It is likely that rather than causing chemotaxis, these agents can induce chemokinesis, (non-directional cell movement). This would explain the lack of statistically significant

results for persistence, which is a measure of cell commitment to a particular direction.

The methods used to analyse the data were adapted from Sapey et al. (2011), however there were some differences, such as the length of time between each image captured, in this study it was every 12 seconds (Sapey et al. had a 20 second time lapse betwwen each frame taken). The results therefore may have been more informative if a great time gap had been used. The number of volunteers for this study was 5 per adipokine concentration, whereas Sapey et al. used up to 20. An increase in the number of volunteers would potentially give a more reliable and accurate result.

The methods used to calculate the chemotactic parameters in this project were from using ImageJ software using the plugin 'Manual Tracking'. This involves manually selecting cells and following their movement through a stack of images. The subjective nature of this method could generate biased and inaccurate results (despite the fact the cells were chosen at random). The cells moved in and out of phase within the images and therefore accurately establishing the location and the centre-most point of the cell can be difficult. Other methods of analysing chemotaxis activity are also available, such as the McCutcheon Index, which measures the length of the path travelled toward the chemoattractant minus the distance travelled away from the chemoattactant, this is then divided by the total distance travelled (McCutcheon 1946). Exploring the different methods of assimilating the data derived from videomicroscopy will aid deeper understanding and provide a more reliable interpretation of the data.

In this project the Insall chamber was used to study the behaviour of neutrophils exposed to different concentrations of adipokines. This chamber was chosen over others available (as outlined in section 1.9) as it was deemed the most accurate in

terms of establishing a gradient within a defined direction. However these gradients are shallow and therefore the results shown in this project showing neutrophil migration are unlikely to reflect the complex three-dimensional environment of the human body.

There are other systems outside the use of chambers that are used for the study of chemotaxis. Polydiemthylsiloxane (PDMS)-based microfluidic channels are thought to provide a better method because there is more control with regard to the spatial and temporal characteristics of the chemical environment to which the cells are exposed compared to the chamber techniques (Pihl et al. 2005). Figure 4.1 shows the schematic representation of the microfluidic device. Future work could involve the use of microfluidic devices to study neutrophil chemotaxis. Other adipokines such as visfatin, which has been shown to induce chemotaxis in monocytes (Moschen et al. 2007), could also be explored to generate a profile characterising the differences between the effects of lean and obese concentrations.

5. Conclusion

The exposure of neutrophils derived from lean healthy male volunteers to adipokine concentrations relfective of lean and obese individuals result in an increase in chemokinesis and potentially chemotaxis. More repeats of these studies and exploration into other methods of analysing chemotaxis data are required in order to validate any significant results.

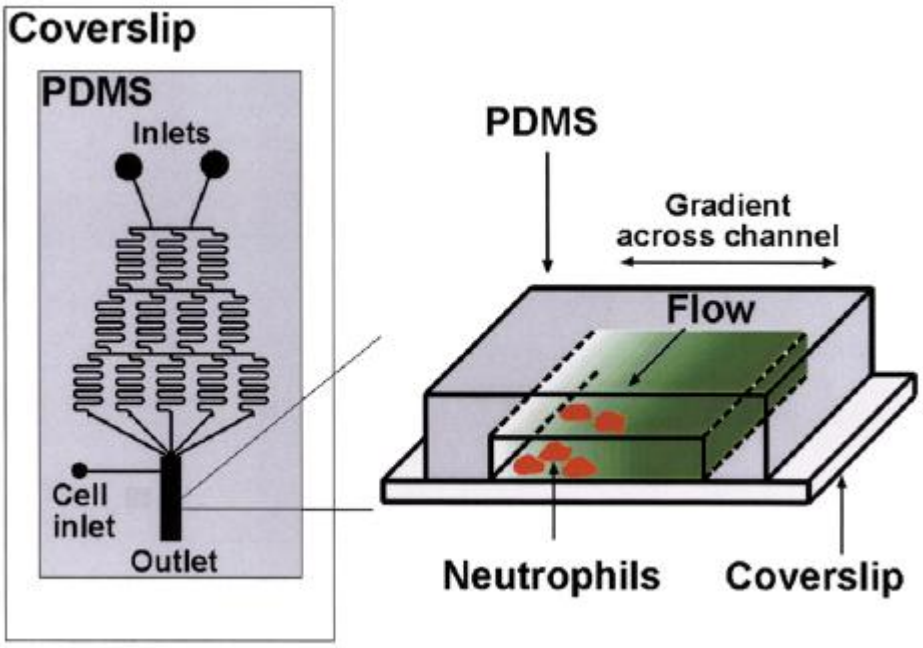


Figure 4.1 An illustration of a microfluidic device. The PDMS piece (which features embedded channels) is bonded to a glass coverslip. Chemoattractant and/or medium is added to the inlets and as the fluid flows through the channels, different concentration gradients are generated for the cells to move towards or away depending on the nature of the agent added (Jeon et al. 2002).

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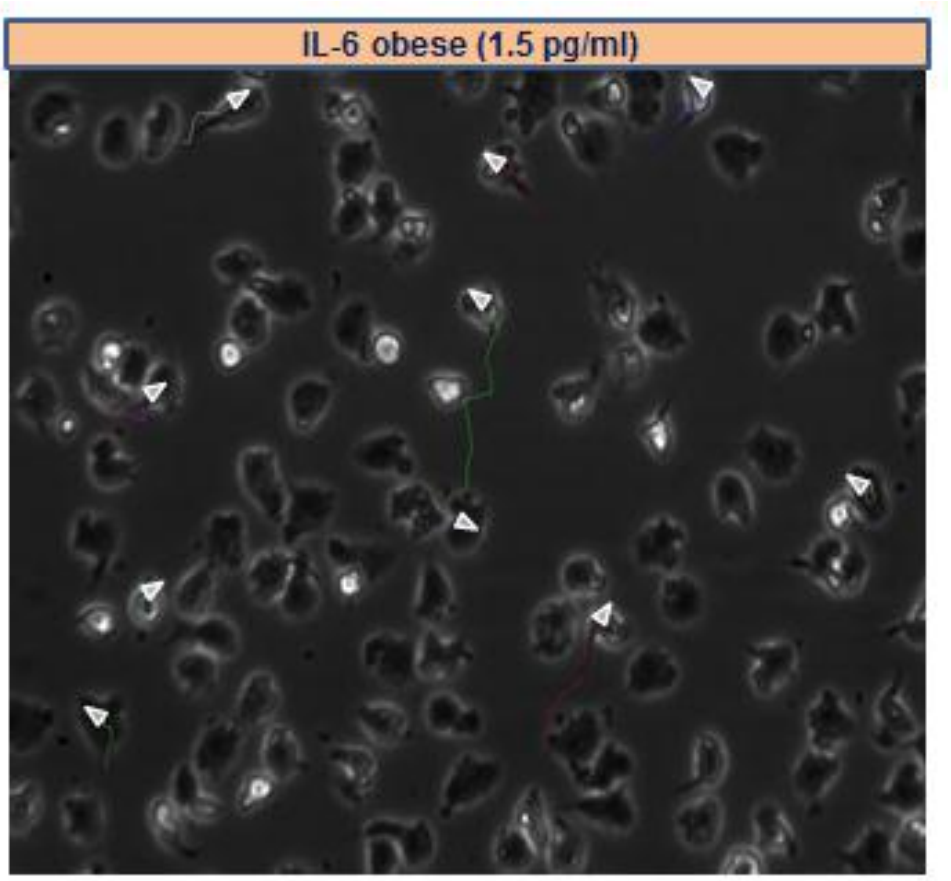
7. Supplementary results



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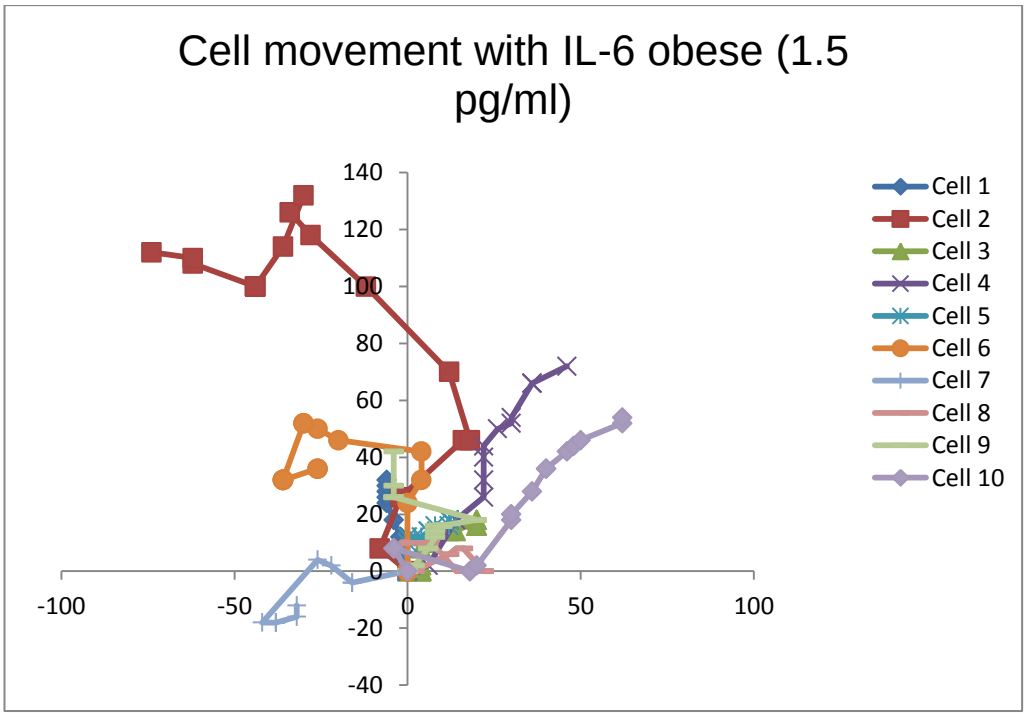
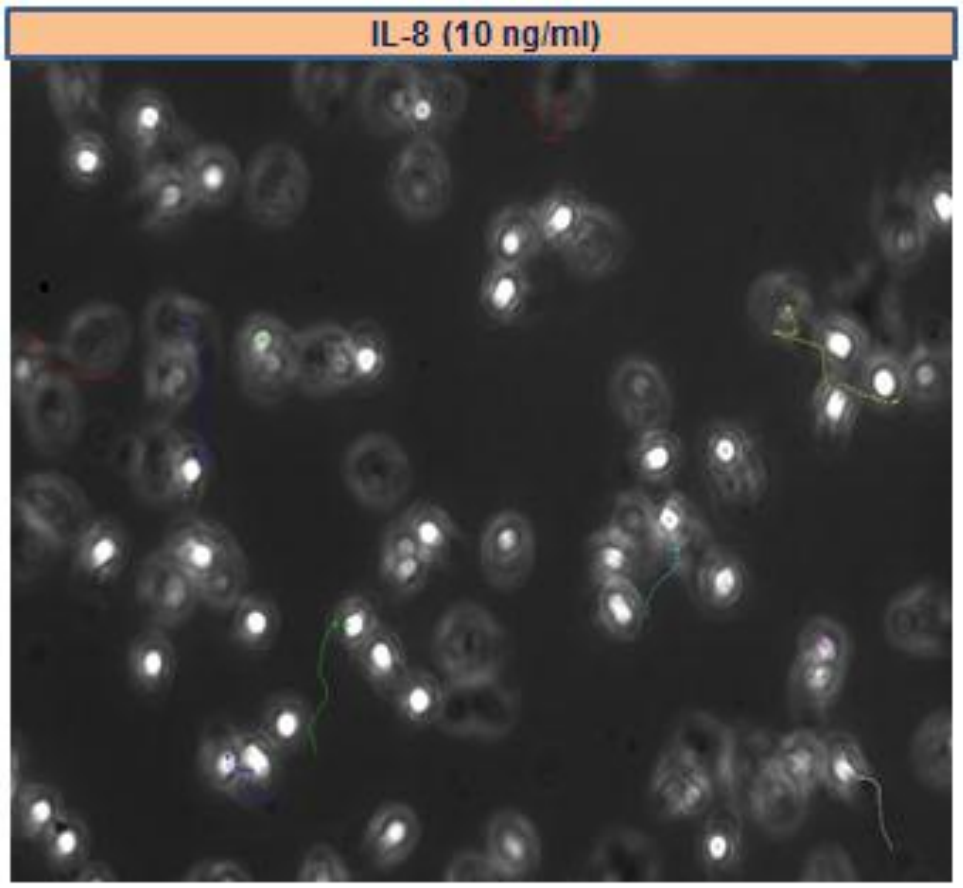


S.1b)

Figure S.1 a) Photograph of tracking paths of neutrophils exposed to IL-6 obese concentration (data is representative of a single volunteer). b) Tracks taken from a) and plotted from a single point of origin.



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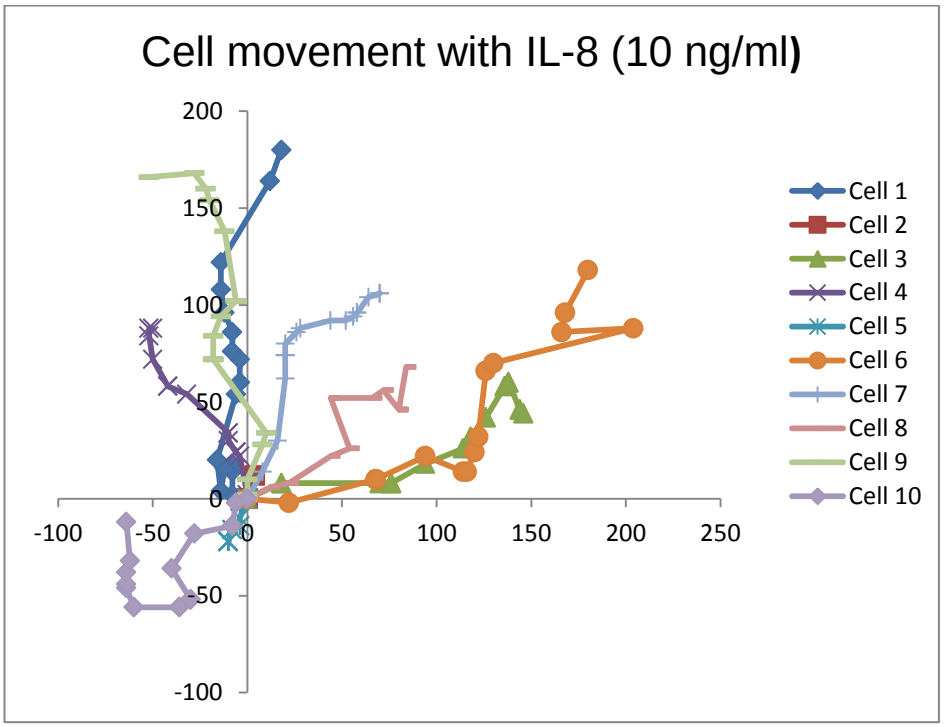
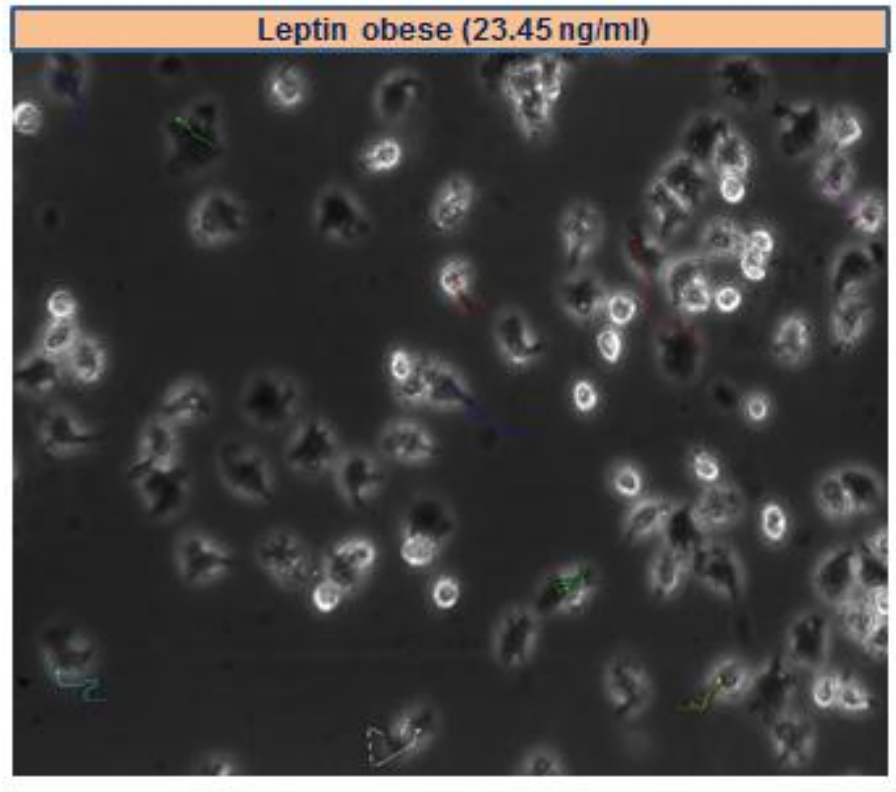


S.2b)

Figure S.2 a) Photograph of tracking paths of neutrophils exposed to IL-8 (data is representative of a single volunteer). b) Tracks taken from a) and plotted from a single point of origin.



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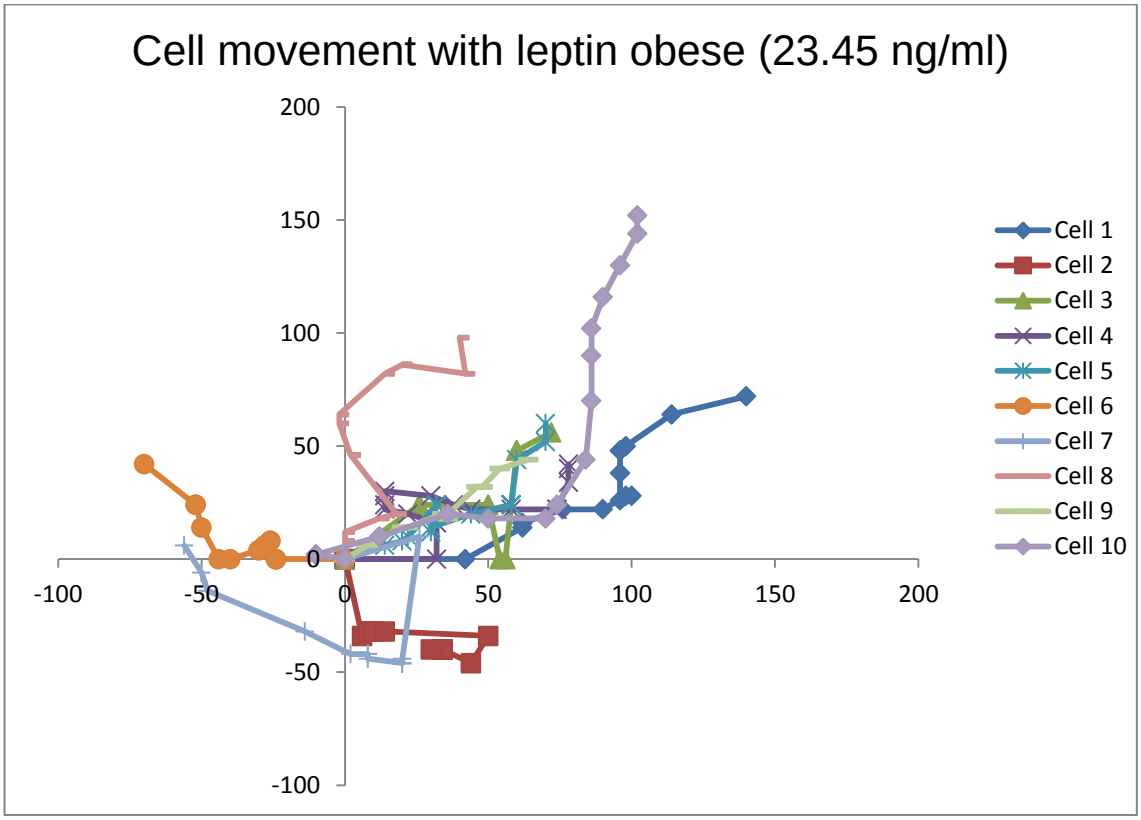
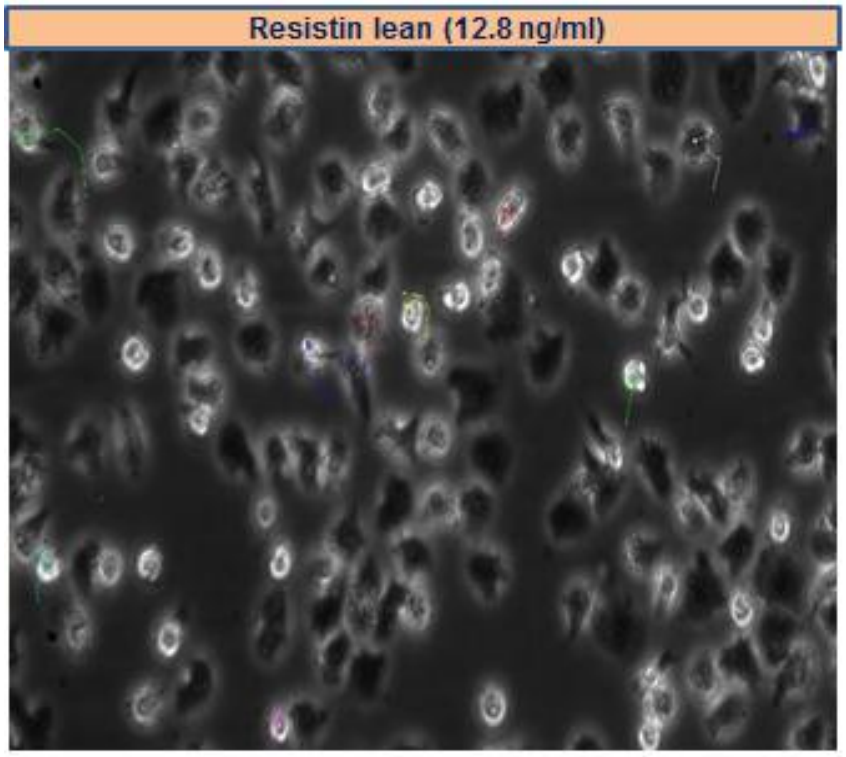
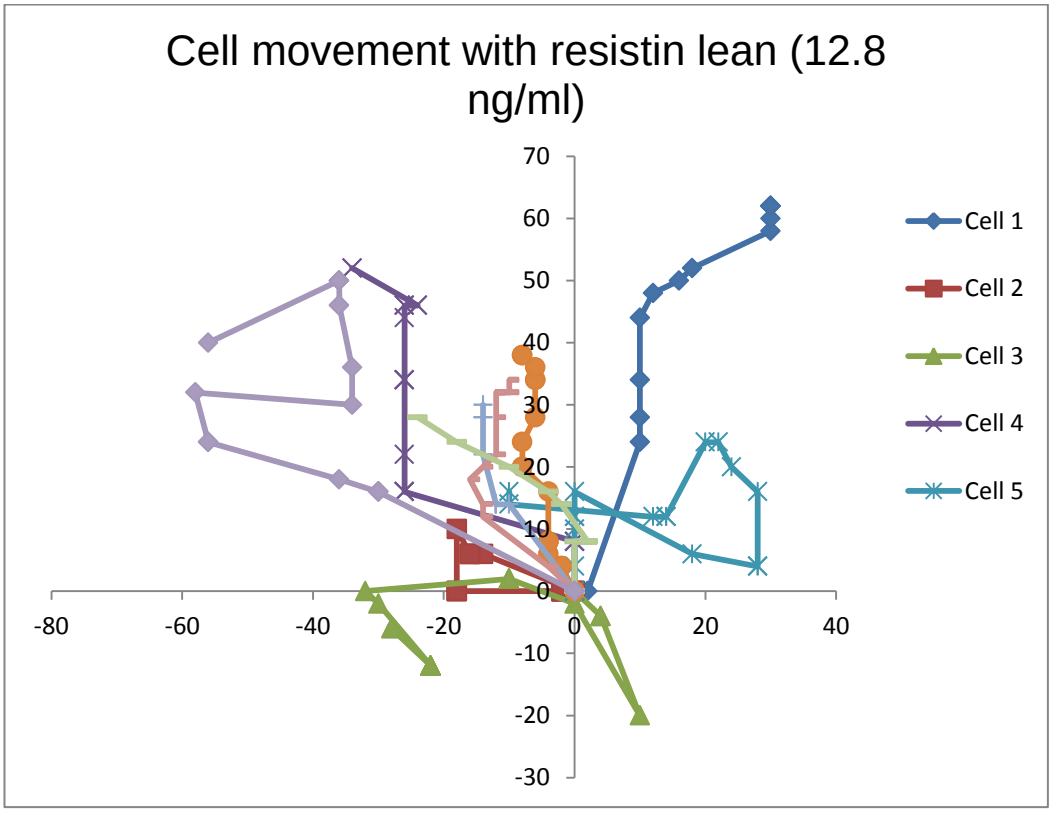


S.3b)

Figure S.3 a) Photograph of tracking paths of neutrophils exposed to leptin obese concentration (data is representative of a single volunteer). b) Tracks taken from a) and plotted from a single point of origin.

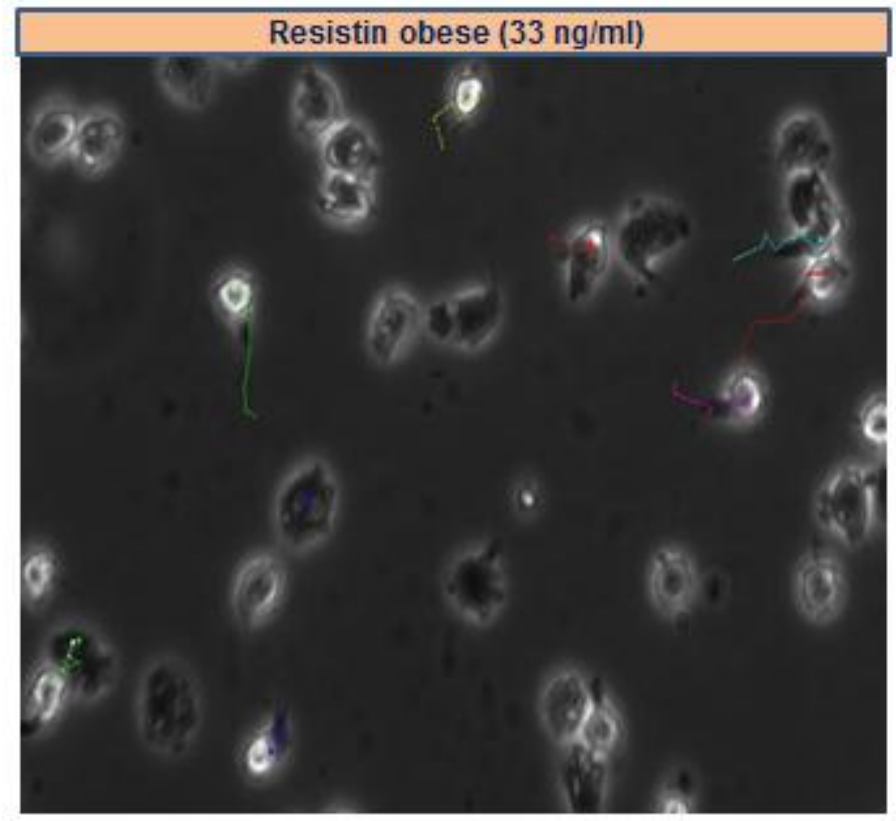


S..4a)



S.4b)

Figure S.4 a) Photograph of tracking paths of neutrophils exposed to resistin lean concentration (data is representative of a single volunteer). b) Tracks taken from a) and plotted from a single point of origin.



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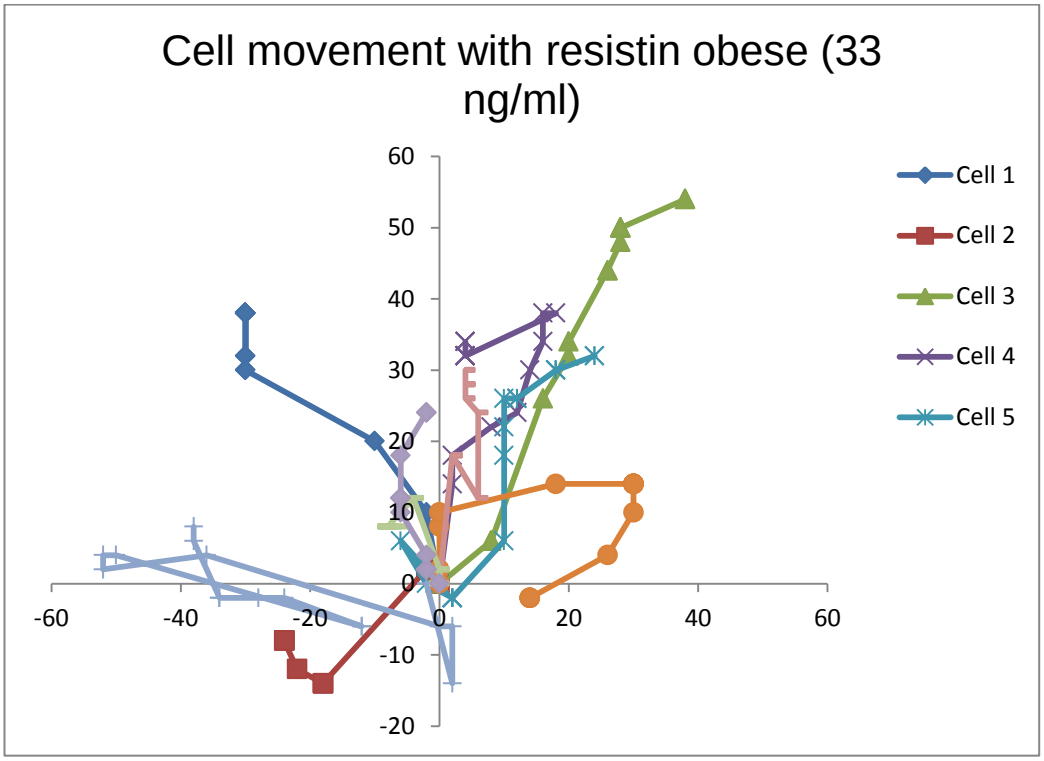

S.5b)

Figure S.5 a) Photograph of tracking paths of neutrophils exposed to resistin obese concentration (data is representative of a single volunteer). b) Tracks taken from a) and plotted from a single point of origin.