# The Effects of C-Reactive Protein (CRP) Isoforms on Inflammation and Wound Healing Processes

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**Chapter 2: General Materials and Methods**



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## List of Abbreviations

| | |
|---|---|
| BCA | Bicinchoninic Acid |
| BMI | Body Mass Index |
| BSA | Bovine Serum Albumin |
| CD16 | FcγRIIIb |
| CD32 | FcγRIIa |
| Cdk | cyclin-dependent kinase |
| CFU | Colony Forming Units |
| CLR | C-type Lectin Receptors |
| cGMP | Cyclic Guanosine Monophosphate |
| CRP | C-Reactive Protein |
| D3 | Day 3 |
| D7 | Day 7 |
| DAMP | Damage-Associated Molecular Patterns |
| DNA | Deoxyribonucleic Acid |
| E2 | Estrogen |
| EDTA | Ethylenediaminetetraacetic Acid |
| EGFR | Epidermal Growth Factor Receptor |
| eNOS | Endothelial Nitric Oxide Synthase |
| FBS | Foetal Bovine Serum |
| FGF | Fibroblast Growth Factor |
| GPCR | G-protein Coupled Receptor |
| HRP | Horse Radish Peroxidase |
| HRT | Hormone Replacement Therapy |
| IFN-γ | Interferon-γ |
| IGF-R | Insulin-like Growth Factor Receptor |
| IgG | Immunoglobulin G |
| IL-1 | Interleukin-1 |
| IL-1β | Interleukin-1β |
| IL-4 | Interleukin-4 |

| | |
|---|---|
| IL-6 | Interleukin-6 |
| IL-8 | Interleukin-8 |
| IL-10 | Interleukin-10 |
| IL-12 | Interleukin-12 |
| iNOS | Inducible Nitric Oxide Synthase |
| JNK | c-Jun N-terminal Kinase |
| LML | SB203580 Inhibitor |
| LPC | Lysophosphatidylcholine |
| LPS | Lipopolysaccharide |
| MAC | Membrane Attack Complex |
| MAP | Mitogen-Activated Protein |
| MAPK | Mitogen-Activated Protein Kinase |
| MCP-1 | Monocyte Chemoattractant Protein-1 |
| mCRP | Monomeric C-Reactive Protein |
| MEK | Ras/Raf/Mitogen-activated kinase/ERK kinase |
| MEK | PD98059 Inhibitor |
| MIF | Migration Inhibitory Factor |
| MKK | MAP Kinase Kinase |
| MMK | Multiple-Mitotic Kinase |
| MMU | Manchester Metropolitan University |
| MRSA | Methicillin-resistant *Staphylococcus aureus* |
| mtNOS | Mitochondrial Nitric Oxide Synthase |
| NADPH | Nicotinamide Adenine Dinucleotide Phosphate |
| nCRP | Native C-Reactive Protein |
| NED | *N*-1-napthylethylenediamine dihydrochloride |
| NF-κβ | Nuclear Factor-κβ |
| NHS | National Health Service |
| nNOS | Neural Nitric Oxide Synthase |
| NO | Nitric Oxide |
| NOS | Nitric Oxide Synthase |
| Nox | NADPH oxidase |

| | |
|---|---|
| OVX | Ovariectomised |
| PAMP | Pathogen-Associated Molecular Patterns |
| PBS | Phosphate Buffered Saline |
| PCh | Phosphocholine |
| PDGF | Platelet Derived Growth Factor |
| PDK1 | Pyruvate Dehydrogenase Kinase, Isozyme 1 |
| PDK2 | Pyruvate Dehydrogenase Kinase, Isozyme 2 |
| PEt | Phosphoethanolamine |
| PI3K | Phosphoinositide-3 kinase |
| $PI(3,4,5)P_3$ | Phosphatidylinositol-3,4,5-triphosphate |
| $PI(4,5)P_2$ | Phosphatidylinositol-4,5-biphosphaste |
| $PIP_2$ | Phosphatidylinositol-4,5-biphosphaste |
| $PIP_3$ | Phosphatidylinositol-3,4,5-triphosphate |
| PKC | Protein Kinase-c |
| PMA | Phorbol 12-myristate 13-acetate |
| PRR | Pattern Recognition Receptor |
| RNS | Reactive Nitrogen Series |
| ROS | Reactive Oxygen Species |
| RPMI 1640 Medium | Roswell Park Memorial Institute 1640 Medium |
| SCCmec | Mec Operon |
| SEM | Standard Error of the Mean |
| TBS | Tris Buffered Saline |
| TGFβ | Transforming Growth Factor β |
| TLR | Toll-like Receptor |
| TNF-α | Tumour Necrosis Factor-α |
| UoM | Univeristy of Manchester |
| VEGF | Vascular Endothelial Growth Factor |

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## Abstract

C-reactive protein (CRP) is a homopentameric acute-phase inflammatory protein that exhibits elevated expression during inflammation. CRP can be found in its pentameric form (nCRP) or monomeric form (mCRP). To date there has been little research investigating the effects of these CRP isoforms in wound healing processes and age-related impaired healing, which is known to have an altered inflammatory response.

The study investigated CRP isoform localisation in murine wounds (detected by immunohistochemistry) and circulating blood (measured by immunoblotting) using models of acute healing in young adults and age-related impaired healing (n=3). Using *in vitro* inflammatory cell assays developed with the monocytic U937 cell line this study investigated the effects of CRP isoforms in nitric oxide release (detected by an immunoassay), phagocytosis (measured by a bacterial recovery assay), apoptosis (detected via DNA fragmentation) and cytokine secretion (measured by a multiplex immunoassay) in both monocyte and macrophage-like cells (n=6). Pharmacological inhibitors were used to determine the potential CRP-induced pathways or mechanisms.

Both isoforms were localised to sites of inflammation, implying that they may play an active inflammatory role during wound repair. Moreover, the ratio of CRP isoforms (mCRP:nCRP) reflected the degree of inflammation, with higher values indicating elevated mCRP localisation and pronounced inflammation in the model of age-related impaired healing. In *in vitro* inflammatory cell assays, nCRP exhibited a more anti-inflammatory effect leading to significantly ($P=0.01$) increased phagocytosis, increased apoptosis, decreased nitric oxide (NO) production, and reduced overall pro-inflammatory cytokine production in monocytes and macrophages. The mCRP isoform significantly ($P<0.001$) increased NO production and overall levels of pro-inflammatory cytokines, thus confirming its pro-inflammatory nature indicated in the literature.

This research highlighted that estrogen mediated CRP-induced responses in a cell type- and status-specific manner, being pro-inflammatory in circulating cells (monocytes) but anti-inflammatory in wound tissue cells (mature activated macrophages). The profile of CRP isoforms shifted in age-related (estrogen-deprived) impaired healing to relatively higher levels of mCRP compared to nCRP, suggesting impaired healing in the elderly may result from the dissociation of nCRP to mCRP following the decline in estrogen with increasing age.

In conclusion, this study has provided novel data showing that both isoforms of CRP may differentially mediate inflammatory responses during wound healing and may play key roles in age-related impaired healing and may ultimately provide potential therapeutic strategies for the treatment of chronic wounds in the elderly.

## Declaration

I declare that no portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or institute of learning.

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# Chapter 1: Introduction

## 1.1 Wound Healing and Inflammation

### 1.1.1 Wound Healing

The healing of acute wounds is a dynamic process consisting of four main overlapping phases; haemostasis, inflammation, proliferation and remodelling, which start as soon as the injury occurs (Diegelmann & Evans, 2004). These phases utilise soluble mediators, blood-derived cells, the extracellular matrix and parenchymal cells (Singer & Clark, 1999).

Following wounding, components in the blood such as platelets enter the injury site and become exposed to collagen and the extracellular matrix. This first phase is made up of two stages: development of the fibrin clot and coagulation (Li *et al*, 2007). Platelets release clotting factors, growth factors and cytokines including platelet derived growth factor (PDGF), that initiates the chemotaxis of neutrophils, macrophages, smooth muscle cells and fibroblasts, and transforming growth factor β (TGFβ) and monocyte chemoattractant protein-1 (MCP-1) that mediate the infiltration of monocytes and macrophages (Dipietro, 1995). TGFβ also stimulates macrophages to release fibroblast growth factor (FGF), PDGF, tumour necrosis factor-α (TNF-α) and interleukin-1 (IL-1) (Bennett & Schultz, 1993). Platelets initiate the formation of a fibrin clot at the wound site to create a provisional matrix and re-establish haemostasis (Singer & Clark, 1999).

After the haemostasis stage, neutrophils are attracted to the wound site by the release of the cytokines in the inflammatory phase of healing such as interleukin-1β (IL-1β), interleukin-6 (IL-6) and interferon-γ (IFN-γ) (Bennett & Schultz, 1993), and begin to phagocytose debris, pathogens, damaged non-functional cells and damaged matrix components (Diegelmann & Evans, 2004). This occurs within 24 hours following the injury. The activation of mast cells also occurs during this phase, resulting in the release of granules containing enzymes, histamine and active amines that disrupt surrounding blood vessels and cause the

extravasion of blood constituents (Singer & Clark, 1999). By allowing the leakage of components from blood vessels, mast cells promote the passage of other cells into the wound site. This leads to the activation of fixed tissue monocytes into specialised wound macrophages when the monocytes are exposed to matrix proteins (Singer & Clark, 1999), the induction of phagocytosis by lymphocytes and the release of growth factors and cytokines such as PGDF and TGFβ (Broughton *et al*, 2006). During this inflammation stage, the wound site is phagocytosed of foreign material, dead cells and debris before fibroblasts arrive at the wound site to begin the overlapping proliferation stage.

A new matrix is generated via deposition of collagen by the fibroblasts and crosslinked so that the overlapping remodelling stage can begin. Collagen provides strength, integrity and structure to the healing wound (Reinke & Sorg, 2012). TGFβ increases the gene transcription of collagen, proteoglycans and fibronectin as well as the decrease in secretion of proteases responsible for matrix breakdown by stimulating the release of protease inhibitors (Diegelmann & Evans, 2004; Broughton *et al*, 2006). Epithelisation is stimulated by endothelial growth factors and forms an epithelial bridge which then dissolves (Singer & Clark, 1999). The pH at the wound site is lowered leading to reduced oxygen tension and increased lactate that induce a new blood supply through angiogenesis and neovascularisation (Witte & Barbul, 1997). This process is stimulated by vascular endothelial growth factor (VEGF), FGF and TGFβ (Singer & Clark, 1999).

The wound closes when the epithelial cells and the new matrix interact. This causes wound contraction; the movement of the tissue towards the direction of the centre of the wound which is supported by the release of nerve growth factor and interleukin-8 (IL-8) (Reinke & Sorg, 2012).

There are four main responses after an injury. Normal repair occurs when equilibrium is re-established without issue. The second is excessive healing; where the host creates too much connective tissue leading to fibrosis and altered structure. The third is deficient healing where not enough connective tissue is generated leading to weak and unstable wound repair (Reinke & Sorg, 2012). The

final type is regeneration, where there is a loss of structure and function due to the injury but the host has the ability to regenerate the affected tissues and their functionality. Humans have lost this regeneration ability in general but some tissues such as the liver, nerves and foetal tissue can still exhibit this trait (Reinke & Sorg, 2012).

### 1.1.2 Age-Related Impaired Healing

The definition of impaired healing is a wound, which has failed to proceed through the phases of healing in an orderly and timely process (Lazarus *et al* 1994), and they are characterised by a pronounced inflammatory profile. By general definition, age-impaired healing only relates to the delay in healing rather than an impairment on the quality of healing (Guo & Dipietro, 2010).

In age-impaired wound healing there is a delay in most of the major processes of wound healing and inflammation. Swift *et al* (1999) discovered that aged mice showed a delay in angiogenesis with a decline in capillary growth as well as a delay in re-epithelialization and collagen synthesis. This study also identified that wounds on the aged mice contained less of the angiogenic mediators fibroblast growth factor-2 and VEGF than wounds in young mice (Gosain & DiPietro, 2004). In aged wounds, there is a different inflammatory profile when compared to young wounds such as delayed T-cell infiltration, alterations in cytokine production and reduced macrophage phagocytic capacity (Swift *et al*, 1999).

### 1.1.3 Chronic Wounds

Chronic wounds form when healing is deficient and when inflammation is not resolved in the wound healing process. Most chronic wounds are associated with ischaemic vascular disease, diabetes mellitus, venous stasis disease or sustained pressure over bony joints leading to ischaemic events in local tissues (Guo & DiPietro, 2010). In the UK, 200,000 patients have a chronic wound with the

National Health Service (NHS) budgeting an estimated £2.3 billion - £3.1 billion annually to treat these wounds. However, one in three chronic wounds are reported to be unhealed after six months, and one in five for over a year (Posnett & Franks, 2008). In the USA, chronic wounds affect 3-6 million people with 85% of these being over the age of 65 (Guo & DiPietro, 2010) indicating that the issue increases with age.

By definition, a chronic wound is a wound that fails to proceed through the phases of wound healing in the appropriate order and within an acute wound healing time frame. Although various types of chronic wound have different aetiologies, all chronic wounds share similar features. These include excessive and prolonged inflammation and tissue breakdown (Frykberg & Banks, 2015), production of pro-inflammatory cytokines, proteases and reactive oxygen species, often with the addition of persistent infection (Frykberg & Banks, 2015).

### 1.1.4 Inflammation

Inflammation is the innate protective response of the body in response to stimuli such as injury, pathogens or damaged cells. The inflammatory stages of wound repair are subdivided into cellular and vascular responses with the main function of this process being to cease the stimuli that are causing the inflammation and to clean up the damage afterwards (Li *et al*, 2007). This process consists of many other smaller processes such as phagocytosis and apoptosis, in order for the tissue to repair itself (Li *et al*, 2007).

There are five main features of inflammation. Calor and Ruber, meaning heat and swelling, occur due to vasodilation and increased blood flow. Tumor (swelling) occurs due to increased vascular permeability allowing plasma fluid to leak out into the tissues. Dolor (pain) occurs because of the increased fluid pressure around nerve endings in the tissue. The last feature is *functio laesa*, a loss of function of the tissue while there is an attempt at repair, caused by a combination of the other four features (Freire & Van Dyke, 2013).

Inflammation is the process of the recruitment of circulating leukocytes from the blood, their accumulation in tissues and their activation to destroy microbes. Many of these reactions need cytokines; produced by dendritic cells, macrophages, neutrophils, monocytes as well as other cell types from the innate immune system (Koh & DiPietro, 2011).

There needs to be a balance with inflammation. The main function of this process is to remove damaged cells and debris to ensure successful resolution of the injury and to prevent progressive bacterial infection (Medzhitov, 2010). However, excessive inflammation can lead to numerous degenerative diseases such as chronic wounds, rheumatoid arthritis and atherosclerosis. This is termed chronic inflammation and is associated with tissue destruction observed in chronic wounds (Koh & DiPietro, 2011).

Acute inflammation tends to have an immediate onset caused by injury or bacterial infection and is driven by neutrophils, eosinophils and mononuclear cells. Acute inflammation within the wound healing process typically resolves within a few days (Eming *et al*, 2007). Chronic inflammation, in contrast, tend to have a delayed onset and can last months or years. Chronic wounds are characterised by excessive and prolonged inflammation (Gethin, 2012). Chronic inflammation is characterised as abundant neutrophil infiltration with associated reactive oxygen species and destructive proteolytic enzymes such as collagenase and elastase that cause the degradation of the dermal matrix (Gethin, 2012). The usual outcome of chronic inflammation in various disease states is tissue degeneration, fibrosis or necrosis (Gethin, 2012). In chronic wounds for example, there is pronounced tissue breakdown due to inflammatory mediators (Medzhitov, 2010).

### 1.1.5 Role of Monocytes and Macrophages in Inflammation

One of the major cell types in inflammation are the monocytes. Monocytes are a subset of white blood cells that circulate through the human body within the cardiovascular system. These cells have the potential to migrate into tissues and differentiate into macrophages or dendritic cells when there is infection or tissue damage (Auffray *et al*, 2009). Evidence shows that the monocyte contribute to the inflammation process by sensing changes in environment caused by exposure to pathogens or tissue damage (Xiong & Pamer, 2015).

When recruited to the site of infection or tissue damage, monocytes become activated and in response produce numerous factors including TNF-α and inducible nitric oxide synthase (iNOS) which is dependent upon TLR-MYD88 signalling and IFN-γ. Activation allows monocytes to participate in the clearance of pathogens (MacMicking *et al,* 1995). The production of iNOS leads to the production of nitric oxide (NO) from phagocytic cells, including monocytes and macrophages, which is involved in the killing of bacteria through the induction of DNA damage and the disruption of bacterial metabolism (Nathan & Shiloh, 2000).

Inflammatory monocytes play a role in the activation of other immune cells in the process. Monocytes can present bacterial antigens to other cells in the immune process, such as T-cells, and activate them to participate in the immune response (Xiong & Pamer, 2015). Monocytes are released from the bone marrow into circulation and over the duration of a few days they seed into the tissues around the body, including the spleen where they are stored (Murray & Wynn, 2011). When the monocytes extravasate though the endothelium they are differentiated into macrophages thereby increasing the pool of tissue-resident macrophages in preparation for an immune response (Murray & Wynn, 2011).

Macrophages are a subset of white blood cells that have differentiated from monocytes. These cells can be located throughout the tissues in the human body. The main role of macrophages is to ingest and process both foreign materials and dead cells through phagocytosis, as well as activating other macrophages though

the release of inflammatory signals such as cytokines (Hart, 2002b). These can include 'professional' macrophages which have receptors for signals that are not normally found in healthy tissues. These include scavenger receptors on their surface which bind apoptotic and necrotic cells, opsonised pathogens and cell debris (Mantovani *et al*, 1972).

Macrophages are divided into subpopulations based upon their localisation in the body and their function. For example, this can range from osteoclasts in the bone, alveolar macrophages in the lung and Kupffer cells in the liver. These subpopulations can then be further classified into subsets based upon the macrophage phenotype (Mosser & Edwards, 2008). Classically activated macrophages (M1 macrophages) function in the defence of the host against viruses, bacteria and fungi as well as having anti-tumour effects. Alternatively activated macrophages (M2 macrophages) have an anti-inflammatory function mainly in the wound healing process (Sutterwala *et al,* 1997; Sutterwala *et al,* 1998). Regulatory macrophages secrete interleukin-10 (IL-10) in response to Fc-γ receptor ligation. Tumour associated macrophages suppress antitumor immunity. It has been shown however that these subsets of macrophages are flexible and will re-programme their function dependent upon the environment and the signals that are produced (Mosser & Edwards, 2008).

In macrophage-depleted wounds, tissue debridement and new-tissue deposition is severely impeded (Trowbridge & Emling, 1997) suggesting that macrophages are involved in acute wound debridement but also have a crucial role in inducing tissue formation (Trowbridge & Emling, 1997). Macrophages conduct phagocytosis and release proteinases into their surroundings to clear wound debris, pathogens and apoptotic neutrophils (Trowbridge & Emling, 1997).

When macrophages are exposed to the environment at the site of an acute wound they start to synthesise a variety of intercellular signalling molecules (growth, regulatory and chemotactic factors) which recruit other inflammatory cells, together with cells required for new tissue synthesis (Hart, 2002a).

## 1.2 C-Reactive Protein

### 1.2.1 Structure and Function

There is increasing evidence that C-reactive protein (CRP), an inflammatory protein, may play a role in the inflammation and wound healing processes. During the cutaneous wound healing process a number of cytokines and inflammatory mediators are secreted by inflammatory cells, including TNFα, IL-6 and migration inhibitory factor (MIF), alongside an increased expression of C-reactive protein (Black *et al*, 2004).

CRP is a homopentameric acute-phase inflammatory protein that exhibits elevated expression during inflammatory conditions such as rheumatoid arthritis, some cardiovascular diseases and infection (Du Clos & Mold, 2004). As an acute phase protein this means that the plasma concentration of the protein increases or decreases by at least 25% during inflammatory disorders (Gabay & Kushner, 1999). The highest concentrations of CRP are found in the serum and it has been shown that during some infections levels can increase up to 1000-fold (Thompson *et al*, 1999). However when the stimuli ends, values decrease exponentially over 18-20 hours, close to the half-life of CRP (Ridker, 2003). CRP plasma levels have been reported to increase from around 1μg/ml to over 500μg/ml within 24-72 hours of severe tissue damaging effects such as trauma and progressive cancer (Ciubotaru *et al*, 2005). IL-6 is considered to be the main inducer of the *CRP* gene, with IL-1 enhancing the effect (Szalai *et al*, 1998). However even though IL-6 is necessary for the induction of the *CRP* gene, it cannot achieve this alone (Weinhold *et al*, 1997).

CRP is a phylogenetically/highly conserved plasma protein that was initially discovered in 1930 by Tillet and Francis while investigating the sera of patients suffering the acute stage of *Pneumococcus* infection (Tillet & Francis, 1930). CRP was named for its specificity of calcium binding for phosphocholine (PCh), which is also a part of the activation of the complement pathway of innate immunity (Volankis, 2001). CRP has many homologs in vertebrates and some invertebrates (Black *et al*, 2006) and is a member of the pentraxin family which also includes

other structurally related molecules such as serum amyloid A (Gewurz *et al*, 1982). Transcriptional induction of the CRP gene mainly occurs in the hepatocytes in the liver in response to increased levels of inflammatory cytokines, especially IL-6 (Boras *et al,* 2014). The role of CRP is that it can activate the classical complement pathway through the recognition of phosphorycholine and activating C1q in the pathway (Schwalbe *et al*, 1992).

There are many factors that can alter baseline CRP levels including age, gender, smoking status, weight, lipid levels and blood pressure (Hage & Szalai, 2007). The average levels of CRP in the serum in a healthy Caucasian is around 0.8mg/L but this baseline can vary greatly in individuals due to other factors, including polymorphisms in the CRP gene (Devaraj *et al*, 2006). The human CRP gene can be found at 1q23.2 on the long arm of chromosome 1 and to date there have been no allelic variations or genetic deficiencies discovered for this gene, though some polymorphisms have been identified (Hage & Szalai, 2007). The baseline levels of CRP are regulated by genetic variations in the number of dinucleotide repeats found in the intronic region of the gene, with the suggestion that up to 50% of baseline variance is due to these variations (Eisenhardt *et al,* 2009).

There is no significant seasonal variation in base-line CRP concentration; however, twin studies show a significant heritable component in base-line CRP values that is independent of age and body mass index (BMI) (Pepys & Hirschfield, 2003). Pankow *et al* (2001) found evidence that inter-individual variation in blood CRP levels is 35-40% heritable. Raised CRP levels are typically associated with disease but liver failure is one condition observed to impair CRP production. Very few drugs reduce elevated CRP levels unless they treat the underlying pathology that is causing the acute-phase stimulus (Pepys & Hirschfield, 2003).

### 1.2.2. Isoforms of C-reactive Protein

The pentameric protein, termed native CRP (nCRP) is characterised by a discoid configuration of five identical non-covalently bound subunits, each 206 amino acids long with a molecular mass of about 23kDa (Figure 1.1). These five subunits lie in the same orientation around a central pore and arranged in a characteristic 'lectin fold' with a two-layered beta sheet (Eisenhardt *et al,* 2009). Each subunit lies with the PCh binding site facing the 'recognition' face of the nCRP molecule (Boncler & Watala, 2009). The molecule has a ligand binding face which has a characteristic feature of having two calcium ions per protomer. These ions are greatly important for the stability and binding of ligands. The 'opposite' face interacts with the C1q aspect of the complement pathway as well as interacting with Fc receptors (Du Clos & Mold, 2004).

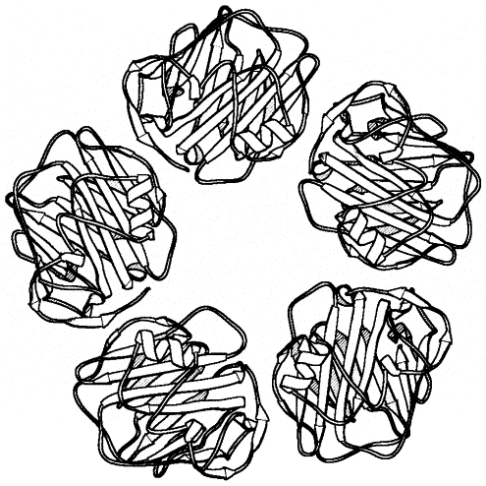


**Figure 1.1 – Structure of human pentameric C-reactive protein as viewed down the 5-fold symmetry axis.** The effector face is on top with the opposite face where calcium and PCh binding sites underneath (Taken from Shrive *et al,* 1996)

The pentameric protein is synthesised primarily in liver hepatocytes but has also been reported to be synthesised in other cell types such as smooth muscle cells (Calabró *et al*, 2003), macrophages (Devaraj *et al,* 2009), endothelial cells (Pasceri *et al,* 2000), lymphocytes (Kuta & Baum, 1986) and adipocytes (Calabró *et al*, 2005). CRP is first synthesised as monomers and then assembled into the pentamer

in the endoplasmic reticulum of the cell. In hepatocytes, the pentameric protein is retained in the endoplasmic reticulum by binding to two carboxylesterases, gp60a and gp50b (Macintyre *et al*, 1994). While in a resting state, the CRP is released slowly however in response to an increase in cytokine levels, the binding to these carboxylesterases decreases and secretion time is reduced, thus allowing an influx of CRP to be seen in response to an inflammatory stimulus such as bacterial infection or injury (Du Clos & Mold, 2004). The stimulation of CRP synthesis mainly occurs in response to pro-inflammatory cytokines, most notably IL-6 and to a lesser degree IL-1 and TNF-α (Zhang *et al,* 1996).

Pentameric CRP can be irreversibly dissociated, with the resultant free subunits (Figure 1.2) termed monomeric (or modified) CRP (mCRP). The dissociation of nCRP into free subunits has been observed at either high concentrations of urea (Potempa *et al,* 1987) or high temperatures in the absence of calcium (Potempa *et al,* 1983), although the precise process is not fully documented. Taylor and Van den Berg (2006) found that by increasing the temperature of the nCRP to 90°C allowed for total dissociation of the protein, but near 100% monomerisation can also be achieved at 70°C, with the process starting after 10 minutes and taking nearly an hour to complete. This process was sped up to 5 minutes at 63°C when there was an absence of calcium. Dissociation has also occurred under exposure of nCRP to lysophosphatidylcholine (LPC), a lipid messenger exposed on cell membranes, which is generally found in apoptotic cells or activated platelets (Kim et al., 2002).

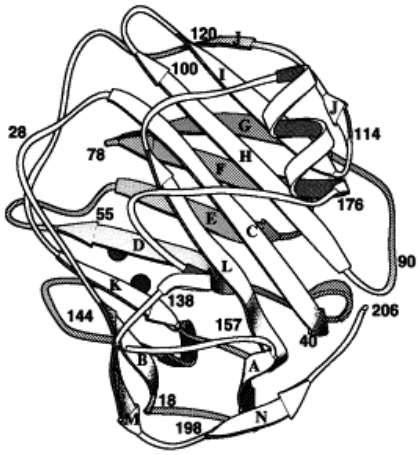



**Figure 1.2- Structure of human monomeric C-reactive protein (mCRP).** The β-strands are labelled A-N with the positions of key amino acid residues indicated. Also two ligated calcium ions are shown as spheres. (Taken from Shrive *et al,* 1996)

The mCRP molecules are distinguished from nCRP by their different antigenic, biological and electrophoretic activities (Kresl *et al*, 1998) and by the fact they express different neoepitopes (Khreiss *et al,* 2004). The two isoforms of CRP have been shown to have distinct biological functions in the inflammatory process. For example, Khreiss *et al* (2004) provided evidence that nCRP suppresses adherence of platelets to neutrophils, whereas mCRP enhances these adhesive interactions. This difference in function can be explained by that the two isoforms having different types of Fcγ-receptor involved in the signalling process; mCRP utilises the low-affinity immune complex binding immunoglobulin G (IgG) FcγRIIIb (CD16b) on neutrophils, FcγRIIIa (CD16a) on monocytes while nCRP binds to the low-affinity IgG receptor FcγRIIa (CD32) (Bharadvaj *et al,* 1999; Stein *et al,* 2000; Tebo & Mortensen, 1990).

Evidence shows that nCRP tends to exhibit a more anti-inflammatory function relative to the mCRP isoform. It is suggested that this occurs by the nCRP binding at sites of tissue injury to limit the generation of the membrane attack complex (MAC) and C5a, thus inhibiting complement activation (Thiele *et al,* 2014). On the other hand, mCRP had marked pro-inflammatory properties *in vitro* and *in*

*vivo* by promoting monocyte chemotaxis as well as the recruitment of circulating leukocytes to areas of inflammation via Fcy-RI and Fcy-RIIa signalling (Thiele *et al,* 2014).

### 1.2.3. C-Reactive Protein and Complement

Complement is one of the major defences of the human immune system which is involved in the clearance of foreign particles and organisms once they have been recognised by antibody. The complement pathway is made up of 35 plasma or membrane proteins that is an important system in immunity and the defence of the host against microbial infection. The components of the complement pathway can be activated in three different pathways to trigger a cascade of proteins, which are used to help bind microbial surfaces for the immune system to recognise and activate phagocytosis (Li *et al*, 2007). The classical pathway is triggered by a target bound antibody whereas the lectin pathway is triggered by microbial repetitive polysaccharide structures and the alternative pathway is triggered by the recognition of other foreign surface structures. Even though the triggers are different, the three pathways merge at a pivotal activation of the C3 and C5 convertases. The majority of the components are synthesised in the liver, C1 in the intestinal epithelium and factor D in the adipose tissue (Li *et al*, 2007).

CRP has been extensively researched in its role to activate the complement pathway. In 1974, Kaplan and Volanakis first described the ability of CRP to activate the classical complement pathway using C-polysaccharide and phospholipid ligands (Kaplan and Volanakis, 1974). Siegel *et al* (1974) also showed the same result using CRP-protamine complexes. The activation of complement by CRP is considered a crucial step since when complement was depleted, the effects of CRP were abrogated (Thiele *et al*, 2014).

The opposite face of the CRP molecule, complexed with polyvalent ligand or chemically cross-linked, binds to C1q and activates the classical complement pathway (Du Clos, 2004). This binding however is different from IgG binding as CRP

is localised to collagen-like regions rather than the globular head groups of C1q (Jiang *et al,* 1991). C1q is a large 460kDa molecule made up of six identical subunits, each made up of 3 structurally similar but distinct polypeptide chains (Agrawal *et al,* 2001). This process requires the use of calcium ions for the stable formation of the C1 complex (Paul, 2008). CRP is most effective during the early classical pathway activation of C1, C4 and C2 (Mold *et al*, 1999). This is because the ligand-bound interaction with C1q leads to the formation of C3 convertase, triggering the complement activation of C1-C4 but with little activation of the late complement proteins C5-C9 (Eisenhardt *et al*, 2009).

Activation of complement by CRP varies from activation by antibody in that CRP has a selective activation of early components without the need to form the MAC. In addition to activating the classical complement pathway, it has been indicated that CRP can inhibit the alternative complement pathway by decreasing C3 and C5 convertase activities and inhibiting the complement amplification loop. This is achieved by the recruitment of factor H to the cell surface as well as preventing C5 converatase cleaving C5 to recruit neutrophils and prevent the formation of the MAC (Marnell *et al*, 2005). As levels of CRP increase this causes decreased binding of C3b and C5b-9 to liposomes, possibly also explaining the lack of C5-C9 consumption by CRP during classical pathway activation (Mold *et al*, 1999).

### 1.2.4. Confliction in C-reactive Protein Research

There have been numerous research papers looking at the roles and effects of CRP as a whole however there can be a source of conflict when addressing these papers.

There are two main issues that need to be considered when researching previous studies in CRP. The first is that a majority of papers refer to CRP as a single entity; disregarding the fact that there are two isoforms of the protein which have distinct structures and functions. This means it can be sometimes impossible to determine which of the isoforms had the causative effect in these studies.

The second issue is taken into consideration with some older studies. These papers state that CRP was measured. It is known now that these papers were measuring nCRP due to a lack of antibodies and assays that could be used to detect mCRP. It can also be assumed that more recent papers have the same issue as their antibodies used to detect mCRP are not commercially available and can only be accessed by a few laboratories.

Taking these two points into consideration it is assumed that most previous studies that refer to CRP have analysed the native form of the protein unless otherwise stated.

### 1.2.5. C-Reactive Protein and Estrogen

There is emerging research that hormone replacement therapy (HRT) causes CRP levels to increase significantly in postmenopausal women. This has many effects including an increased risk of cardiovascular events (Kurtz, 2011). It was also observed that in older men and postmenopausal women, estrogen caused a variable response in CRP levels whereas testosterone had no effect providing evidence of a link between estrogen and CRP levels (Corcoran *et al,* 2010). Other studies have shown that a correlation between CRP and estrogen levels exist. Ridker *et al* (1999) found that apparently healthy postmenopausal women had nearly two-fold increased levels of CRP when they were taking HRT. As CRP is a marker of cardiovascular risk, this suggested that those on HRT were at a higher risk of cardiovascular implications.

Most of the research conducted into the interactions of CRP and estrogen is based upon cardiovascular risk, with no investigations of wound healing performed to date.

### 1.2.6. C-reactive Protein in Disease Pathology

The majority of research into CRP has focused upon the role of CRP and its isoforms their effects upon the risk of cardiovascular risk and stroke. However, correlations between CRP levels and other disease states have also been found. Studies conclude that CRP levels increase due to inflammation. CRP is used as a clinical marker of inflammation, as elevated serum levels are a strong independent predictor of cardiovascular disease in asymptomatic individuals (Ridker *et al*, 2002). CRP levels have been linked to prognosis in patients with atherosclerotic disease, congestive heart failure, atrial fibrillation, myocarditis, aortic valve disease and heart transplantation, confirming that it has an active role in the pathophysiology of cardiovascular diseases (Osman *et al,* 2006).

High sensitivity assays, such as nephelometric assays, are used to detect baseline levels of CRP and are used to detect patients who are at risk of cardiovascular disease. An individual with a CRP level higher than 3mg/L has an increased risk of coronary heart disease (Kushner, 1990) and this risk increases in those with type 2 diabetes (Soinio *et al*, 2006).

Increased levels of CRP have also been found in patients suffering from appendicitis, cholecystitis, pancreatitis and meningitis (Clyne & Olshaker, 1999). In patients suffering with the symptoms of appendicitis, those with CRP levels greater than 25mg/L after 12 hours were given a definitive diagnosis of acute appendicitis (Albu *et al,* 1994). When symptoms of cholecystitis were combined with a CRP level of over 30mg/L this led to an accurate diagnosis, with 78% sensitivity indicating CRP was a more sensitive marker than erythrocyte sedimentation rate and white cell count in the diagnosis (Juvonen *et al,* 1991). In terms of pancreatitis, CRP levels of more than 210mg/L were able to discriminate between mild and severe etiologies, with 83% sensitivity and 85% specificity (Wilson *et al,* 1989).

Serum CRP levels have been able to accurately diagnose bacterial meningitis with levels also corresponding to the resolution of symptoms after treatment with antibiotics (Peltola, 1982). When cerebrospinal fluid was analysed for CRP the test

had a sensitivity of 100% with 94% specificity and was more accurate than neutrophil count and gram stain in the diagnosis of infection (Corrall *et al,* 1981). However it is stated that there is the chance for false positive and negatives with this test. False negatives arise if the test is conducted too early in the infection process or if there is poor diffusion of CRP into the cerebrospinal fluid while false positives are generated during viral meningitis, brain abscesses and other sources of inflammation. This test is also not able to distinguish between bacterial and viral infections so cannot aid diagnosis (Gershôm *et al*, 1986; Macfarlane & Narla, 1985).

While studies have shown correlations between CRP levels and various aetiologies, the precise role of CRP isoforms in disease development and progression remains largely unknown. Thus, investigations are required to determine the effects of each CRP isoform on specific cellular processes.

### 1.2.7 C-Reactive Protein and Inflammation

CRP is mainly classed as an acute marker of inflammation and very little research has been conducted into the role that CRP plays in inflammation, particularly in relation to wound healing. CRP levels are known to increase dramatically in response to injury, infection and inflammation.

CRP is the principal downstream mediator of the acute phase response following an inflammatory event and is primarily synthesised by IL-6-dependent hepatic biosynthesis (Pradhan *et al*, 2001). The main role of CRP in inflammation tends to focus around the activation of the C1q molecule in the complement pathway leading to the opsonisation of pathogens. Although CRP can initiate the fluid phase pathways of the host defence by activating the complement pathway, it can also initiate cell-mediated pathways by activating complement as well as to binding to Fc receptors of immunoglobulin G (Pradhan *et al,* 2001). CRP binds to Fc receptors with the resulting interaction leading to the release of pro-inflammatory cytokines (Du Clos, 2000). CRP also has the ability to recognise self and foreign molecules based on pattern recognition, something that other activators of

complement such as IgG cannot achieve since these molecules only recognise distinct antigenic epitopes (Du Clos, 2000).

## 1.3 The Effect of CRP Isoforms on Inflammation Processes

Evidence suggests that CRP is not only just a marker of inflammation but also plays an active role in the process. Most previous research only refers to CRP as a singular molecule and does not identify between the two isoforms. This would offer an explanation as to why there is conflicting evidence in literature, with reports of both pro-inflammatory and anti-inflammatory effects attributed to the protein. Thus to provide greater clarity in the field, this study investigated the differential effects of each CRP isoform on specific inflammatory processes. Since antibodies for mCRP are not commercially available, very few laboratories in the world are able to conduct studies to investigate the mCRP isoform. However, since non-commercial antibodies specific for each isoform were available during this project, the study was in a unique position to be able to investigate both CRP isoforms and differentiate between their effects on inflammatory processes.

### 1.3.1. Inflammatory Signalling Pathways and Inhibition

Cells require the use of various intracellular pathways in order to regulate cellular processes such as proliferation, survival and gene expression. It can be suggested that CRP utilises these signalling pathways to exert some of the effects observed in previous research.

### 1.3.1.1. The Phosphoinositide-3 Kinase (PI3K) Pathway and C-Reactive Protein

The phosphoinositide-3 kinase (PI3K) pathway is an intracellular signalling pathway important in regulating the cell cycle and is important in processes involved in cellular quiescence, proliferation, cancer and longevity (Cantley, 2002). The PI3 kinases are a group of enzymes that catalyse phosphorylation of one or more inositol phospholipids in the 3' position of the inositol ring. The main focus of these enzymes is to catalyse the production of phosphatidylinositol-3,4,5-triphosphate ($PI(3,4,5)P_3$) from the plasma membrane lipid phosphatidylinositol-4,5-biphosphaste ($PI(4,5)P_2$) (Cantley, 2002). The enzyme is a heterodimer consisting of a 110kDa catalytic subunit and a 85kDa regulatory subunit allowing it to selectively phosphorylate inositol lipids at the 3' position (Norman *et al,* 1996). A simplified version of the PI3K pathway can be seen in Figure 1.3.

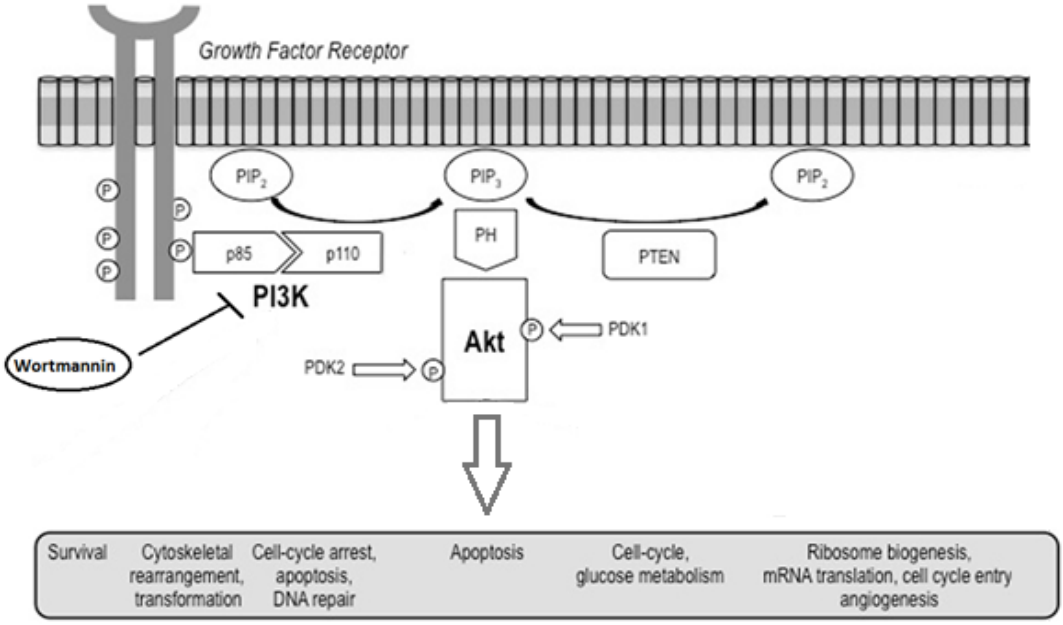



**Figure 1.3 – A simplified PI3 kinase pathway.** Growth factors bind to the receptor tyrosine kinase which activates PI3K. This leads to the conversion of $PIP_2$ to $PIP_3$, leading to the phosphorylation of Akt. When phosphorylated Akt acts upon multiple substrates. Wortmannin inhibits PI3K (see Section 1.3.1.2 for further details). Modified from Porta *et al* (2014).

*PI3K - phosphoinositide-3 kinase; $PIP_2$ - phosphatidylinositol-4,5-biphosphaste; $PIP_3$ - phosphatidylinositol-3,4,5-triphosphate ; PDK1 - Pyruvate Dehydrogenase Kinase, Isozyme 1; PDK2 - Pyruvate Dehydrogenase Kinase, Isozyme 2*

There are three classes of PI3K (I, II and III) and each of them have different isoforms. Each class of kinase has a different selectivity to ensure there is the generation of several types of molecular messenger produced in order to activate tyrosine growth factors such as epidermal growth factor receptors (EGFR) and insulin-like growth factor receptors (IGF-R) as well as cell adhesion molecules such as integrins, G-protein coupled receptors (GPCR) and oncogenes (Domin & Waterfield, 1997).

#### 1.3.1.2. Inhibition of the PI3K Pathway by Wortmannin

The PI3K pathway can be irreversibly inhibited by wortmannin; a fungal metabolite isolated from *Penicillium wortmanni* (Brian *et al,* 1957). This natural product had early signs of being anti-inflammatory and anti-proliferative (Wiesinger *et al,* 1974); however the most potent target of wortmannin were the class I PI3 kinases. These kinases are inhibited with an $IC_{50}$ in the range of 1-10nM (Knight, 2010). There is some evidence that class II PI3 kinases are inhibited by wortmannin but not as strongly as the class I group (Walker *et al,* 2000).

Wortmannin inhibits PI3 kinases by covalent inactivation of the enzyme (Walker *et al*, 2000) as seen in Figure 1.3. This is achieved by the electrophillic furan ring of the wortmannin molecule being targeted by a lysine residue in the kinase active site. This leads to the ring opening forming a stable enamine. The lysine residue is conserved in all PI3 kinases so this amino acid conservation leads to inhibition of multiple kinases. The wortmannin molecule also binds to the kinase with high affinity through the use of multiple hydrogen bonds and hydrophobic interactions (Walker *et al,* 2000).

### 1.3.1.3. The Ras/Raf/Mitogen-Activated Kinase/ERK Kinase Pathway (MEK pathway) and C-Reactive Protein

The Ras/Raf/Mitogen-activated kinase/ERK kinase pathway (MEK pathway) is an extracellular regulated kinase cascade which couples signals from cell surface receptors to transcription factors to help in the regulation of gene expression (Chang *et al,* 2003). This pathway plays a role in the proliferation of cell and the prevention of apoptosis. There are many molecules involved in this pathway (Figure 1.4) including small GTP-binding proteins such as Ras, Raf which is a serine/threonone kinase and their downstream transcription factor targets such as nuclear factor-κβ (NF-κβ), AP-1 and c-Myc (Chang *et al,* 2003).

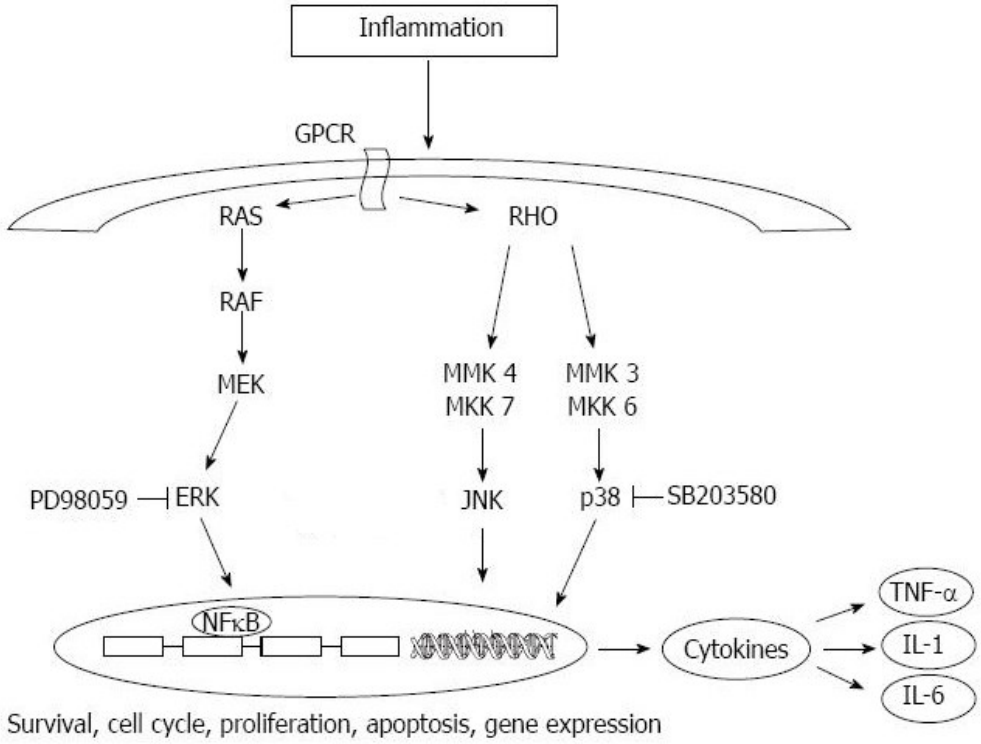



**Figure 1.4 The Ras/Raf/Mitogen-activated kinase/ERK kinase pathway (MEK pathway).** This pathway shows both the classical activation pathway though the activation of RAS and the non-classical pathway through the activation of RHO. PD98059 is a MEK pathway inhibitor while SB203580 is a p38 MAP kinase inhibitor. Modified from Irrera *et al (*2014).

*GPCR –G-protein coupled receptor; NF-κβ – Nuclear Factor-κβ; MMK – multiple-mitotic kinase; MKK – MAP Kinase Kinase; JNK – c-Jun N-terminal kinase; TNF-α – Tumour Necrosis Factor-α; IL-1 –Interleukin 1; IL-6 – Interleukin-6; MAP – mitogen-activated protein*

The MEK pathway is heavily upregulated in conditions such as cancer, where mutations lead to the overexpression of receptors. This pathway also has a role in the regulation of apoptosis through the post-translational phosphorylation of apoptotic regulatory molecules (McCubrey *et al,* 2007).

#### 1.3.1.4 Mitogen Activated Protein Kinases (MAPK) and C-Reactive Protein

Mitogen activated protein kinases (MAPKs) are a group of kinases involved in cell differentiation, apoptosis and autophagy and are related to the MEK pathway. There are four mitogen activated protein (MAP) kinases which have been identified and can be activated by a variety of methods (Zhao & Adjei, 2014). These kinases are regulated by a phosphoprotein relay system in which a series of three proteins are consecutively phosphorylated and activated. The MEK pathway is the most characterised MAPK pathway which is classed as the classical pathway involving the Ras, Raf, MEK 1 and 2 proteins and ERK proteins (Zhao & Adjei, 2014). The non-classical pathway involves other proteins including the p38-MAPK proteins.

MAP kinases are implicated as key regulators of inflammatory diseases as well as increasing evidence that they can contribute to progression of some disorders such as acute pancreatitis (Irrera *et al,* 2014).

The p38 mitogen-activated protein kinases react to stress stimuli such as heat shock and osmotic shock but can also be activated in response to pro-inflammatory stimuli such as cytokine levels. There are four of these kinases; p38α, β, γ and δ. Activation of these kinases is necessary for cell processes such as gene expression, cell survival and cell division (Irrera *et al,* 2014).

### 1.3.1.5. Inhibition of the MAPK Pathway by MEK

MEK 1 and 2 are homologous proteins that are ideal targets for inhibition. Both of these proteins have a kinase domain, a MAPK-docking region, a negative regulatory region, a proline rich insert and a nuclear export sequence (Liang *et al,* 2011). In addition to this structure there is a pocket structure adjacent to the ATP-binding pocket. When inhibited, conformational changes occur to revert the protein into a catalytically inactive state. This non-competitive mechanism does not exert its inhibition on the ATP-binding pocket and therefore does not have the same adverse effects as other competitive inhibitors (Zhao & Adjei, 2014). Inhibitors of this process, such as PD98059, tend to be selective, reversible and cell permeable and inhibit MAP kinase activation and subsequent phosphorylation of the MAP kinase substrates. They achieve this by preventing the activation of MEK1 and MEK2 by upstream kinases; however, they do not inhibit activated MEK or the non-classical pathway through the p38 MAPK pathway (Alessi *et al, 1995).*

### 1.3.1.6. Inhibition of the p38 MAP Kinase Pathway by LML

Inhibitors of the p38 MAP kinases, such as SB203580, tend to target the β isoforms while not inhibiting the ERK and JNK pathways (Cuena *et al,* 1995). Inhibition is achieved through direct binding of the inhibitor to the kinase. These selective inhibitors work by binding to the ATP binding pocket of the kinase. However, they do not inhibit the phosphorylation of p38 by upstream kinases (Kumar *et al,* 1999).

### `1.3.1.7 C-Reactive Protein and Inhibition by Nystatin

Nystatin is an inhibitor which is isolated from *Streptomyces* and is considered a polyene antibiotic. Nystatin is used to treat fungal infections (Kristanc *et al*, 2012) since it targets the plasma membrane, especially ergosterol in cell membrane of fungi, which is the equivalent of cholesterol in human cell

membranes. As bacteria are devoid of sterols, nystatin is not effective against these types of infections (Kristanc *et al*, 2012).

Nystatin disrupts the plasma membrane of fungal cells, leading to membrane leakage by forming barrel-like pores in the lipid bilayer, thus allowing potassium and other cell constituents to pass across the membrane and cause cell death (Razonable *et al,* 2005*;* Coutinho & Prieto, 2003). The formation of pores in the bilayer leads to the disturbance of electrochemical gradients and an increase in plasma membrane permeability. This permeability allows ions and small molecules no bigger than glucose to enter into the cell (Holz & Finkelstein, 1970; Marty & Finkelstein, 1975).

Nystatin also induces the secretion of IL-1β, interleukin-8 (IL-8) and TNF-α in toll-like receptor 2 (TLR2)-expressing (but not TLR2-deficient) cells in a dose dependent manner, resulting in a pro-inflammatory effect (Razonable *et al,* 2005). The exact mechanism by which nystatin achieves this is still unknown (Kristanc *et al*, 2012).

## 1.4 Aims

The aim of this project was to determine the effects of the two isoforms of CRP on specific inflammatory processes, particularly in relation to wound healing. The study utilised various *in vitro* models of inflammation, together with murine models of wound healing to investigate endogenous production or exogenous supplementation of CRP isoforms.

## 1.5 Objectives

The specific objectives of the project were:

- To determine the localisation of the CRP isoforms in murine histological samples of the inflammatory phase of wound healing.
- To determine endogenous levels of CRP isoforms in unactivated and activated monocyte- and macrophage-like cells grown in culture using *in vitro* models of inflammation, infection and ageing.
- To analyse the effects of exogenous supplementation of CRP isoforms on monocyte/macrophage:
    - growth and viability
    - apoptosis
    - nitric oxide production
    - cytokine production
    - phagocytosis
- To analyse the effects of pH and calcium concentration on the conversion of nCRP to mCRP.
- To investigate the effects of estrogen on circulating and wound levels of the CRP isoforms using a murine model of age-related impaired healing.

# Chapter 2: General Materials and Methods

## 2.1 Ethics

Ethical approval for the study was granted by Manchester Metropolitan University (MMU), including the use of murine blood and wound protein samples kindly provided by Dr M Hardman, University of Manchester (UoM) in strict accordance with UK Home Office regulations and UoM ethical approval.

## 2.2 Procedure

### 2.2.1 Cell Culture

The commercial U937 cell line was used to generate monocyte/macrophage-like cells in an *in vitro* model of inflammation. U937 cells were activated with lipopolysaccharide (LPS) from the cell wall of *Escherichia .coli* to model responses following bacterial infections and differentiated into a macrophage-like phenotype using phorbol 12-myristate 13- acetate (PMA). Cell viability was assessed using trypan blue exclusion in which cellular uptake of the dye indicates cell death.

### 2.2.2 C-reactive Protein Purification

Commercial C-reactive protein (CRP) was purchased in its native form. However it was imperative to confirm the purity of the native isoform since the protein can dissociate into the monomeric form. The commercial CRP also contained sodium azide to prevent microbial contamination of the protein during transit and storage but it removal was required prior to use in cell culture. Thus, the commercial native CRP sample was purified to ensure exclusion of the monomeric

isoform and remove the sodium azide. The purification was conducted in a two-step process. The first step utilised the float-a-lyzer dialysis system that incorporates a low protein-binding synthetic membrane with a molecular weight cut-off of 50 KDa. This dialysis allowed the larger pentamer to be retained in the unit while smaller contaminants (including mCRP and sodium azide) diffused across the membrane. The dialysis buffer contained calcium in order to prevent the dissociation of the pentamer to the monomer, thus maintaining the purity of nCRP. The second step utilised a phenyl hitrap that consists of a column containing phenyl sepharose beads for high-resolution hydrophobic interaction chromatography. This column contained hydrophobic groups that bind proteins in aqueous solutions in a manner that is dependent on protein structure.

To create the mCRP isoform the pentameric protein was dissociated. This was achieved through the chelation of the protein using ethylenediaminetetraacetic acid (EDTA) and urea to break the covalent bonds of the pentamer. Dialysis was performed to remove contaminants from the chelated protein using a dialysis buffer lacking calcium to aid the process of dissociation. Samples were then dot blotted (Section 2.2.3) to ensure purity before being used in future investigations.

After purification both isoforms were run through an endotoxin removal kit. This step removed any trace amounts of LPS in order to ensure any biological effects induced by the CRP isoforms during subsequent investigations were not simply due to contamination with LPS. The column of the kit contained resin which combine cellulose beads and other preservatives as an affinity ligand to bind and selectively remove endotoxins.

The protein concentration of CRP samples were then quantified using a bicinchronic acid detection kit which utilises the chemical reaction of converting copper II sulphate solution into a $CU^{2+}$ complex in alkaline conditions followed by further reduction to $Cu^{1+}$. This sensitive assay allows the quantification of proteins against a set of known samples. An example standard curve for this process can be

located in Appendix 1. During the chemical reaction, a colour change from blue to purple was proportional to the amount of protein present in the sample.

### 2.2.3 Dot Blot

Nitrocellulose membrane is designed as a matrix to be utilised in the detection of protein. The membrane immobilises the protein though hydrophobic interactions as well as high salt with low methanol concentrations. The protein binds the membrane with high affinity and enables a variety of detection methods to be used, including chemiluminescence.

Samples were blotted onto the membrane and the membrane was then treated with a blocking buffer containing a non-target protein which binds to all non-specific bindings sites on the membrane, thus blocking subsequent signal generation at sites lacking the protein of interest. The membrane was then incubated with primary antibody specific for the C-reactive protein. After incubation, the membrane was washed to ensure that only bound antibody remained and the membrane was then incubated in secondary antibody that binds at the exact location as the primary antibody. Since the primary antibody was raised in mouse, the secondary antibody was raised against mouse in rabbit. The secondary antibody was made up in milk solution to reduce background signal and prevent non-specific antigen recognition by the secondary antibody. The secondary antibody also had horseradish peroxidase (HRP) attached to it which would allow the chemiluminescence kit to bind to the secondary antibody in order to generate a signal that can be detected visually. After incubation, further washes were conducted before treated with the chemiluminescence kit that works by binding to the HRP of the secondary antibody and undergoing a chemical reaction by which light is emitted. This chemiluminescent reaction was captured so that the protein can be detected and quantified. Example dot blots can be seen in Appendix 2.

## 2.3 Materials

### 2.3.1 Cell Culture

Human monocyte cell line U937 *(Health Protection Agency Culture Collections, Salisbury)*

RPMI-1640 Media *(Lonza, Slough)*

Foetal Bovine Serum *(Sigma-Aldrich, Dorset)*

Penicillin-Streptomycin *(Lonza, Slough)*

Lipopolysaccharide *(Sigma-Aldrich, Dorset)*

Phorbol 12-myristate 13-acetate *(Applichem, Darmstadt)*

0.4% Trypan Blue *(Lonza, Slough)*

Trypsin *(Thermo Fisher Scientific, Loughborough)*

### 2.3.2 C-reactive Protein Purification

C-reactive Protein, human >95% native, 5mg/ml (*MyBioSource, San Diego)*

EDTA/urea buffer:

- 8M Urea (Fisher Scientific, Cheshire)
- 10mM Ethylenediaminetetraacetic acid (Sigma-Aldrich, Dorset)

Dialysis buffer for purifying native C-reactive protein, adjusted to pH 7.4

- 50mM Tris-hydrochloride *(Fisher Scientific, Cheshire)*
- 2mM Calcium Chloride *(Sigma-Aldrich, Dorset)*

Dialysis buffer for purifying monomeric C-reactive protein, adjusted to pH 7.3

- 13mM Tris-hydrochloride (*Fisher Scientific, Cheshire)*
- 50mM Sodium Chloride (*Sigma-Aldrich, Dorset)*

Dialysis Tubing 21.5mm *(Laboratories Supplies, Nottingham)*

50,000 Da Float-a-Lyzer (*Medicell, London)*

Phenyl Hi-trap *(Scientific Laboratories Supplies, Nottingham)*

E-Toxate Endotoxin Kit *(Sigma-Aldrich, Dorset)*

1% Sodium Deoxycholate *(Sigma-Aldrich, Dorset)*

Bicinchronic Acid Detection Kit *(Sigma-Aldrich, Dorset)*

### 2.3.3 Inhibitors

100nM Wortmannin *(Sigma-Aldrich, Dorset)* made up in RPMI-1640 media

100µM SB203580 (LML inhibitor) *(MedChem Express, Stockholm)* made up in RPMI-1640 media

100µM PD98059 (MEK inhibitor) *(MedChem Express, Stockholm)* made up in RPMI-1640 media

25µg/ml Nystatin *(Sigma-Aldrich, Dorset)* made up in RPMI-1640 media

### 2.3.4 Dot Blot

Nitrocellulose membrane *(Scientific Laboratories Supplies, Nottingham)*

C-reactive protein buffer, adjusted to pH 7.4

- 140mM Sodium Chloride *(Sigma-Aldrich, Dorset)*
- 20mM Tris-hydrochloride (*Fisher Scientific, Cheshire)*
- 2mM Calcium Chloride *(Sigma-Aldrich, Dorset)*

Primary antibody: Mouse anti-human monomeric-CRP made up 1:10 ratio with blocking buffer

- Mouse anti-human monomeric-CRP 8C10 culture supernatant *(this was a kind gift from Dr LA Potempa)*

Primary antibody: Mouse anti-human native-CRP made up 1:10 ratio with blocking buffer

- Mouse anti-human native-CRP 1D6 culture supernatant *(this was a kind gift from Dr LA Potempa)*

TBS/Tween buffer, adjusted to pH 7.4

- 10mM Tris-base *(Fisher Scientific, Cheshire)*
- 140mM Sodium Chloride *(Sigma-Aldrich, Dorset)*

- 0.1% Tween-20 *(Sigma-Aldrich, Dorset)*

Blocking buffer, adjusted to pH 7.4

- 1% Albumin from Bovine serum *(Sigma-Aldrich, Dorset)* in TBS-Tween

Milk Solution, adjusted to pH 7.4

- 5% semi-skimmed milk powder in TBS-Tween

Polyclonal rabbit, anti-mouse immunoglobulins/horseradish peroxidase (HRP) *(Dako, Cambridge)*

- 1:1000 dilution in milk solution

EZ-ECL chemiluminescence detection kit *(Geneflow, Lichfield)*

## 2.4 Methods

### 2.4.1 Cell Culture

The commercial human monocyte cell line U937 was cultured in RPMI-1640 media supplemented with 10% foetal bovine serum (FBS) and 2% penicillin-streptomycin under aseptic conditions at 37°C and 5% carbon dioxide ($CO_2$). Cell growth and viability was assessed using 0.4% trypan blue dye in a 1:1 ratio on a Biorad TC10 automated cell counter. Cell viability was maintained above 90% for all experimental assays.

### 2.4.2 Cell Activation

The U937 cells were activated with 0.05mg/ml lipopolysaccharide (LPS) and incubated for a period of 24 hours to model bacterial infection. Cell activation for the phagocytosis assay differs from this process; see Section 8.6 for more details on this.

### 2.4.3 Cell Differentiation

The U937 cells were differentiated from monocyte-like cells to macrophage-like cells using 50ng/ml phorbol-12-myristate 13-acetate (PMA) and incubated for 72 hours.

### 2.4.4 C-Reactive Protein Isoform Purification

The C-reactive protein isoforms were purified from samples of commercial native C-reactive protein.

#### 2.4.4.1 Monomeric C-Reactive Protein

The mCRP was purified using the Potempa method (Potempa *et al,* 1983). The commercial sample was chelated with 10mM EDTA and 8M urea and incubated at 37°C for 2 hours. The sample was then transferred to dialysis tubing and placed in TBS buffer (0.01 Tris-HCl and 0.05M NaCl) for 24 hours. The sample was checked for purity by dot blot, run through an endotoxin kit and quantified using bicinchoninic acid (BCA) analysis.

#### 2.4.4.2 Native C-Reactive Protein

The nCRP was purified using a 50,000Da float-a-lyzer (Medicell, London) and left overnight in 2mM $CaCl_2$ buffer. The sample was collected and run through a Hitrap hydrophobic interaction chromatography column (GE Healthcare, Buckinghamshire). The sample was tested for purity by dot blot, run through an endotoxin kit and quantified using BCA analysis.

### 2.4.5 Endotoxin Removal

Endotoxins were removed from the purified samples using the E-Toxate Kit. The column was conditioned using 1% sodium deoxycholate and basal media. The sample was allowed to run into the column and then left for two hours before collection.

### 2.4.6 Protein Quantification

The purified protein samples were quantified using BCA analysis according to the manufacturer's instructions. A standard curve was generated using a protein standard consisting of albumin from bovine serum (BSA). Samples were incubated with the working buffer for 30 minutes at 37°C prior to being read on a plate reader at 595nm. The total protein concentration of unknown samples were quantified from the standard curve by interpolation and then normalised prior to storage at -80$^{o}$C for subsequent analysis.

### 2.4.7 Dot Blot

Firstly, 5µl of each of the samples were blotted onto two separate nitrocellulose membranes. BSA at a concentration of 1mg/ml was used as a negative control while known concentrations of mCRP and nCRP were used as positive controls to generate a standard curve. The samples were allowed to dry and are treated with blocking buffer (1% BSA in TBS-Tween, pH 7.4) for one hour at room temperature with 60 rpm rotation. One membrane was then treated with anti-nCRP antibodies (1:10 monoclonal anti-native-CRP 1D6 culture supernatant: blocking buffer) and the other with anti-mCRP antibodies (1:10 monoclonal anti-monomeric-CRP 8C10 culture supernatant: blocking buffer). The membranes were left overnight at 5°C with 50rpm rotation. The primary antibody was then removed and the membranes were washed five times at five minutes each with TBS-Tween buffer. The membranes were then treated with the secondary antibody polyclonal

rabbit anti-mouse immungloblins/HRP in 5% milk/TBS-Tween solution for one hour with 60rpm rotation. The membranes were washed again five times at five minutes each and then treated with the EZ-ECL chemiluminescence detection kit. The image is captured using the Genesnap program (Version 7.07, Syngene) in a Syngene GBox with a 1 minute exposure time.

### 2.4.8 CRP Treatment of Cells

Cells were treated for each assay with either 100µg/ml mCRP, 250µg/ml mCRP, 500µg/ml nCRP or 1000µg/ml nCRP for 24 hours prior to other treatments or collection of results as described in the particular method section.

### 2.4.9 Inhibitors

Cells were treated with CRP isoforms as previously described (Section 2.4.4) but were also simultaneously treated with the four inhibitors mentioned in Section 2.3.3 at the CRP isoform treatment stage and incubated for 24 hours at 37$^{o}$C and 5% $CO_2$.

# Chapter 3: The Effect of calcium and pH on C-reactive protein stability

## 3.1 Introduction

Research has shown that as pentameric CRP dissociates there is a change in structure and biological function. The dissociation of nCRP can be achieved in the presence of high concentrations of urea (Potempa *et al,* 1987) or at high temperatures (Potempa *et al,* 1983). Both of these processes need to be conducted in the absence of calcium. The process by which nCRP dissociates in the absence of calcium is not fully understood. Calcium-dependent binding of CRP to Pch provides an indication that calcium ions play a role in the function of CRP. However, nCRP can spontaneously dissociate in samples containing little or no calcium (Thiele *et al,* 2014). Potempa *et al* (1983) tested the theory that calcium is required for CRP stability and provided evidence that the dissociation of the native protein into its monomeric form can be enhanced by the removal of calcium.

There has been little research conducted into the effects of changing concentrations of calcium on CRP dissociation. Wu *et al* (2002) showed that the spontaneous dissociation of CRP could be promoted by removing calcium but also by lowering the pH to acidic concentrations *in vitro*. Other *in vivo* methods of dissociation include the binding of nCRP to lysophosphatodylcholine monolayers or altered cell membranes (Wang & Sui, 2001; Ji *et al* 2007) and can occur on the surface of activated platelets or apoptotic monocytes (Eisenhardt *et al,* 2009; Molins *et al,* 2011). This dissociation requires the binding of nCRP to lysophosphatodylcholine, which is expressed on the cell surface. This is generated from phosphatidylcholine generated via the activation of calcium-independent phospholipase A2 (Wu *et al,* 2015).

These studies would indicate that CRP stability is only assured at an optimal pH and calcium concentration and that fluctuations would cause the generation of

the more pro-inflammatory mCRP. This could have implications in terms of physiology including inflammatory responses during wound repair.

As humans age there is a reduction in the concentration of circulating calcium. In a normal healthy adult person during youth the concentration of calcium in blood is around 10.2mg/dl, which then decreases to around 9mg/dl in the elderly (Lansdown, 2002). There is a range of calcium concentrations throughout the skin; from 0.5mM in the basal layer to more than 1.4mM in the stratum granulosum (Lansdown, 2002). As calcium decreases with age this has an inhibitory effect on the generation of estrogen (Weitzmann & Pacifici, 2006), which in turn mediates the wound healing process. If the concentration of calcium is decreased, healing is generally impaired.

Calcium is a regulator in the wound healing process, acting as a signalling molecule between cells by regulating cellular organisation and directed migration of cells (Graham *et al,* 2013). Calcium is also used to aid the healing of wounds through the use of calcium alginate dressings; a gel substance generated from seaweed, which helps to keep wound sites moist and prevent bacterial infection while promoting the healing process (Doyle *et al,* 1998), with healing occurring up to four times faster than those without dressings (Sayag *et al*, 1996). Although these dressings cannot be used in all wound types, it can be used to treat diabetic foot ulcers, burns and surgical incisions. These types of dressing can also be made into 'ropes' and used to pack more open and chronic wounds (Doyle *et al,* 1998).

Wu *et al* (2002) indicated that pH also plays a vital role in the dissociation of nCRP. The blood pH of a healthy human is usually between 7.35 and 7.45 and is tightly regulated to remain between these two levels. Cell function and viability are dramatically affected by pH fluctuations beyond these levels. If the pH of the blood decreases below pH 7 or increases above pH 7.8 then death quickly occurs.

If the pH of blood decreases below 7.35 it causes metabolic acidosis. This condition is due to an overabundance of acid or a loss of bicarbonate from the blood and generally arises when the kidneys and lungs are unable to keep the pH in

balance (Kraut & Madias, 2010). There are several causes of metabolic acidosis and it can be acute or chronic in nature (Kraut & Madias, 2010). Diabetes is a major contributor to acidemia since the lack of insulin allows for the build-up of ketones. As the kidneys are typically damaged, the ketones cannot be processed correctly, leading to the acidification of blood. Another contributor is prolonged vomiting and diarrhoea causing hyperchloremic acidosis. This occurs when there a loss of sodium bicarbonate from the blood. Another major contributor, lactic acid acidosis, is caused by a build-up of lactic acid in the blood. This can be caused by chronic alcohol use, heart failure, seizures, cancer low blood sugar but also a lack of oxygen or prolonged exercise (Kraut & Madias, 2010).

If pH increases above 7.45 this is termed metabolic alkalosis. The main cause of metabolic alkalosis is a loss of electrolytes in the blood. This can be caused through excess vomiting, an overuse of diuretics, adrenal disease or a sudden decrease in potassium or sodium concentrations. Other contributors to akalemia are the use of laxatives, alcohol abuse and the accidental ingestion of bicarbonate (Galla, 2000).

There has been little research on the effect of altering pH within these ranges on the dissociation of nCRP to mCRP. Wu *et al* (2002) used pH 2 to cause spontaneous dissociation, but this was conducted using rabbit CRP and the pH was too acidic to model physiological levels observed in blood.

## 3.2 Aims

The aim of this preliminary assay was to determine the effects of pH and calcium concentration on nCRP dissociation into mCRP. The findings were then used to establish the stability of nCRP in changing physiological conditions, which could be found in disease states or impaired healing in the elderly.

## 3.3 Objectives

The fundamental objectives of this section were:

- To assess the effects of calcium concentration on the dissociation of nCRP into mCRP.
- To assess the effects of pH on the dissociation of nCRP into mCRP.

## 3.4 Summary of Procedure

A stability assay was used to model physiological conditions in healthy and disease states. By conducting the assay across a range of pH and calcium levels, it can be used to model the effect of pH and calcium changes on CRP profile during the wound healing process. The assay used a fixed concentration of purified nCRP and measured the amount of mCRP generated through dissociation under various pH and calcium conditions.

The pH levels used in this assay were representative of physiological levels of pH found in normal human blood (pH 7.4) as well as those in acidosis (pH 7.0) and alkalosis (pH 7.8) as well as levels beyond normal human physiological conditions (pH 6.0 and 8.0). The calcium concentrations used were representative of physiological concentrations in human blood in a healthy state as well as the diseased states acidosis and alkalosis.

## 3.5 Materials

C - reactive protein, human >95% native, 5mg/ml (*MyBioSource, San Diego)* Purified as previously stated in Section 2.4.4.

Nitrocellulose membrane *(Scientific Laboratories Supplies, Nottingham)*

C-reactive protein buffer, adjusted to pH 7.4 (pH is varied for stability assay)

140mM Sodium Chloride *(Sigma-Aldrich, Dorset)*

20mM Tris-hydrochloride (*Fisher Scientific, Cheshire)*

2mM Calcium Chloride *(Sigma-Aldrich, Dorset)* – Calcium concentration is varied for stability assay

Primary anti-monomeric-CRP made up 1:10 ratio with blocking buffer

Anti-monomeric-CRP 8C10 culture supernatant *(this was a kind gift from Dr LA Potempa)*

TBS/Tween buffer, adjusted to pH 7.4

10mM Tris-base *(Fisher Scientific, Cheshire)*

140mM Sodium Chloride *(Sigma-Aldrich, Dorset)*

0.1% Tween-20 *(Sigma-Aldrich, Dorset)*

Blocking buffer, adjusted to pH 7.4

1% Albumin from Bovine serum *(Sigma-Aldrich, Dorset)* in TBS-Tween

Milk Solution, adjusted to pH 7.4

5% semi-skimmed milk powder in TBS-Tween

Polyclonal rabbit, anti-mouse immunoglobulins/HRP *(Dako, Cambridge)* 1:1000 dilution in milk solution

EZ-ECL chemiluminescence detection kit *(Geneflow, Lichfield)*

## 3.6 Methods

### 3.6.1 Native C-reactive Protein Isoform

*The n*CRP isoform was purified and quantified as previously described in Section 2.4.4.

### 3.6.2 Stability Assay Sample Preparation

A sample of nCRP isoform was normalised to a final concentration of 500µg/ml. The nCRP was then treated with a buffer similar to the CRP buffer used in previous experiments (Section 2.3.4) but the buffers varied in calcium concentration (0mM, 0.5mM, 1mM and 1.5mM final concentration) and pH (pH 6.8, pH 7.0, pH 7.4, pH 7.8 and pH 8.0). Samples (n=4) were treated for 24 hours at 37$^{o}$C (human body temperature) and then evaluated by dot blot to determine CRP isoform concentrations.

### 3.6.3 Dot Blots

Dot blots were conducted as previously described in section 2.4.7. An example dot blot is located in Appendix 3. A set of control standards (Negative control, 10µg/ml, 20µg/ml, 30µg/ml and 50µg/ml mCRP) were used to allow for a standard cruve to be produced. An example standard curve is located in Appendix 4.

### 3.6.4 Statistical Analysis

Images from the Bio-Rad ChemiDoc$^{TM}$ Touch Imaging System were analysed using the Bio-Rad Image Lab Software (Version 5.2.1). Graphs were drawn using Microsoft Excel 2013 and all statistics performed using IBM SPSS Statistics (Version 22). Data were analysed with the Shapiro-Wilkes normality test before t-tests or Mann-Whitney tests were conducted upon all the data.

## 3.7 Results

Figure 3.1 indicates the effect of calcium concentration and pH on the stability of nCRP by analysing how much mCRP was produced. Figure 3.1 shows that at each pH, less mCRP is produced in a concentration dependent manner as calcium concentration increases. Most mCRP is produced in the absence of calcium.

The amount of mCRP produced at pH 7.4 was generally lower than produced at the other pH values when calcium levels were at or below 1mM. Most mCRP was produced at both pH 7 and pH 7.8 at or below 0.5mM calcium, with slightly lower mCRP produced at pH 6 and pH 8 (than at pH 7 and 7.8) when calcium levels were at or below 1mM.

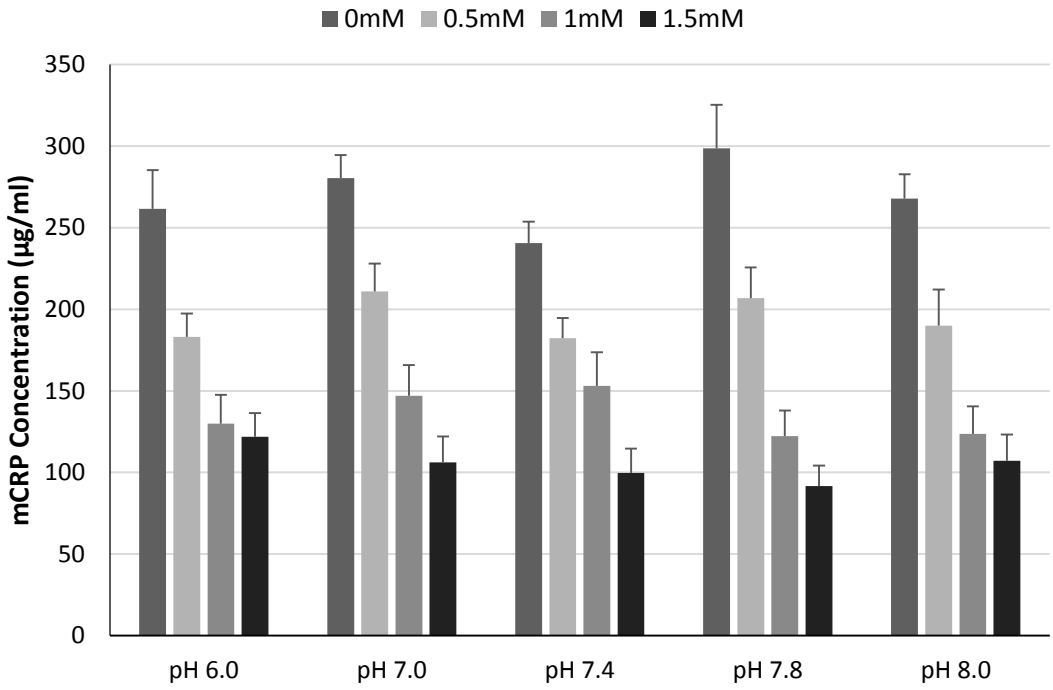



**Figure 3.1 – The effect of pH and calcium concentration on the production of mCRP from nCRP.** Error bars represent standard error of the mean.

## 3.8 Discussion

### 3.8.1 Stability Assay

When one molecule of nCRP dissociates it leads to the generation of five monomeric units (Potempa *et al,* 1983). The results of the assay indicated that not all of the nCRP dissociated but the amount of mCRP increased as the pH deviated from pH 7.4 or as calcium levels reduced. This could have led to an overall pro-inflammatory effect if enough of the nCRP molecules dissociate to change the balance into a higher ratio of mCRP to nCRP.

#### 3.8.1.1 The Effect of Calcium Concentration on the Stability of nCRP

Figure 3.1 provides evidence that calcium plays a key role in the stability of nCRP. At all five pH levels there was a higher amount of mCRP produced when calcium was absent than when there was 1.5mM calcium present. This corroborates all studies that have stated that mCRP is produced in the absence of calcium (Potempa et al, 1983; Wu et al, 2002).

The physiological concentration of calcium in a healthy, young adult is around 1mM. These findings indicate that increasing the amount of calcium stabilises the pentamer further and inhibits its dissociation. Thus as calcium levels decline through increasing age, there is an increased risk of spontaneous dissociation of the pentamer into the more pro-inflammatory mCRP isoform (Thiele et al, 2014) resulting in impaired healing due to a prolonged and excessive inflammatory response due to mCRP accumulation at the wound site. The results suggest that calcium supplementation in elderly subjects may to some extent, reverse the loss of nCRP stability due to increasing age.

Furthermore, the addition of calcium into dressings for chronic wounds such as diabetic foot ulcers (Doyle et al, 1998) may in part be due to the high calcium concentration preventing the generation of mCRP at the wound site, thus reducing inflammation. When a calcium alginate dressing comes into contact with a wound,

there is a release of calcium ions into the wound site. The calcium aids coagulation and the alginate helps to regulate blood flow (Timmons, 2009) but it has been suggested that the calcium could also be acting in an anti-inflammatory manner. This study has provided a novel mechanism by which this type of wound dressing may be anti-inflammatory. Further studies are now required to confirm whether such dressings inhibit the dissociation of nCRP into mCRP in a clinical setting.

The use of calcium in dressings for chronic wounds may also aid the function of CRP. The binding of PCh by nCRP requires the binding of two calcium ions per subunit (Mullenix & Mortensen, 1994). This binding leads the triggering of the complement pathway as well as the opsonisation of bacteria and dead cells (Mold *et al*, 1999). The addition of calcium to chronic wounds also leads to the activation of these pathways and improved healing by enhancing the host immune system detect and remove bacteria and dead cells by phagocytosis.

#### 3.8.1.2 The Effect of pH on the Stability of nCRP

The pH had a moderate effect on the production of mCRP, with little change in the presence of calcium but a more pronounced effect in the absence of calcium. This finding suggests that presence of calcium (rather than pH) is the critical stability factor for nCRP within a pH range that reflects normal and disease states. As the pH deviated away from pH 7.4 in the absence of calcium the amount of mCRP increased. This confirms that the normal (healthy) pH of blood (pH 7.4) is the most suitable pH for maintaining nCRP stability. However, more mCRP was produced in acidic conditions (indicative of metabolic acidosis) than in alkaline conditions, which supports Wu *et al*'s (2002) study that used acidic conditions to dissociate rabbit CRP.

The findings of this study indicate that wound dressings that maintain a pH around pH 7.4 are most optimal at maintaining the stability of the more anti-inflammatory nCRP isoform.

## 3.9 Future Work

The assay could also be repeated at a series of different temperature to determine if slight temperature fluctuations can alter the stability of nCRP. It is known that high temperatures above 75$^{o}$C can cause some dissociation of nCRP but could smaller temperature changes similar to physiological values observed in fever following acute infections affect the stability of nCRP.

Another aspect to be analysed would be to collect blood samples from patients with metabolic acidosis and alkalosis and determine if their mCRP levels are significantly affected. Samples from elderly subjects taking calcium supplements would be valuable to determine whether they have lower levels of mCRP and improved healing.

## 3.10 Summary

In summary, calcium concentration was the major factor mediating the stability of the pentameric nCRP isoform; with higher calcium concentrations maintain nCRP stability. However as pH deviated away from normal (healthy) values there was greater propensity for nCRP instability, particularly as calcium levels declined. Acidic conditions caused more mCRP production than alkaline conditions. This study indicated that the elderly are at greater risk of impaired healing and pronounced inflammation due a drop in calcium levels with increasing age.

This preliminary study indicates that further research needs to be conducted in order to fully understand how the tissue environment (including pH and calcium levels) might mediate mCRP concentration *in vivo*.

# Chapter 4: The Localisation of C-Reactive Protein Isoforms in Monocytes and Macrophages during the Inflammatory Phase in Murine Wounds

With special thanks to J Hynes and Dr J Ashworth for their contribution to the collection of data in this chapter.

## 4.1 Introduction

There is increasing evidence that C-reactive protein (CRP) may have a functional role in the inflammatory process. It is well established that CRP is an acute marker of inflammation and that its concentration increases in the circulation during inflammatory events.

There has been some attempt to localise CRP to sites of inflammation and tissue damage. It has been demonstrated that CRP has been deposited in various conditions, both naturally occurring and in experimental conditions (Kushner & Kaplan, 1961; Kushner *et al*, 1963; Parish, 1977; Gitlin *et al*, 1978). However, there are some issues with the published research on the localisation of CRP. These studies investigated CRP without considering the 2 different isoforms separately. It is now established that there are two isoforms of CRP and that they have differing structure and biological function so there could also be differential localisation of the protein as well. When these studies were conducted it was not well established that there were two isoforms of the protein and available antibodies would have been raised to detect the pentameric nCRP alone. The second issue is that these studies localised the protein in cases of inflammation with specific tissue damage. Gitlin *et al* (1978) concluded that CRP was localised to the nuclei of cells within the synovium of rheumatoid arthritis patients but the cell type could not be identified at the time.

It has been suggested that following tissue injury, CRP binds to damaged cell membranes and by activating complement contributes to the inflammatory

response (Kaplan & Volanakis, 1974) with CRP molecules becoming associated with terminal complement complexes, especially in atherosclerotic lesions (Torzewski *et al*, 1998). Lagrand *et al* (1997) provided evidence that CRP localises to infarcted heart tissue and promotes local complement activation, triggering further damage to the heart tissue. However, other studies indicate no significant localisation of CRP in a number of pathologies, suggesting that CRP is found predominantly in the fluid phase rather than deposited in tissues at sites of inflammation or tissue injury (Vigushin *et al*, 1993).

There has been little research conducted on the localisation of CRP in inflammatory cells and wounds to date. There is a correlation between the localisation of CRP in neutrophil infiltrates, especially in lesions of vasculitis and allergic encephalomyelitis (Du Clos *et al,* 1981; Parish, 1971). However, the localisation of distinct CRP isoforms in acute wounds of skin has not been conducted previously.

## 4.2 Aims

The aim of this study was to determine the localisation of CRP isoforms in inflammatory cells of murine cutaneous wounds.

## 4.3 Objectives

The fundamental objectives of this assay were:

- To analyse the effect of 'hot' and 'cold' antigen retrieval of CRP isoforms
- To provide evidence that CRP is localised to sites of inflammation in cutaneous murine wounds
- To assess the localisation patterns of CRP isoforms in inflammatory cells of murine wounds
- To assess the effect of impaired healing on the localisation of CRP isoforms using a murine model of age-related impaired healing.

## 4.4 Summary of Procedure

Immunohistochemistry uses an antigen-antibody interaction to detect tissue and cellular constituents either by direct labelling of the antibodies or by a second labelling method (Bancroft & Gamble, 2008). The Vectastain Elite ABC kit uses an unlabelled primary antibody to bind to the target protein, followed by the binding of a biotinylated secondary antibody. This is then followed by the binding of a preformed avidin and biotinylated horseradish peroxidase (HRP) macromolecular complex, allowing the target protein to be visualised by a coloured dye due to the development of a peroxidase substrate when bound to the HRP complex. For example, Novared produces a dark red colour in the presence of the HRP complex.

Prior to visualisation of an antibody- labelled, a process called antigen retrieval is vital to ensure the antigen is exposed to the antibody. Antigen retrieval breaks crosslinks between proteins and any fixative in the sample that could mask the antigen (Shi *et al,* 2001). A simple antigen retrieval method is the use of high temperature and an acidic buffer. However, for comparison this study also used a 'cold' antigen retrieval method to ensure that the hot antigen retrieval was not

inadvertently affecting results by dissociating the pentameric nCRP resulting in false-positive mCRP staining.

## 4.5 Materials

Wax embedded murine wound samples – *Kindly provided by the University of Manchester*

Anti-monomeric-CRP 8C10 culture supernatant primary antibody *( kind gift from Dr LA Potempa)*

Anti-native-CRP 1D6 culture supernatant primary antibody *(kind gift from Dr LA Potempa)*

Vectastain Elite ABC kit (mouse IgG) (Vector Labs, Burlingame)

- Normal horse blocking serum
- Horse anti-mouse biotinylated IgG
- Avidin-biotinylated horseradish peroxidase (HRP) complex (ABC kit reagent)

Vectastain Elite ABC kit (rat IgG) (Vector Labs, Burlingame, CA)

- Norrmal horse blocking serum
- Horse anti-rat biotinylated IgG
- Avidin-biotinylated horseradish peroxidase (HRP) complex (ABC kit reagent)

Tris-buffered Saline (TBS), adjusted to pH 7.5

- 10mM Tris-base *(Fisher Scientific, Cheshire)*
- 140mM Sodium Chloride *(Sigma-Aldrich, Dorset)*
- 0.1% Tween-20 *(Sigma-Aldrich, Dorset)*

Anti-CD105 antibody (Abcam, Cambridge)

Anti-CD34 antibody (Abcam, Cambridge)

Haematoxylin (VWR International, Leicestershire)

Novared (Vector Labs, Burlingame)

Ethylenediaminetetraacetic acid (EDTA)-Tween buffer, adjusted to pH 8.0

- 0.01M EDTA *(Sigma-Aldrich, Dorset)*
- 0.01%Tween (*Sigma-Aldrich, Dorset)*

Glycine, EDTA, Tween Buffer adjusted to pH 3.5

- 0.01M EDTA *(Sigma-Aldrich, Dorset)*

- 0.05M Glycine *(Sigma-Aldrich, Dorset)*
- 0.01% Tween *(Sigma-Aldrich, Dorset)*

Xylene (VWR International, Leicestershire)

3% Hydrogen Peroxide (Sigma-Aldrich, Dorset)

Methanol (Sigma-Aldrich, Dorset)

Ethanol (Sigma-Aldrich, Dorset)

Pertex Mounting Media (Cellpath PLC, Powys)

Vectabond Coated Slides (Vector Laboratories, Burlingame)

## 4.6 Methods

### 4.6.1 Animals and Wounding

Collaborators from the University of Manchester provided the wax embedded murine wounds for this study. The murine wound samples were collected from 10 week old female Balb/C wild-type mice that were either INTACT (possessing ovaries) or had undergone ovariectomy (OVX) 1 month previously. Wounds from OVX mice are an established murine model of age-related impaired healing whilst wounds from INTACT mice represent a model of acute wound healing during youth (Ashcroft *et al* 1997). Isofluorane was used as an inhaled anaesthetic and the dorsum was shaved and cleaned with alcohol. Mice were wounded on their dorsum making two equidistant 1 cm full thickness skin incisional wounds, placed 1cm from the base of the skull and 1cm from either side of the midline. These incisions were made through the skin and panniculus carnosus muscle. Healing of the wounds was by secondary intention and subsequent removal was by excision (6 mm excisional wounds) at 12 hours, 24 hours, 3 days and 7 days post-wounding. The excised wounds were bisected and processed such that the midpoint of each wound was used for embedding in paraffin wax and histological

analysis following an established protocol (Bancroft & Gamble, 2008). For each time point the data represented 3 mice (n =3) and 5 separate images were collected for analysis from each wound.

### 4.6.2 Immunohistochemical Chemical Staining Protocol

Murine wound samples at specified time points during the wound healing process (12h, 24h, D3, D7 post-wounding) from both INTACT and OVX were stained for the nCRP and mCRP isoforms using the Vectastain Elite ABC mouse IgG kit, an avidin-biotin peroxidase system, and monoclonal mouse anti-human nCRP (2C10) and anti-mCRP (8C10) primary antibodies. Paraffin wax embedded 5µm thick sections were cut from murine wounds using a microtome and mounted on Vectabond coated slides. Slides were deparaffinised using xylene, rehydrated through a graded ethanol series and then incubated in 3% hydrogen peroxide ($H_2O_2$) in methanol to block endogenous peroxidases. Antigen retrieval involved either incubation in a 0.01M EDTA and 0.1% Tween buffer for 6 hours at $60^0$C (referred to as cold antigen retrieval) or incubated in an 0.01M EDTA, 0.05M Glycine and 0.1% Tween buffer for 20 minutes following 5 minutes heating in a microwave (referred to as hot antigen retrieval). After antigen retrieval, slides were washed for 15 minutes in TBS-Tween. Slides were then incubated in normal horse serum for 1 hour to block non-specific binding sites followed by an overnight incubation at $4^0$C in primary antibody (2C10 or 8C10) diluted 1:10 with TBS containing normal horse serum. To minimise cross reactivity, a secondary staining kit was used which was specifically designed to stain for mouse antibodies on mouse tissue. Negative controls were used to determine any non-specific binding of the antibodies. The negative control was treated with TBS containing normal horse serum instead of primary antibody with no other changes to the staining protocol. After the overnight incubation, the slides were washed in TBS-Tween for 15 minutes followed by incubation in secondary biotin-conjugated horse anti-mouse antibody for 1 hour then washed again in TBS-Tween for 15 minutes. Slides were then incubated in an avidin-biotin peroxidase reagent for 30 minutes at room

temperature followed by a final wash in TBS-Tween for 30 minutes. Slides were stained with Novared for 2 minutes followed by counterstaining with haematoxylin. Slides were dehydrated through a graded ethanol series before mounting in Pertex. For each time point the data represented 3 mice (n =3) and 5 separate images were collected for analysis from each mouse.

### 4.6.3 Dual Staining Protocol

Murine wound samples (n=3) from D3 and D7 wild type mice were stained for nCRP and mCRP using the Vectastain Elite ABC mouse IgG kit and monoclonal mouse anti-human nCRP (2C10) and mCRP antibodies (8C10). Sequential sections were stained for neutrophils and macrophages using monoclonal anti-neutrophil and MAC3 antibodies respectively. This was conducted using a Vectastain Elite ABC rat IgG kit, an avidin-biotin peroxidase system using the previously described immunohistochemical staining protocol (*3.6.2*). Sequential sections were stained with appropriate primary antibodies and resulting images overlaid to determine co-localisation of inflammatory cell markers and CRP isoforms. A negative control was used to determine any non-specific binding of antibodies during the dual staining process. For the negative control, the primary antibody was replaced with TBS containing normal horse serum with no other changes to the staining protocol. For each time point the data represented 3 mice (n =3) and 5 separate images were collected for analysis from each mouse.

### 4.6.4 Inflammatory Phase Analysis

For histological sections at 12h, 24h, D3 and D7 post-wounding staining for both CRP isoforms was analysed from 5 pre-defined areas within the wound. Images were captured using the Axio Imager M1 microscope (Zeiss, Germany) with 630x magnification. Area 1 was representative of mid-wound and areas 2, 3, 4 and 5 represented right and left areas just under the epidermis and just above the fat layer in the dermis ensuring a representative proportion of the whole wound was

analysed. The amount of inflammatory cell staining was analysed using Image J software (Version 1.49 Rasband W., National institutes of Health, USA). Positive staining of inflammatory cells was expressed as a ratio of total inflammatory cells, thus providing a basis for comparison of the native and modified CRP. For sections at D3 and D7 post-wounding, inflammatory cells were manually stratified based on their morphology into neutrophils and macrophages respectively.

### 4.6.5 Statistical Analysis

Statistical analysis was performed using SPSS (Version 22). A Shapiro-Wilks normality test was performed followed by appropriate paired or unpaired t-tests for normally distributed data or Wilcoxon-Mann-Whitney U tests for non-parametric situations. Tests were chosen depending on the distribution of each dataset generated and $P<0.05$ was considered statistically significant. Graphs were generated using Microsoft Excel 2013, with error bars representing the standard error of the mean (SEM).

## 4.7 Results

### 4.7.1 Comparison of Hot and Cold Antigen Retrieval Technique

Antigen retrieval allows for the antigens in the wax-embedded samples to become exposed and available for primary antibody binding (Shi *et al*, 2001). This study looked at the difference between two techniques, the 'hot' and 'cold' antigen retrieval. This was conducted in order to determine the ability of the 2 retrieval processes to reveal the desired antigens and to assess the potential dissociation of the nCRP that may be caused by typical hot antigen retrieval methods. The data from this study indicated that there was no significant difference (nCRP $p=0.9056$, mCRP $p=0.4074$) between the staining of the CRP isoforms using the two techniques (Figures 4.1A and 4.1B). This showed that both methods consistently retrieved the

CRP antigens and that the hot retrieval method did not disrupt the integrity of the nCRP isoform.

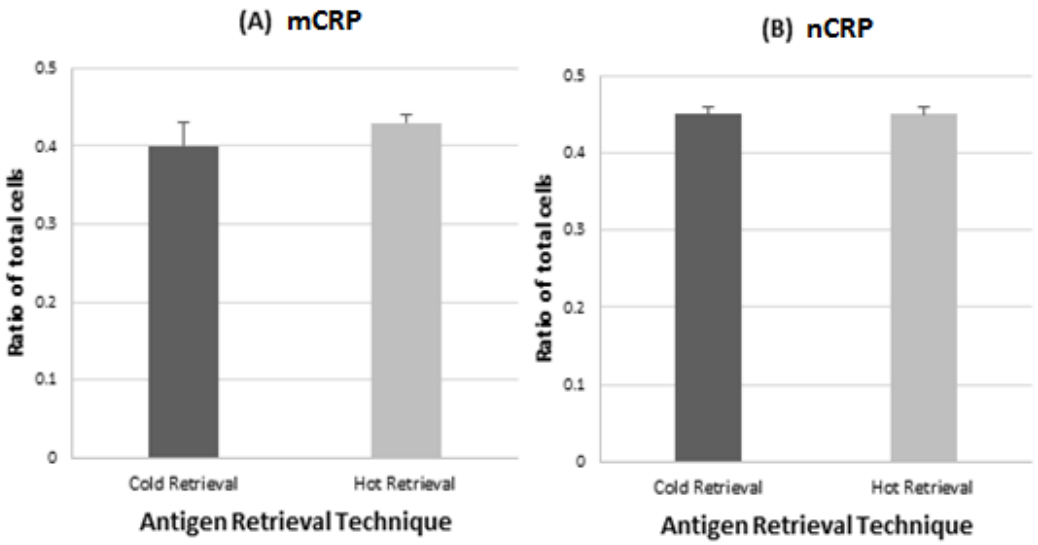



**Figure 4.1: Comparison of hot and cold antigen retrieval techniques of CRP isoforms at D7 post-wounding.** (A) Comparison of the two antigen retrieval techniques in detecting mCRP-positive inflammatory cells. (B) Comparison of the two antigen retrieval techniques in detecting nCRP-positive inflammatory cells. Error bars represent the SEM.

### 4.7.2 Presence of CRP Isoforms During the Inflammatory Phase of Wound Healing

During the early and late inflammation phases, both mCRP and nCRP were detected within inflammatory cells. This would indicate that the source of these isoforms would most likely be the most predominant cell types at these time points; principally neutrophils and monocytes (Kaplanski *et al,* 2003).

#### 4.7.2.1 Detection of CRP Isoforms in Murine Wound in the Early Inflammatory Phase

The early inflammatory phase CRP detection was measured in the cutaneous murine wounds samples obtained at 12 hours and 24 hours post-wounding. The samples at both time points were sectioned and stained for mCRP (Figures 4.2B and 4.3B) or nCRP (Figures 4.2C and 4.3C).

At 12 hours post-wounding, mCRP-positive staining was 588 cells/$mm^2$ whilst nCRP-positive staining was 437 cells/$mm^2$, equating to be around 19% and 15% of the total inflammatory cell count respectively (Figure 4.2A). Thus, the proportion of total inflammatory cells demonstrating positive staining of nCRP or mCRP was significantly ($P<0.01$) lower than the proportion of inflammatory cells lacking CRP isoform localisation. However, no significant ($P=0.1165$) difference between mCRP and nCRP was identified in relation to the proportion of positively stained cells. Lastly, the ratio of mCRP:nCRP was 1.3 at 12 hours post-wounding. At 24 hours post-wounding, mCRP-positive staining was 1765 cells/$mm^2$ and 1619 cells/$mm^2$ were positive for nCRP. This was calculated to be around 45% and 40% of the total cells present respectively (Figure 4.3A). Again, the proportion of total inflammatory cells demonstrating positive staining of nCRP or mCRP was significantly ($P<0.01$) lower than the proportion of inflammatory cells lacking CRP isoform localisation. However, no significant ($P=0.1310$) difference between mCRP and nCRP was identified in relation to the percentage of positively stained cells. The ratio of mCRP:nCRP reduced to 1.1 at 24 hours post-wounding.

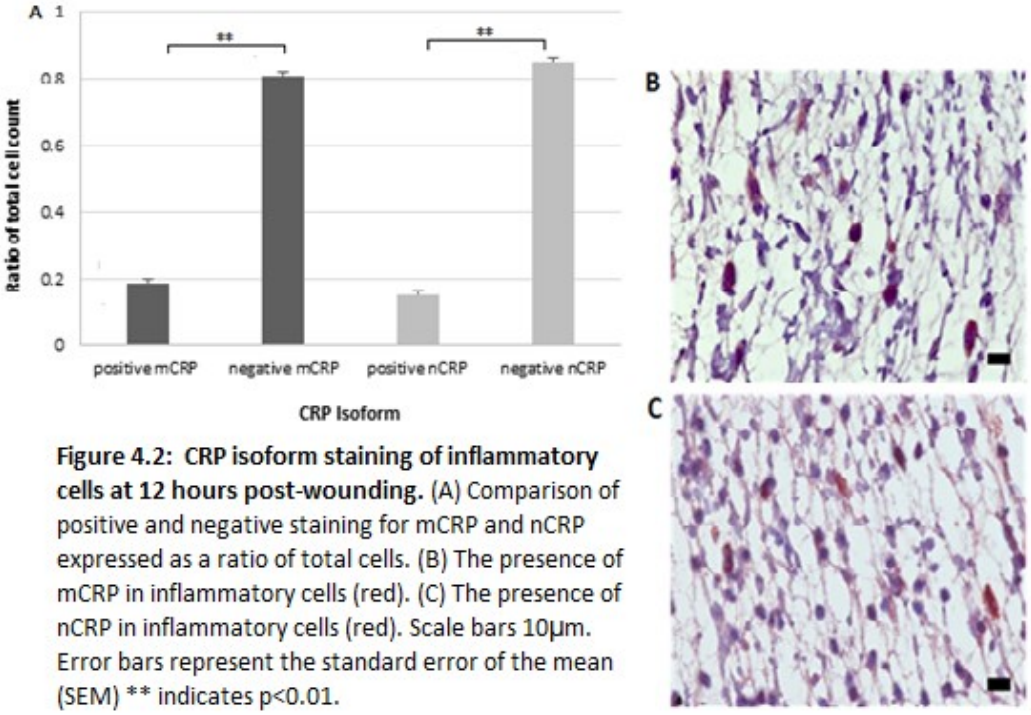



**Figure 4.2: CRP isoform staining of inflammatory cells at 12 hours post-wounding.** (A) Comparison of positive and negative staining for mCRP and nCRP expressed as a ratio of total cells. (B) The presence of mCRP in inflammatory cells (red). (C) The presence of nCRP in inflammatory cells (red). Scale bars 10μm. Error bars represent the standard error of the mean (SEM) ** indicates p<0.01.

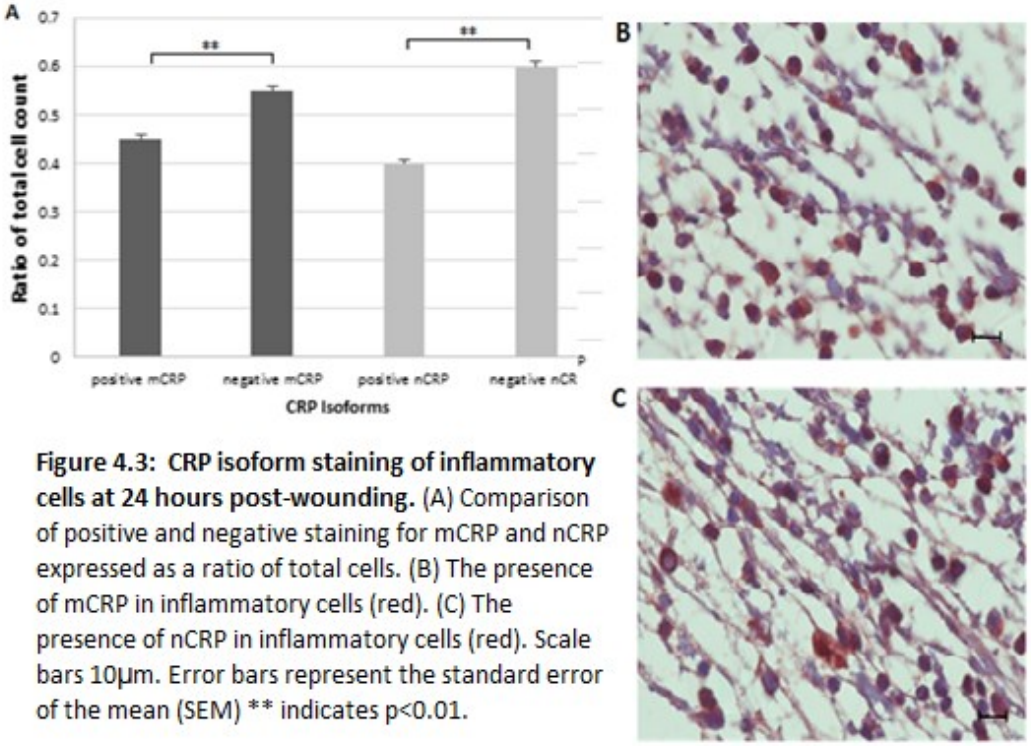



**Figure 4.3: CRP isoform staining of inflammatory cells at 24 hours post-wounding.** (A) Comparison of positive and negative staining for mCRP and nCRP expressed as a ratio of total cells. (B) The presence of mCRP in inflammatory cells (red). (C) The presence of nCRP in inflammatory cells (red). Scale bars 10μm. Error bars represent the standard error of the mean (SEM) ** indicates p<0.01.

#### 4.7.2.2 Comparison of 12 and 24 Hours Post-Wounding

Detection of both CRP isoforms was analysed at the early phase of the inflammatory process to compare the amount of positive staining between the early inflammatory time points (12 hours and 24 hours). The amount of mCRP detected increased significantly ($p<0.001$) from 19% to 45% between these two early time points (Figure 4.4A) and nCRP increased significantly ($p<0.001$) from 15% to 40% (Figure 4.4B), suggesting a rapid increase in the proportion of both isoforms. Both CRP isoforms effectively doubled over the 12 hour period in terms of cell concentration and proportion of total inflammatory cells. No difference between mCRP and nCRP was detected at each of the time points in the early phase of inflammation but the ratio of mCRP:ncRP reduced from 1.3 at 12 hours post-wounding to 1.1 at 24 hours post-wounding.

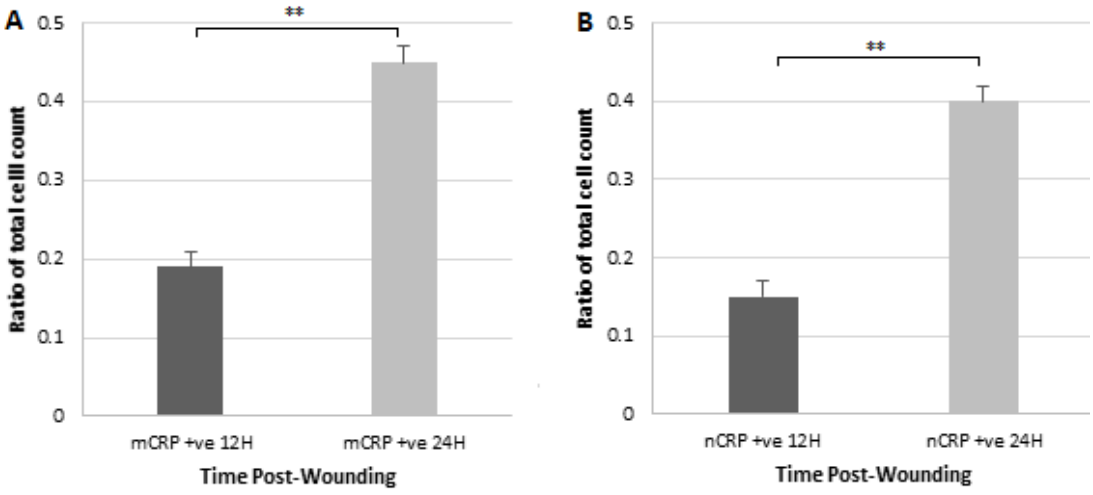



**Figure 4.4: Comparison of CRP positive inflammatory cells at 12 and 24 hours post-wounding.** (A) Comparison of mCRP positive inflammatory cells at 12 and 24 hours post-wounding expressed as a ratio of total cells. ** represents $P<0.001$. (B) Comparison of nCRP positive inflammatory cells at 12 and 24 hours post-wounding expressed as a ratio of total cells. ** represents $P<0.001$. Error bars represent the standard error of the mean (SEM). ** indicates $p<0.01$.

#### 4.7.2.3 Day 3 Post-Wounding.

Neutrophils typically peak in numbers by day 3 (D3) post-wounding (Kaplanski *et al*., 2003), suggesting neutrophils should form a substantial proportion of the total inflammatory cell count taken at this time point.

Murine cutaneous wound samples at D3 post-wounding were sectioned and stained for CRP isoforms (Figures 4.5B and 4.5C). The mean cell density in these D3 wounds was 4267 cells/mm$^2$, indicating a significant ($P<0.01$) increase in total inflammatory cell density compared to wounds at 24 hours post-wounding. The concentration of mCRP and nCRP positive cells was 3286 cells/mm$^2$ and 2347 cells/mm$^2$ respectively. To allow for a comparison of these results, these data were expressed as a proportion of the total inflammatory cell count (Figure 4.5A). The proportion of total inflammatory cells demonstrating positive staining of nCRP or mCRP was significantly ($P<0.01$) greater than the proportion of inflammatory cells lacking CRP isoform localisation. The results indicated significantly ($p<0.001$) more mCRP positive staining than nCRP positive staining at D3 post-wounding, with 77% of the total cells being stained mCRP positive compared to the 55% stained positively for nCRP. This represented another significant ($P<0.01$) increase in the proportion of CRP localisation from 24 hours post-wounding (45% mCRP-positive; 40% nCRP-positive) to 3 days post-wounding (77% mCRP-positive; 55% nCRP-positive). The ratio of mCRP:nCRP also increased from 1.1 at 24 hours post-wounding to 1.4 at D3 post-wounding.

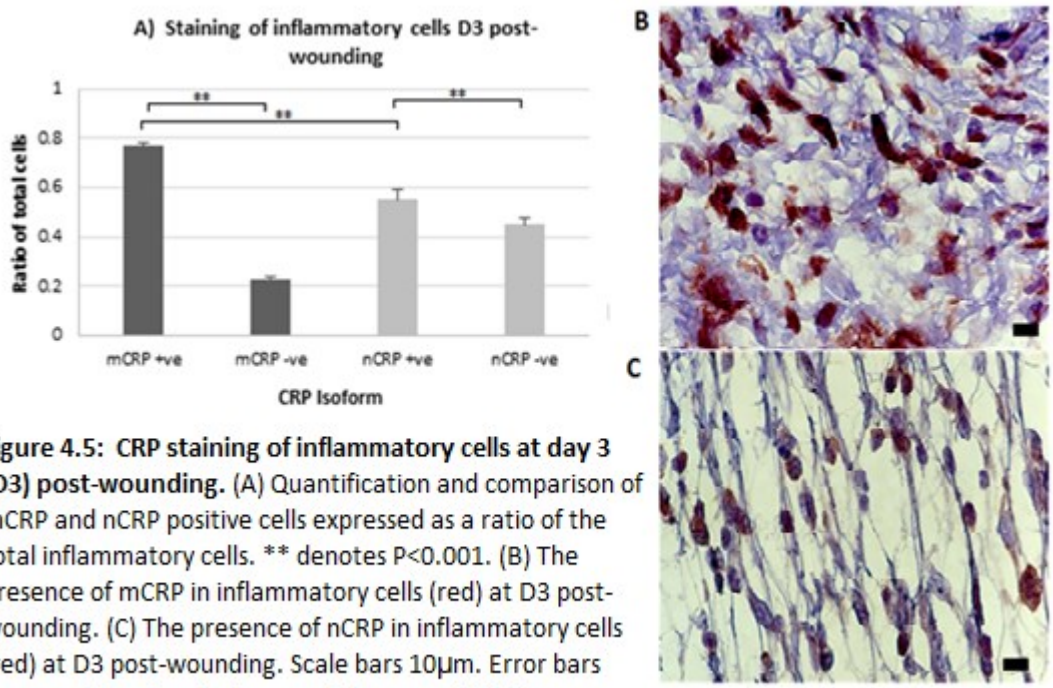



**Figure 4.5: CRP staining of inflammatory cells at day 3 (D3) post-wounding.** (A) Quantification and comparison of mCRP and nCRP positive cells expressed as a ratio of the total inflammatory cells. ** denotes P<0.001. (B) The presence of mCRP in inflammatory cells (red) at D3 post-wounding. (C) The presence of nCRP in inflammatory cells (red) at D3 post-wounding. Scale bars 10µm. Error bars represent the standard error of the mean (SEM).

### 4.7.3 Detection of CRP Isoforms in Murine Wound in the Late Inflammatory Phase

#### 4.7.3.1 D7 Post-Wounding

To analyse CRP isoform levels at the later inflammatory phase, cutaneous murine wound samples from day 7 (D7) post-wounding were sectioned and stained for mCRP and nCRP (Figures 4.6B and 4.6C)

The results indicated that at D7 post-wounding, 42% of total cells stained positively for mCRP and 45% stained positive for nCRP, with no significant (p=0.0977) difference in the proportion of positive staining between the two CRP isoforms (Figure 4.6A). The proportion of total inflammatory cells demonstrating positive staining of nCRP or mCRP was significantly (P<0.01) lower than the proportion of inflammatory cells lacking CRP isoform localisation.

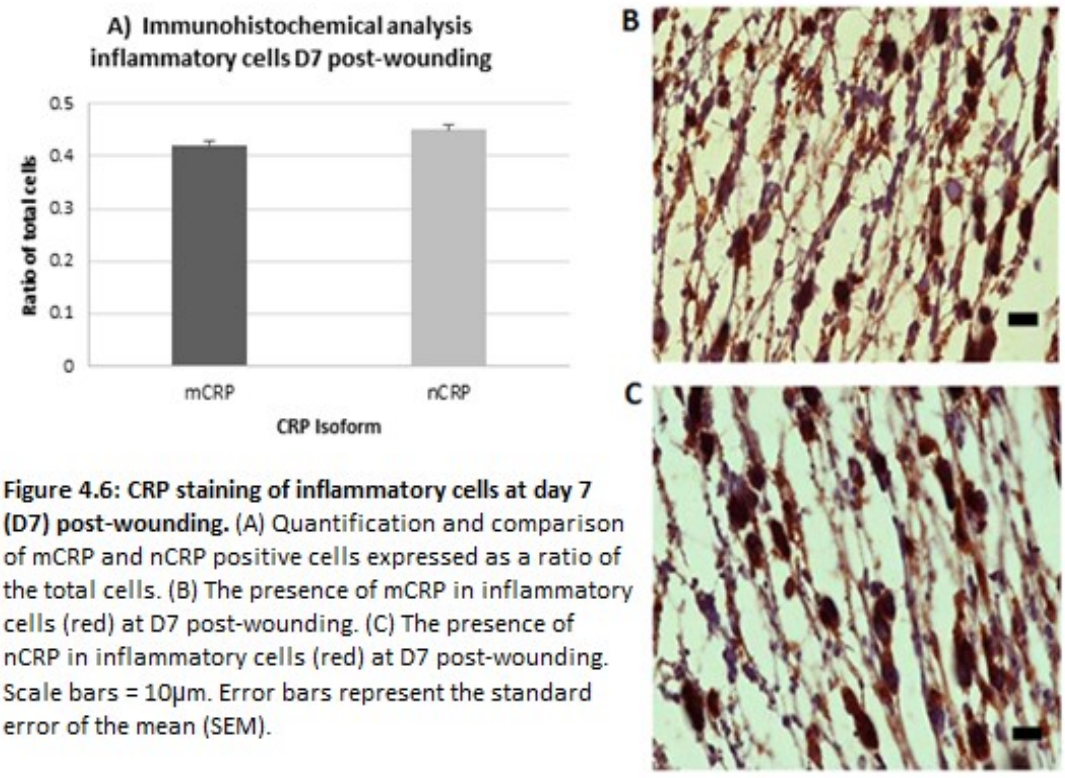



**Figure 4.6: CRP staining of inflammatory cells at day 7 (D7) post-wounding.** (A) Quantification and comparison of mCRP and nCRP positive cells expressed as a ratio of the total cells. (B) The presence of mCRP in inflammatory cells (red) at D7 post-wounding. (C) The presence of nCRP in inflammatory cells (red) at D7 post-wounding. Scale bars = 10μm. Error bars represent the standard error of the mean (SEM).

#### 4.7.3.2 Comparison of Staining in Inflammatory Cells D3 and D7 Post-Wounding

To complete the analysis of CRP isoform staining in the inflammatory process a comparison of the positively stained cells at D3 and D7 post-wounding was performed (Figure 4.7A and 4.7B).

When compared to D3 there was a significant increase in the number of total inflammatory cells. The total cell density increased significantly ($P<0.01$) from 4270 cells/mm$^2$ at D3 to around 5000 cells/mm$^2$ at D7. However, the proportion of total inflammatory cells with positive staining significantly decreased for both mCRP (77% to 42%) and nCRP (55% to 45%) between the two time points, with the ratio of mCRP:nCRP also decreasing from 1.4 at D3 post-wounding to 0.9 at D7 post-wounding.

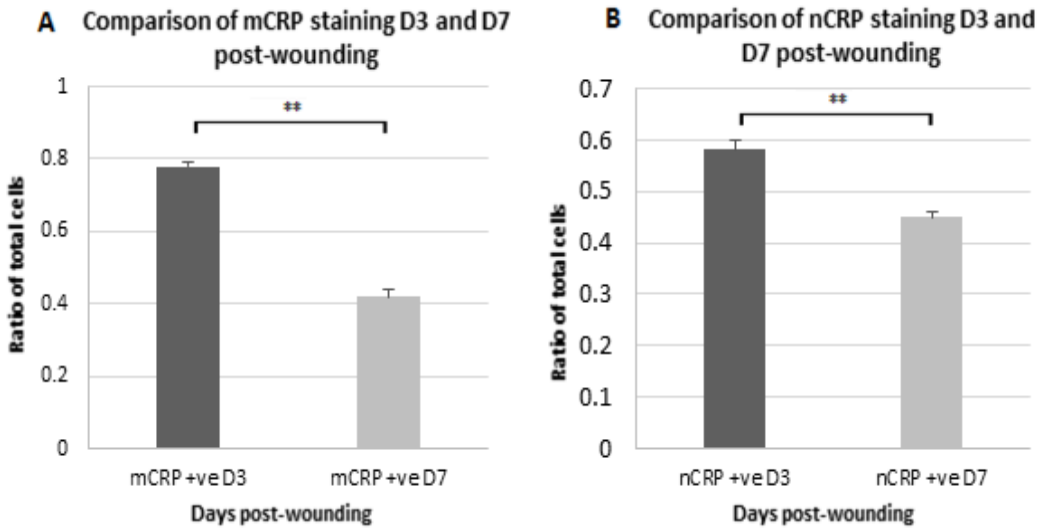



**Figure 4.7: Comparison of CRP positive staining of inflammatory cells at day 3 (D3) and day 7 (D7) post-wounding.** (A) Quantification and comparison of mCRP positive inflammatory cells at D3 and D7 post-wounding, expressed as a ratio of total cells. ** denotes P<0.001. (B) Quantification and comparison of nCRP positive inflammatory cells at D3 and D7 post-wounding, expressed as a ratio of total cells. ** denotes P<0.001. Error bars represent the standard error of the mean (SEM).

#### 4.7.3.3 Localisation of CRP Isoforms in Neutrophils at D7 Post-Wounding

Neutrophils are usually around 6-10µm in diameter and appear smaller and rounder than monocytes (Trowbridge & Emling, 1997). Based on these morphological differences this cell type could be easily distinguished from other cell types in D7 wound sections (Figures 4.8B and 4.8C). The mean total neutrophil cell density D7 post-wounding was approximately 3000 cells/mm$^2$ based on morphology and positive staining was expressed as a ratio of total neutrophils (Figure 4.8A). The results showed there was no significant difference (p=0.1308) between the staining of the CRP isoforms in neutrophils at D7 post-wounding with mCRP being detected in 28% of neutrophils and nCRP in 31% of neutrophils. This shows that most neutrophils were not producing either CRP isoform at this time point during the healing process. This is in direct contrast to D3 post-wounding in which most cells became positively stained, with significantly (p<0.001) more mCRP staining observed

than nCRP (Figure 4.5). The ratio of mCRP:nCRP staining in neutrophils at D7 post-wounding was 0.9.

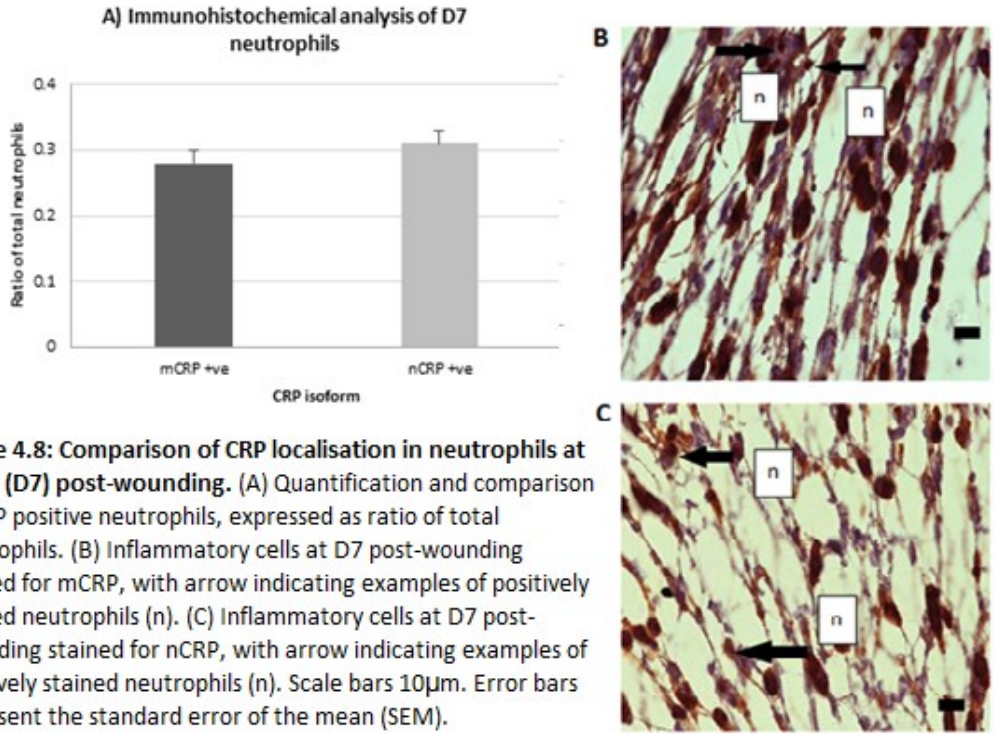



**Figure 4.8: Comparison of CRP localisation in neutrophils at day 7 (D7) post-wounding.** (A) Quantification and comparison of CRP positive neutrophils, expressed as ratio of total neutrophils. (B) Inflammatory cells at D7 post-wounding stained for mCRP, with arrow indicating examples of positively stained neutrophils (n). (C) Inflammatory cells at D7 post-wounding stained for nCRP, with arrow indicating examples of positively stained neutrophils (n). Scale bars 10µm. Error bars represent the standard error of the mean (SEM).

#### 4.7.3.4 CRP Localisation in Macrophages at D7 Post-Wounding

Numbers of tissue macrophages typically peak at around D7 post-wounding (Kaplanski *et al,* 2003). Tissue macrophages are much larger in size (typically around 10-30µm) with a more lobular shape (Figures 4.9B and 4.9C) than neutrophils.

The cell types were stratified according their morphology and the corresponding cell counts performed as described previously. The CRP isoform staining is expressed as ratio of total macrophages and the comparisons were made between CRP isoforms (Figure 4.9A).

No significant (p=0.6203) difference was identified between the two isoforms of CRP with 80% of macrophages staining positive for mCRP and 79% for nCRP (Figure 4.9A). At D7 post-wounding the mean inflammatory cell density was calculated at around 5000 cells/mm$^2$ with around 2000 cells/mm$^2$ of those being morphologically identified as macrophages. Of these cells 1600 macrophages/mm$^2$ (80%) stained positive for mCRP and 1580 (79%) macrophages/mm$^2$ stained positively for nCRP.

This evidence suggests that most macrophages at D7 post-wounding were simultaneously producing both CRP isoforms due to the fact that both isoforms were detected in the majority of macrophages within the sample. The ratio of mCRP:nCRP in macrophages when identified by morphology was 1.0 at D7 post-wounding.

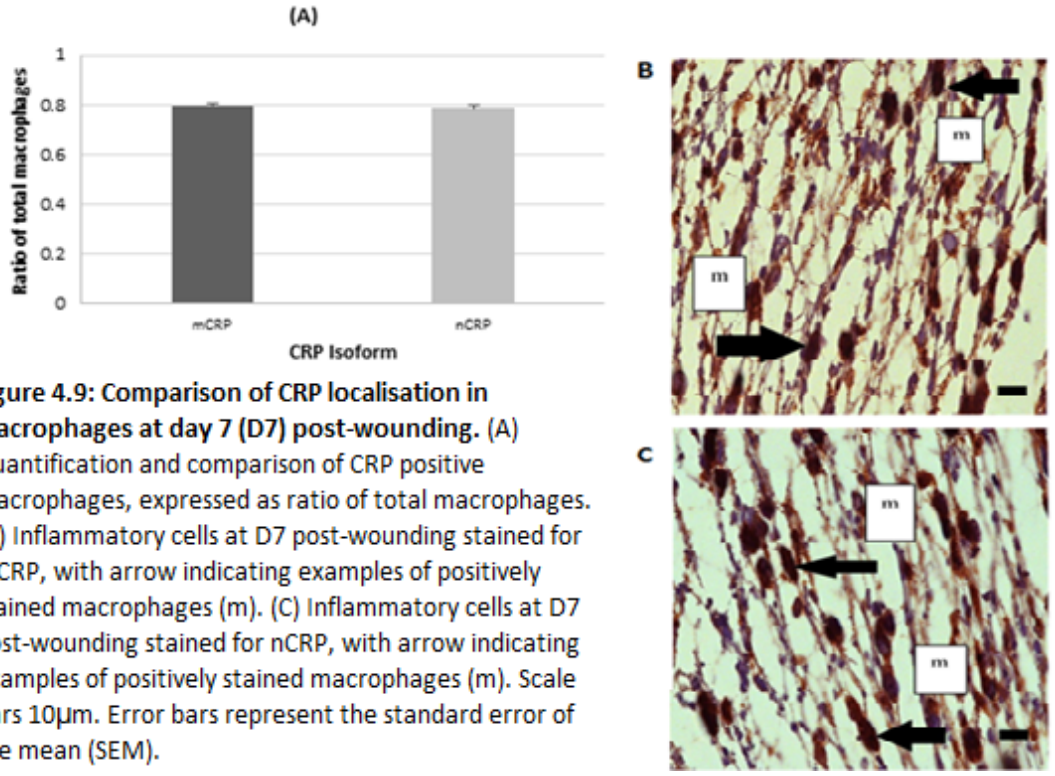



**Figure 4.9: Comparison of CRP localisation in macrophages at day 7 (D7) post-wounding.** (A) Quantification and comparison of CRP positive macrophages, expressed as ratio of total macrophages. (B) Inflammatory cells at D7 post-wounding stained for mCRP, with arrow indicating examples of positively stained macrophages (m). (C) Inflammatory cells at D7 post-wounding stained for nCRP, with arrow indicating examples of positively stained macrophages (m). Scale bars 10µm. Error bars represent the standard error of the mean (SEM).

### 4.7.4 Dual Staining

Dual staining was conducted for the detection of specific cell types at D7 post-wounding at the same time as determining positive CRP isoform staining. Serial sections of wound samples were stained with antibodies specific to the two CRP isoforms as well as a second antibody to stain for cell-specific markers for neutrophils or macrophages. Images taken from each section were overlaid and areas of dual staining were highlighted using Adobe Photoshop image analysis software.

#### 4.7.4.1 Dual staining to Detect CRP in Neutrophils

The dual staining process identified some co-localisation of CRP isoforms in neutrophils at D7 post-wounding (Figures 4.10A and 4.10B). Cell counts were conducted to determine the number of cells positive for only the neutrophil antigen and another count for the number of cells in which both the neutrophil antigen and CRP isoform were detected.

From the total number of inflammatory cells at D7 post-wounding, 22% showed dual detection for mCRP and the neutrophil antigen. This is in comparison to 67% of cells staining positive for mCRP alone and 10% for the neutrophil antibody alone. Similarly, 25% of total inflammatory cells showed dual staining for both nCRP and the neutrophil antigen. This is in comparison to 65% staining positive for nCRP alone and 10% for neutrophil antigen alone. Using dual staining data the ratio of mCRP:nCRP in neutrophils at D7 post-wounding was 0.9. This is in agreement with the measurements obtained by morphological identification of neutrophils (Section 4.7.3.3).

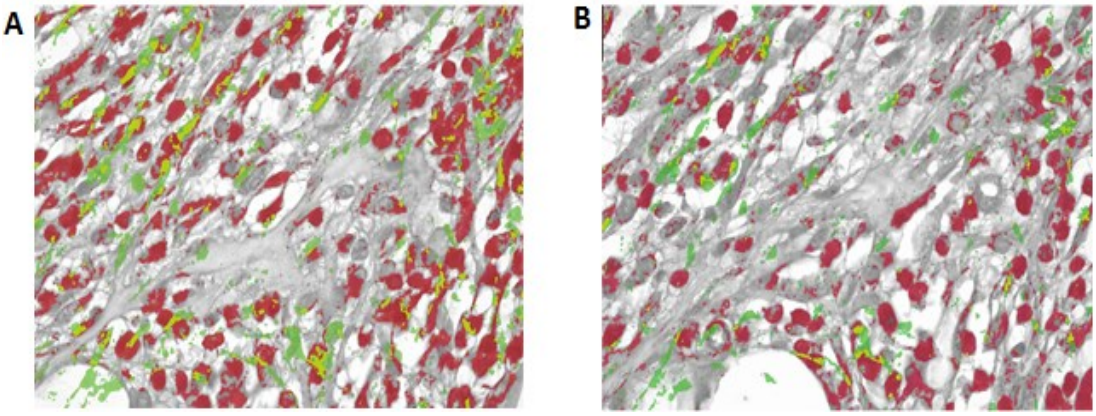



**Figure 4.10: Dual staining of neutrophils and CRP isoforms at day 7 post-wounding.** (A) Staining with mCRP antibody (8C10) (red) overlaid onto an image of staining with an anti-neutrophil antibody (green). Areas of dual staining highlighted (yellow). (B) Staining with nCRP antibody (2C10) (red) overlaid onto an image of staining with an anti-neutrophil antibody (green). Areas of dual staining highlighted (yellow).

#### 4.7.4.2 Dual staining to Detect CRP in Macrophages

The literature suggests the number of macrophages peak at D7 post-wounding. Dual staining with a macrophage antigen specific antibody (Mac3) and CRP specific antibodies identified substantial co-localisation of the macrophage marker and CRP isoforms at D7 post-wounding (Figures 4.11A and 4.11B). Cell counts were conducted to determine the number of cells positive for only the macrophage antigen and another count for the number of cells in which both the macrophage antigen and CRP isoform were detected.

From the total number of inflammatory cells at D7 post-wounding, 53% showed dual detection for the Mac3 marker and mCRP. This is in comparison to 30% of cells staining positive for mCRP alone and 17% for the Mac3 marker alone. Also 41% of the total cells showed dual staining for both nCRP and the Mac3 marker. This is in comparison to 33% staining positive for nCRP alone and 26% for the Mac3 marker alone. Using dual staining the ratio of mCRP:nCRP in macrophages at D7 post-wounding was 1.3.

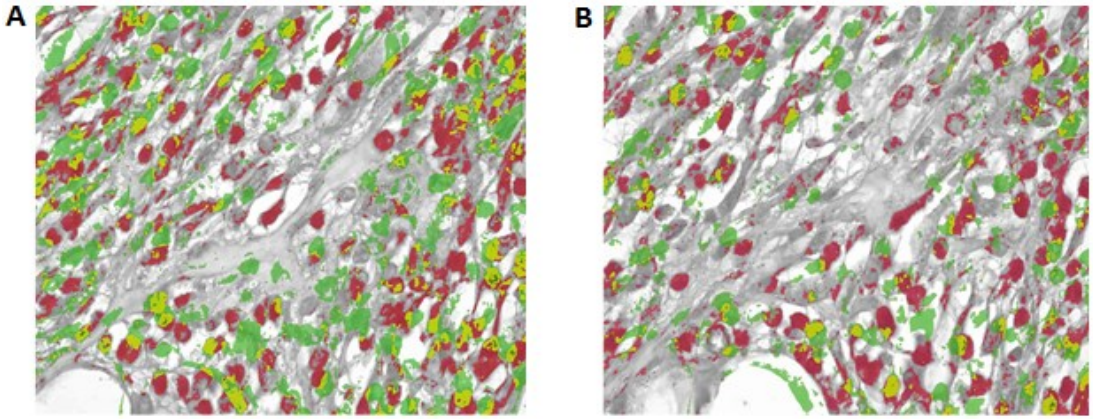



**Figure 4.11: Dual staining of macrophages and CRP isoforms at day 7 (D7) post-wounding.** (A) Staining with mCRP antibody (8C10) (red) overlaid onto an image of staining with an anti-macrophage antibody (green). Areas of dual staining highlighted (yellow). (B) Staining with nCRP antibody (2C10) (red) overlaid onto an image of staining with an anti-macrophage antibody (green). Areas of dual staining highlighted (yellow).

### 4.7.5. Presence of CRP Isoforms During Impaired Healing

#### 4.7.5.1 Comparison of CRP Isoforms During Inflammatory Phase of Acute Wounds in a Model of Age-Related Delayed Healing

This study utilised a well-defined model of impaired wound-healing using ovariectomised (OVX) mice in comparison to non-impaired acute healing found in INTACT mice. This OVX model is known to show increased inflammation, larger wounds and decreased matrix deposition (Ashcroft *et al* 1997). This section of this study analysed the presence of CRP isoforms in both INTACT and OVX wound healing states at day 3 (D3) and day 7 (D7) post-wounding.

#### 4.7.5.1.1. Presence of CRP Isoforms in Non-Impaired (INTACT) Acute Wounds at D3 Post-Wounding.

Counts of inflammatory cells at D3 post-wounding from wound sections of INTACT mice were presented earlier (Figure 4.5). In summary, there was significantly ($p<0.001$) more mCRP staining than nCRP at D3 post-wounding, with mCRP present in 77% of the total inflammatory cells whereas nCRP was only detected in 55% of total inflammatory cells. The ratio of mCRP:nCRP in wounds of INTACT mice at D3 post-wounding was 1.4. The absolute concentration of inflammatory cells staining positive for mCRP and nCRP were 3290 and 2350 cells/$mm^2$ respectively.

#### 4.7.5.1.2 Presence of CRP Isoforms in a Model of Age-Related Impaired Healing at D3 Post-Wounding

The presence of CRP isoforms were analysed in samples of impaired (OVX) healing at D3 post-wounding (Figures 4.12B and 4.12C) with detection levels expressed as a ratio of the total inflammatory cell count (7650 cells/$mm^2$). The data show a significantly ($p=0.013$) higher number of inflammatory cells staining positive for mCRP than nCRP, with 49% staining positive for mCRP compared to 30% for nCRP (Figure 4.12). The ratio of mCRP:nCRP in the model of impaired murine healing at D3 post-wounding was 1.6. The absolute concentration of inflammatory cells staining positive for mCRP and nCRP were 3750 and 2300 cells/$mm^2$ respectively.

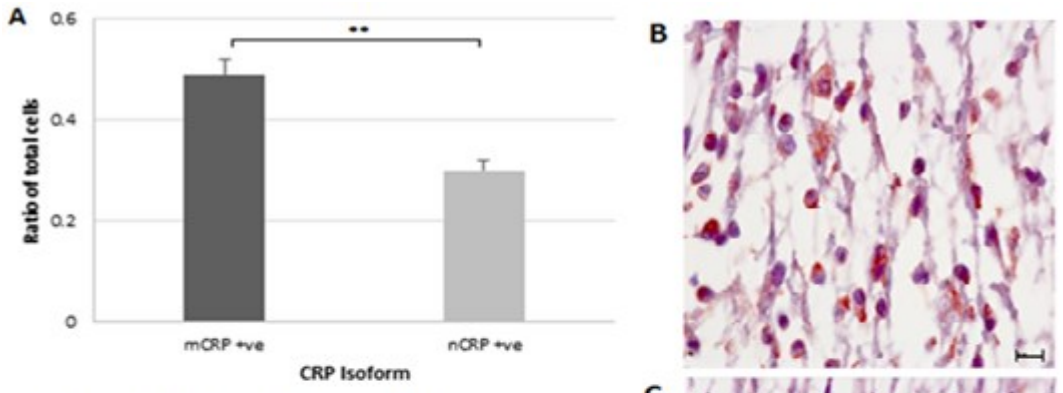



**Figure 4.12: Comparison of staining on inflammatory cells at day 3 (D3) post-wounding in ovarectomised (OVX) mice.** (A) Comparison of positive staining for mCRP and nCRP in OVX mice. (B) Inflammatory cells showing the presence of mCRP (red) within a D3 wound from an OVX mouse. (C) Inflammatory cells showing the presence of nCRP (red) within a D3 wound from an OVX mouse. Scale bars = 10μm. Error bars represent the standard error of the mean (SEM). ** indicates $p<0.01$.

#### 4.7.5.1.3 Presence of CRP Isoforms in a Model of Age-Related Impaired Healing at D7 Post-Wounding

The presence of CRP isoforms were analysed in samples of impaired (OVX) healing at D7 post-wounding (Figures 4.13B and 4.13C) with detection levels expressed as a ratio of the total inflammatory cell count (8630 cells/mm$^2$). The data show a significantly higher (P=0.03) number of inflammatory cells staining positive for mCRP than nCRP, with 37% staining positive for mCRP compared to 24% for nCRP (Figure 4.13A). The ratio of mCRP:nCRP in the model of impaired murine healing at D7 post-wounding was 1.5. The absolute concentration of inflammatory cells staining positive for mCRP and nCRP were 3190 and 2070 cells/mm$^2$ respectively.

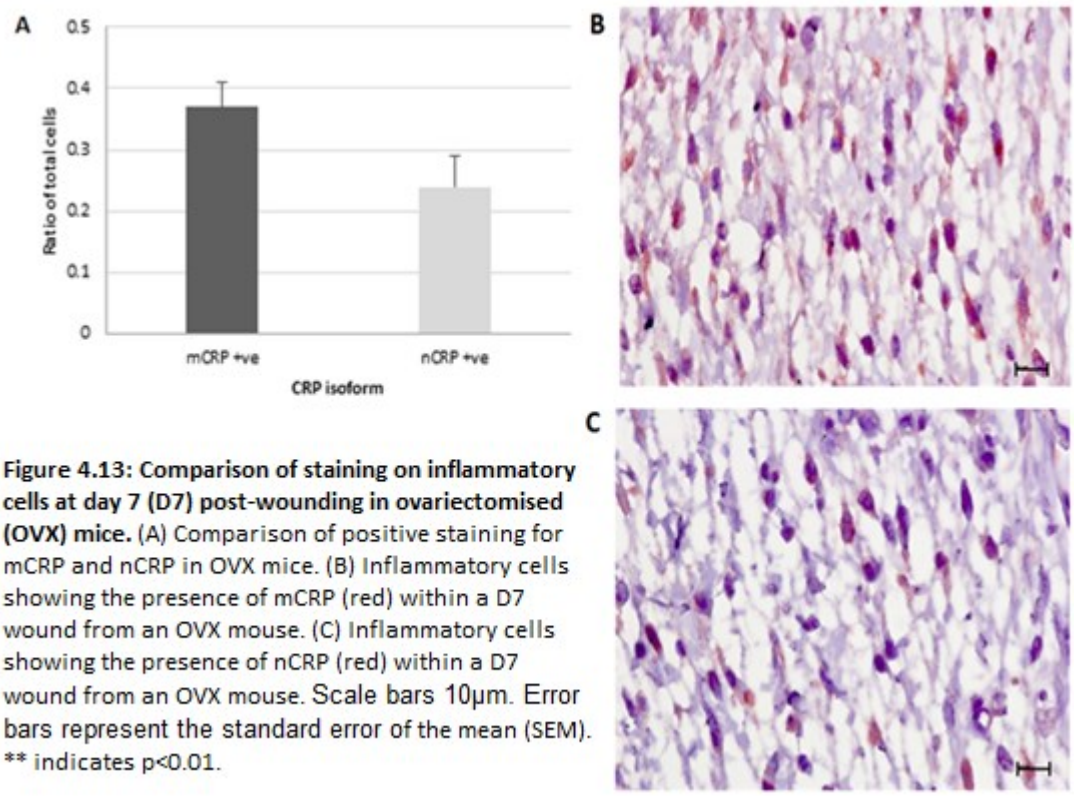



**Figure 4.13: Comparison of staining on inflammatory cells at day 7 (D7) post-wounding in ovariectomised (OVX) mice.** (A) Comparison of positive staining for mCRP and nCRP in OVX mice. (B) Inflammatory cells showing the presence of mCRP (red) within a D7 wound from an OVX mouse. (C) Inflammatory cells showing the presence of nCRP (red) within a D7 wound from an OVX mouse. Scale bars 10μm. Error bars represent the standard error of the mean (SEM). ** indicates $p<0.01$.

### 4.7.6 Comparison of CRP Isoforms in Non-Impaired and Estrogen-Deprived Healing States

The ratio of mCRP:nCRP in the model of impaired murine healing at D3 post-wounding was 1.6 compared to 1.4 found in acute wounds of INTACT mice at D3 post-wounding. This finding suggests a more pro-inflammatory response in the model of impaired healing due to higher relative levels of mCRP. The concentration of positively-staining mCRP reflects this finding, with a significantly higher cell density of mCRP-positive cells (3750 cells/$mm^2$) in OVX wounds than in INTACT wounds (3290 cells/$mm^2$) at D7 post-wounding.

Counts of inflammatory cells at D7 post-wounding from wound sections of INTACT mice were presented earlier (Figure 4.13). In summary, there was no significant difference between mCRP and nCRP staining at D7 post-wounding, with mCRP and nCRP present in 42% and 45% of total inflammatory cells respectively. The ratio of mCRP:nCRP in wounds of INTACT mice at D7 post-wounding was 0.9. The

absolute concentration of inflammatory cells staining positive for mCRP and nCRP were 2100 and 2250 cells/mm$^2$ respectively.

The ratio of mCRP:nCRP at D7-post wounding in model of impaired healing was 1.5 compared to 0.9 found in acute wounds of INTACT mice at D7 post-wounding. These findings suggest a more pro-inflammatory response in the model of impaired healing due to higher relative levels of mCRP. The concentration of positively-staining mCRP reflects this finding, with a significantly higher cell density of mCRP-positive cells (3190 cells/mm$^2$) in OVX wounds than in INTACT wounds (2250 cells/mm$^2$) at D7 post-wounding.

## 4.8 Discussion

### 4.8.1 C-Reactive Protein Isoforms and Inflammation

The role of c-reactive protein (CRP) and its isoforms has not been fully elucidated in the terms of wound healing and inflammation. Previous papers have agreed that CRP is a marker of inflammation and that there is some function being performed however there is much debate over that function with arguments for both anti-inflammatory and pro-inflammatory effects. The two isoforms have been shown to have both pro-inflammatory and anti-inflammatory effects upon numerous cells types including neutrophils and monocytes as well as other cells that have particular roles in the wound healing and inflammation processes (Khreiss *et al*, 2002; Khreiss *et al,* 2004; Khreiss *et al,* 2005; Eisenhardt *et al,* 2009).

The results of this study have indicated a temporal and spatial profile of both nCRP and mCRP during in both early and late inflammatory stages as well as in non-impaired and impaired wound profiles. These results have indicated that both mCRP and nCRP are present in the inflammatory cells of murine wounds with significantly differing levels observed in the various cell types and time points.

It has been well established that levels of CRP can increase up to 1000 fold on the onset of inflammation hence why it is considered an acute marker of inflammation and is used for investigative purposes in cardiovascular disease (Eisenhardt *et al,* 2009). Thus, there are numerous studies on CRP levels and the risk of cardiovascular events and very little on other biological effects of the two isoforms including inflammation processes, such as nitric oxide release and phagocytosis (Eisenhardt *et al,* 2009).

#### 4.8.1.1 The Effect of CRP Isoforms in the Early Inflammatory Stages

The inflammatory process in wound healing follows a set of predetermined stages. Neutrophils are always the first cell type that enters the wound site via diapedesis (Kapalanski *et al,* 2003) with numbers increasing dramatically over 12-24 hours and peaking at around day 3 (D3) post-wounding. This study provides evidence that at 12 hours post-wounding around 19% of the inflammatory cells within the wound stained positively for mCRP and 15% stained positively for nCRP, with no significant difference ($p=0.1165$) in the levels of detection between the two CRP isoforms. By 24 hours this had increased to 45% of cells being positively stained for mCRP and 40% being stained positively for nCRP with no significant difference ($p=0.2393$) being detected between the two isoforms. The increase between the two time points however was significant for both isoforms ($p<0.001$).

The results at day 3 (D3) post-wounding showed a different effect. Positive staining of mCRP was detected in 77% of inflammatory cells which was significantly ($p<0.001$) higher than the proportion of cells staining for nCRP (55%). Zouki *et al* (1997) stated that both CRP isoforms can downregulate L-selectin (CD62L) detection in neutrophils, of which L-selectin is expressed on leukocytes to mediate in leukocyte capture and rolling (Nagaoka *et al*, 2000). During activation L-selectin is shed leading to the up-regulation of Mac-1 (CD11b/CD18). This causes the enhancement of adhesion and trans-endothelial migration of neutrophils via their endothelial counter-ligands ICAM-1 and ICAM-2 (Zouki *et al,* 2001). The mCRP

isoform can increase the detection of Mac-1 and ICAM-1 (Khreiss *et al* 2004; Zouki *et al* 2001). This would suggest that high levels of mCRP in the day 3 (D3) post-wounding cells would increase adhesion molecule expression on the cell surface of neutrophils, leading to the increased efficiency of the neutrophil transmigration.

#### 4.8.1.2 The Effect of CRP Isoforms in the Late Inflammatory Stages

At day 7 (D7) post-wounding there was a significantly higher inflammatory cell count than that seen at day 3 (D3), which is not unexpected in the inflammatory process. However, the amount of positive staining for each of the CRP isoforms significantly reduced during this time. The amount of mCRP staining decreased by 49% and the nCRP reduced by 24% during this period. This may be due in part to the change in cell type between the 2 time points, with the apoptosis of neutrophils (Li *et al,* 2007) following their peak at D3 and the subsequent recruitment of tissue macrophages (Kaplanski *et al,* 2003). This change is due to a change in the expression profiles of cytokines and chemokines during the inflammatory process; usually caused by an increase in macrophage inflammatory protein-1 and monocyte chemoattractant protein-1 (MCP-1) (Kaplanski *et al,* 2003; Dipietro, 1995).

At day 7 (D7) post-wounding, the data indicated that around 80% of the inflammatory cells present had positive staining for both isoforms. These results show that some inflammatory at D7 are capable of simultaneously producing both CRP isoforms. Such co-localisation could possibly mediate the chemotaxis of monocytes (Torzewski *et al,* 2000) or the inhibition of neutrophil chemotaxis (Khreiss *et al,* 2002) noted at this time point.

The differentiation of monocytes to tissue macrophages is a process that is mediated by the release of interleukin-4 (IL-4) during tissue injury (Mosser & Edwards, 2008) and activation of the macrophages can occur by various agents. Macrophage-activating agents can include neutrophils, red blood cells, bacterial antigens as well as platelet derived factors, chemokines and cytokines, especially MCP-1, IL-1 and IFN-γ (Dipietro, 1995). Tissue debris consisting of extracellular

matrix components such as fibronectin and collagen can also activate wound macrophages (Dipietro, 1995).

Most published research to date does not take into account the two CRP isoforms and their differential biological functions, but some roles are beginning to emerge. It has been indicated that the mCRP isoform could be a potential stimulant for the activation of monocytes to phagocytic macrophages when combined with the activation of Mac-1 (Eisenheardt *et al,* 2009). It has also been suggested that mCRP is a potent inducer of reactive oxygen species (Eisenhardt *et al,* 2009). This elucidates a further role of mCRP in the phagocytic process. A further pro-inflammatory response of mCRP is reported that mCRP has the ability to increase monocyte adhesion to a fibrinogen matrix *in vitro* (Eisenhardt, 2009). Eisenhardt *et al* (2009) also explained that the nCRP isoform could partially inhibit the effects of mCRP on monocytes and suggested that this was due to a regulatory attenuating function caused by the pro-inflammatory effects of mCRP. This is also indicated in this study since at day 7 (D7) in acute INTACT wounds, the levels of the CRP isoforms were similar (mCRP at 42% and nCRP at 45%). Inflammation peaks and then subsides after Day 7, suggesting that the pro-inflammatory effects of mCRP decline its level becomes similar to that of the nCRP (i.e. ratio of mCRP:nCRP becomes close to 1). Beyond this point (ratio less than 1) the anti-inflammatory effects of the nCRP would start to come into effect. This difference in biological function could also be suggested due to the fact that both isoforms bind to separate receptors (mCRP binds CD16 whereas nCRP binds CD32 and CD64) (Khreiss *et al* 2002). These differing levels of CRP isoforms at different time points post-wounding suggest that CRP isoforms may simultaneously work together in playing a role in the inflammation process.

### 4.8.2 C-Reactive Protein Isoforms and Model of Age-Related Impaired Healing

Previous studies have shown that age is a factor effecting CRP levels in the body indicating that the CRP isoforms have the potential to be involved in the impairment of the normal wound healing process (Gomez *et al*, 2005). Impaired healing is defined as healing that has failed to progress throughout the normal stages of healing within the usual timeframes and is usually accompanied by an altered and pronounced inflammatory profile which tends to be pro-inflammatory in nature (Lazarus *et al,* 1994).

This study analysed the detection of the CRP isoforms in an established model of age-related impaired murine healing using mice that have undergone ovariectomy (OVX) prior to wounding (Ashcroft *et al, 1997*).

The ratio of mCRP:nCRP should be considered when analysing the effects of the CRP isoforms on the wound healing process. The mCRP isoform is considered to be more pro-inflammatory (Khreiss *et al,* 2004) than the nCRP isoform. In the age-related impaired (OVX) model of healing, both time points indicated a higher mCRP:nCRP ratio and therefore a higher number of mCRP-positive cells than nCRP-positive cells. This high ratio at both D3 and D7 indicates that the inflammation in impaired healing is excessive and prolonged, while corresponding wounds from INTACT mice have a lower ratio of mCRP:nCRP indicating fewer cells with the pro-inflammatory CRP isoform. It can be suggested that in the INTACT wounds this lower ratio of the two CRP isoforms leads to a resolution of inflammation, thus allowing the wound to heal more rapidly and successfully (Eming *et al*, 2007).

The exact role of the CRP isoforms have not been fully investigated to date. However, this study has shown for the first time that both CRP isoforms are located to inflammatory cells of murine wounds and that levels change with time post-wounding, cell type and the effects of aging. These data indicate that the CRP isoforms may have a role in the inflammation process that needs further investigation.

## 4.9 Summary & Future Work

This study has shown that both CRP isoforms are present in inflammatory cells throughout the inflammatory phase of acute wound healing. Furthermore, differential staining in a model of age-related impaired healing suggests the ageing process may affect the profile of CRP isoforms present in wounds. Future work may include further characterisation of CRP isoforms in murine wounds to pinpoint the precise cell types and cellular structures involved. Techniques such as dual fluorescence microscopy or immunogold labelling could be adopted to enable staining of multiple proteins on a single wound section and confirm the co-localisation of CRP isoforms with specific cell types present in wound sections.

No evidence has been presented to show what role the CRP isoforms may play during the inflammatory phase of wound healing. However, this work has provided a basis to consider the effects of both CRP isoforms on models of wound healing and inflammation processes. It is speculated that the relative amounts of each CRP isoform may mediate inflammation and that a shift in this balance may contribute to impaired healing states and pronounced inflammation. The next steps would be to investigate the effect of CRP isoforms on a range of processes such as cell proliferation, cell death, nitric oxide and cytokine production and phagocytosis.

Finally, this work should be repeated using human acute wounds taken from young and elderly subjects to determine whether the findings from this murine model can be extrapolated to healing in humans. Moreover, confirmation of an altered CRP isoform profile in chronic compared to acute human wounds together with data showing distinct biological roles for CRP isoforms could provide tantalising evidence to implicate CRP isoforms in chronic wound pathology. Such findings could ultimately elucidate novel and focused therapeutic strategies for the treatment of chronic wounds.

# Chapter 5: The Effect of C-Reactive Protein Isoform on Growth and Viability in Monocytes and Macrophages

## 5.1 Introduction

### 5.1.1 Cell Proliferation and Recruitment

The cell cycle is a heavily regulated process which involves many different components. Cell proliferation is an increase in the number of cells through the regulation of cell division, death and differentiation (van den Heuvel, 2005).

Cell division is made up of four main phases which need to be completed in order for cells to divide. This process takes roughly 24 hours for many mammalian cells. The $G_1$ phase (Gap phase) prepares for deoxyribonucleic acid (DNA) replication by synthesising the many enzymes that are needed for the process. The S phase (synthesis phase) allows for the replication of DNA and the packaging of DNA so that the chromosomes become segregated (Scholey *et al*, 2003). This is followed by the $G_2$ phase where protein synthesis occurs, including the production of microtubules to help with mitosis. During this gap phase the cell proof-reads the DNA to ensure proper replication and prepares for division (Park & Koff, 1998; Alberts *et al,* 2008).

The fourth phase, M phase (Mitosis phase), consists of karyokinesis and cytokinesis where nuclear and cytoplasmic division occur resulting in the formation of a new cell membrane. The cells then enter into $G_0$ which is a rest phase until the cell receives growth-promoting signals or pro-differentiation signals (Scholey *et al*, 2003).

This cell cycle is strictly controlled by cyclin-dependent kinases (Cdk) which turn specific proteins on and off through phosphorylation during the different phases of the cell cycle. Various cyclins are produced during the process and when it becomes phosphorylated it forms a cyclin-Cdk complex which then activates the production of the next cyclin (van den Heuvel & Harlow, 1993). The process can be

inhibited by proteins such as p16, p21 and p27 which bind directly to the Cdk-cyclin complexes and inhibit their protein kinase activity (Scholey *et al*, 2003).

The process of proliferation allows for cells to be replaced when they have been lost to injury or death. Cell death can occur in one of two main ways; necrosis or apoptosis.

### 5.1.2 Apoptosis

Cell death is part of the proliferation of cells. Cells that are damaged or ageing need to be removed from the host to retain the balance of cells. All cells can commit a programmed cell death which is termed apoptosis. This is different to necrosis where cell death occurs following injury or inflammation, causing the cell membrane to become disrupted and the release of intracellular contents into the extracellular environment.

In apoptosis, cell death is highly regulated. The cells shrink in size as the cell condenses down (pyknosis), the cytoskeleton of the cell collapses, the nuclear envelope disassembles and nuclear DNA fragments (karyorrhexis) (Elmore, 2007). This allows the cell contents to accumulate in apoptotic bodies in a process called budding. These bodies are then phagocytosed, taken up into phagosomes and degraded (Elmore, 2007). This process allows the cells to die without inducing an inflammatory response since all the intracellular components are recycled by phagosomes (Elmore, 2007).

Apoptosis relies upon a family of proteases called caspases. These proteases have cysteine in the active site that is able to cleave proteins at specific aspartic acids. These caspases are synthesised as an inactive precursor termed procaspases which remain inactive until cleaved at the aspartic acids by another caspase. Once activated, these caspases will cleave other procaspases to amplify the proteolytic cascade (McIlwain *et al,* 2015). These caspases can also cleave cell proteins and nuclear lamina. These processes cleave the proteins that are responsible for

keeping DNAse inactive, meaning that it is released and able to fragment the DNA in the nucleus (McIlwain *et al*, 2015).

Apoptosis is a complete and irreversible process which is self-amplifying and destructive. In the early phases of apoptosis, procaspases are activated by adaptor proteins which brings initiator procaspases into close proximity to one another to form a complex to then be activated by signals from the death receptors on the cell surface. This triggers the recruitment of intracellular adaptor proteins which bind and aggregate procaspase-8 in order for it become cleaved and activated. This capsase-8 can then activate downstream procaspases to induce apoptosis (McIlwain *et al*, 2015).

The death receptor, Fas, is expressed in stressed or damaged cells in order to trigger the caspase cascade and induce apoptosis (Elmore, 2007). DNA damage can also trigger apoptosis through p53; a tumour suppressor gene that is able to activate apoptosis that encodes a transcription factor which can bind DNA in a sequence-specific manner (Ko & Prives, 1996). P53 has control of both the intrinsic and extrinsic pathways of apoptosis and functions by transcribing the gene that encodes the protein responsible for the release of cytochrome C from the mitochondria. These proteins belong to the Bcl-2 family, which includes *Bax*, *Bad* and *Bak* (Fridman & Lowe, 2003).

The members of the Bcl-2 family function in various ways. Some, like *Bad*, are promotors of procaspase activation in order to promote apoptosis while others are death inhibitors. *Bad* will bind to these death inhibiting members of the family while *Bax* and *Bak* stimulate cytochrome C being released by the mitochondria, a step which is crucial for the induction of apoptosis (Amaral *et al,* 2010).

There are many caspases that are involved with this process and each of them have a specific role based upon their biochemical features and role they play in the process. There are ten major caspases which can be split into three main groups. Caspases -2, -8, -9 and -10 are initiators of the apoptotic process; caspases -3, -6 and -7 are effectors and executioners and caspases -1, -4 and -5 are

inflammatory. There are others that have a less defined role in the process. For example, caspase-11 regulates apoptosis and cytokine maturation in instances of sceptic shock; caspase-12 mediates endoplasmic specific apoptosis and caspase-14 is expressed only in embryonic tissues (Elmore, 2007).

Apoptosis is one of the main regulators of the cell cycle. It helps to prevent against cancer by inhibiting the growth of tumour cells but also to remove damaged cells that are no longer useful in the inflammatory process.

### 5.1.3 C-reactive Protein, Cell Proliferation and Apoptosis

There is little research that has been conducted into the effect of C-reactive protein (CRP) on the proliferation process. However there is evidence that CRP has a major role in the apoptosis process.

Firstly, Kim *et al* (2014) suggested that CRP produces pro-apoptotic cytokines and inflammatory mediators which cause the activation of Fc-γ receptors. These receptors allow monocytes, macrophages and vascular smooth muscle cells to produce interleukin-1β (IL-1β) and tumour necrosis factor-α (TNFα) which are pro-apoptotic and lead to the formation of atherosclerotic plaques. This also activates reactive oxygen species (ROS) which activates NADPH oxidase (Nox) and subsequently reactive oxygen species (Ryu *et al,* 2007).

The upregulation of p53 in monocytes is also regulated by CRP which allows for the transcription of proteins that promote apoptosis. It was suggested that CRP affects the cell cycle kinetics of monocytes through CD32 (FcγRII) and therefore triggering the resulting cascade. This induces $G_2$/M arrest in the cell cycle which results in apoptosis (Kim *et al,* 2014).

These CD32 receptors have been shown to trigger apoptotic signals in granulocytes and eosinophils and is expressed in a subset of monocytes that have been shown to polarise to pro-inflammatory macrophages. The results of these studies would suggest that the functional activity of CRP may alleviate macrophage

driven pro-inflammatory responses (Kim *et al*, 2014). CRP bound apoptotic monocytes and macrophages could be potentially removed via FcγR mediated phagocytosis. However CRP can trigger lots of signalling pathways so it cannot be assumed that the consequences are exclusive to CRP signalling (Kim *et al*, 2014).

CRP is elevated in cardiovascular disorders and has is a mediator of atherosclerosis. CRP localises directly in the atherosclerotic plaques where it induces the expression of genes that directly involved in the adhesion of monocytes and the recruitment of intracellular molecules such as E-selectin and monocyte chemoattractant protein-1. CRP has also been shown to play a role in the mediation of low density lipoprotein uptake in macrophages and activating the complement system which plays a role in atherogenesis (Torzewski *et al,* 2000). Apoptosis occurs in these atherosclerotic plaques and the number of apoptotic cells increase as the lesion becomes more advanced. As cells become apoptotic they start to cause plaque disruption. This disruption leads to the expression on growth arrest- and DNA damage-inducible gene 153 (*GADD153*) by cells. *GADD153* can induce $G_1$ arrest or can induce apoptosis in some cancer cell lines (Guyton *et al*, 1996).

Blaschke *et al* (2004) found that CRP can induce the apoptosis of human coronary vascular smooth muscle cells through a caspase-mediated mechanism especially through increased capase-3 activity. CRP was co-localised to the *GADD153* gene in atherosclerotic lesions suggesting that CRP is triggering the caspase cascade by inducing the expression of the *GADD153* gene, and therefore inducing apoptosis.

There is little research on how the two isoforms of CRP interact with the apoptosis process. It is suggested that CRP can exert anti-apoptotic activity but only when the cyclic pentameric structure is lost. This would suggest that the majority of the apoptotic activity of CRP is caused through the native isoform. Apoptotic can be delayed by activating the CD16 receptors on the cell. Native CRP (nCRP) can bind to low affinity IgG FcγRIIa (CD32) and IgG FcγRI (CD64) which leads to depressed functional activities, degranulation and the generation of superoxide by inducible respiratory burst. On the other hand mCRP binds to low affinity IgG FcγRIIIb (CD16)

which is the receptor that can delay apoptosis by triggering cell survival pathway in neutrophils, even at low concentrations (Khreiss *et al,* 2002).

Native CRP does not repress apoptosis in neutrophils (Khreiss *et al,* 2002). However it can bind to the surface of intact apoptotic cells. C-reactive protein has the ability to opsonise apoptotic cells and induce the phagocytosis of these damaged cells. CRP binds to apoptotic cells in calcium dependent manner and trigger the classical complement pathway (Gershov *et al*, 2000). This results in protection against the assembly of terminal complement membrane complex and promotes a non-inflammatory clearance of cells (Khreiss *et al,* 2002).

## 5.2 Aims

The aim of this investigation was to analyse the effects of various concentrations of exogenous CRP isoforms on the growth and viability of monocyte/macrophage models of inflammation. The investigation also established dose response data to guide future work in terms of optimal CRP concentrations and treatment times. Finally, the investigation assessed whether any reduction in viability was due to apoptosis and whether this could be reversed using pharmacological inhibitors of CRP-mediated pathways.

## 5.3 Objectives

The fundamental objectives of this assay were:

- To analyse the effect of treatment time and concentration of exogenous nCRP on the growth and viability of monocytes/macrophages
- To analyse the effect of treatment time and concentration of exogenous mCRP on the growth and viability of monocytes/macrophages
- To determine if a reduction in cell viability was due to apoptosis by detecting genomic DNA fragmentation.
- To determine if apoptosis could be reversed with the use of pharmacological inhibitors of pathways mediated by CRP.

## 5.4 Summary of Procedure

### 5.4.1 Growth and Viability Assay

This assay utilised a dye exclusion method using trypan blue dye. Cells were treated with a range of concentrations and incubation times for each CRP isoform. Following treatments, cells were treated with trypan blue dye to assess viability. The semi-permeable cell membranes of viable (living) cells did not take up the trypan blue dye and appeared colourless by microscopy. However, the permeable cell membrane of non-viable (dead) cells took up the trypan blue dye and appeared blue by microscopy. An automated cell counter was used to obtain the total cell count and a viable cell count based upon the amount of trypan blue staining. This method measured cell death induced by CRP isoform treatments but could not provide information as to whether the cell death occurred as a result of apoptosis or necrosis.

### 5.4.2 Apoptosis Assay

An apoptosis assay was utilised to determine whether apoptosis was occurring in the non-viable (dead) cells determined by the trypan blue staining method. The apoptosis assay relied upon the detection of genomic DNA fragmentation via gel electrophoresis however this method differs slightly from others mentioned in literature in that DNA fragmentation was visualised as smeared bands across the gel rather than individual detectable bands. This indicated the presence of multiple sized DNA fragments and provided evidence for apoptosis. A single (unfragmented) band of genomic DNA indicated the absence of DNA fragmentation and apoptosis, suggesting that cell death (if present) was instead likely to be due instead to necrosis.

## 5.5 Materials

### 5.5.1 Growth and Viability Assay

Human monocyte cell line U937 *(Health Protection Agency Culture Collections, Salisbury)*

RPMI-1640 Media *(Lonza, Slough)*

Foetal Bovine Serum *(Sigma-Aldrich, Dorset)*

Lipopolysaccharide *(Sigma-Aldrich, Dorset)*

Phorbol 12-myristate 13-acetate *(Applichem, Darmstadt)*

Trypsin *(Thermo Fisher Scientific, Loughborough)*

C-reactive Protein, human >95% native, 5mg/ml (*MyBioSource, San Diego)* – Purified as previously stated in Section 2.4.4.

Inhibitors – made up as mentioned in section 2.4.9.

### 5.5.2 Apoptosis Assay

Camptothecin *(Sigma-Aldrich, Dorset)*

Quick Apoptotic DNA Ladder Detection Kit *(Biovision, San Francisco)*

TE Lysis Buffer

Enzyme A Solution

Enzyme B, dissolved in distilled water

Ammonium Acetate Solution

DNA suspension buffer

Phosphate Buffered Saline (PBS) *(Sigma-Aldrich, Dorest)*

70% Ethanol *(Sigma-Aldrich, Dorest)*

### 5.5.3 Gel Electrophoresis

Midori Green *(Geneflow, Lichfield)*

1.2% Agarose Gel *(Melford, Suffolk)*

Electrophoresis Gel tank *(Bio-Rad, Hertfordshire)*

TBE buffer

900mM Tris-base *(Fisher Scientific, Cheshire)*

890mM Boric acid *(Fisher Scientific, Cheshire)*

16mM Ethylenediaminetetraacetic acid (EDTA) *(Sigma-Aldrich, Dorset)*

Bio-Rad ChemiDoc™ Touch Imaging System *(Bio-Rad, Hertfordshire)*

## 5.6 Methods

### 5.6.1 Cell Culture

Cells were cultured as previously stated in Section 2.4.1.

### 5.6.2 CRP Purification

CRP was purified as stated in Section 2.4.4. The samples were then diluted to experimental concentrations using RPMI-1640 media.

### 5.6.3 Growth and Survival Assays

The CRP isoforms were analysed to determine their effects on the growth and survival of U937 cells over a set of treatment times and CRP isoform concentrations. Concentrations of mCRP were 1µg/ml, 5µg/ml, 10µg/ml, 25 µg/ml, 50 µg/ml, 100µg/ml and 250 µg/ml and concentrations of nCRP were 100 µg/ml, 250 µg/ml, 500µg/ml and 1000µg/ml. A negative control was set up of just media treatment to create a baseline. Three replicates of each CRP concentration were set up in cell culture wells with a final cell density of $1x10^6$ viable cells/ml and incubated for a range of time periods (0.5, 1, 3, 6, 15 or 24 hours) at 37$^oC$ and 5% $CO_2$. Following incubation each individual well was analysed using 0.4% trypan blue in a 1:1 ratio of cells to trypan blue and cell counts measured on a Biorad TC10 Automated Cell Counter. Cell counts were taken twice from each well to collect six separate counts for each CRP isoform concentration.

### 5.6.4 Cell Activation

Some U937 cells were activated with 0.05mg/ml lipopolysaccharide (LPS) for a period of 24 hours as a model of bacterial infection before conducting the growth and survival assay (Section *5.6.3)*.

### 5.6.5 Cell Differentiation

Some U937 cells were differentiated into macrophage-like cells using 50ng/ml phorbol-12-myristate 13-acetate (PMA) for 72 hours to model tissue macrophages before conducting the growth and survival assay (Section 5.6.3).

### 5.6.6 Quick Apoptotic DNA Ladder Detection Kit

The cells were collected after a 24 hour treatment (as described in Section 5.6.3) with either media (negative control), camptothecin (apoptosis positive control), 250µg/ml mCRP (mCRP sample), 1000µg/ml nCRP (nCRP sample), or equivalent concentrations of BSA (non-specific protein controls). Cell counts were obtained from each sample as described in Section 5.6.5. The cells were then centrifuged and re-suspended in RPMI-1640 at a concentration of 1 million total cells. Samples were then centrifuged at 1000rpm for 7 minutes and the supernatant removed. The pellet was washed in PBS and then centrifuged again for 5 minutes at 500xg. The supernatant was removed and 35µl of TE lysis buffer was added to the pellet with 5µl of Enzyme A. Following a 10 minute incubation at 37$^{o}$C and 5% $CO_2$ 5ul of Enzyme B was added and incubated for 30 minutes at 50$^{o}$C. This stage was followed by the addition of 5µl of Ammonium Acetate and 50µl of isopropanol before being stored at -20$^{o}$C for 10 minutes. The sample was centrifuged for 5 mins at 500xg to precipitate the DNA. The supernatant was removed and the resulting pellet was washed with 0.5ml 70% ethanol. The trace ethanol was removed and the pellet was allowed to air dry for 10 minutes. The pellet was finally dissolved in 100µl of DNA suspension buffer prior to gel electrophoresis.

### 5.6.7 Gel Electrophoresis

A 1.2% agarose gel containing Midori green was prepared and 40µl of sample was loaded into separate lanes of the gel. The gel was run at 15v/cm. The gel was imaged using the Bio-Rad ChemiDoc$^{TM}$ Touch Imaging System.

### 5.6.8 Statistical Analysis

Graphs were drawn using Microsoft Excel 2013 and all statistics performed using IBM SPSS Statistics (Version 22). The Shapiro-Wilkes normality test was conducted on the data and t-tests or Mann-Whitney tests were conducted upon all the data. Images from the Bio-Rad ChemiDoc™ Touch Imaging System were analysed using the Bio-Rad Image Lab Software (Version 5.2.1).

## 5.7 Results

### 5.7.1 The Effect of CRP Isoforms on the Growth of Monocytes and Macrophages Over 24 Hours

The average growth of the unactivated monocytes after treatment with mCRP was plotted against time. There was a general trend of increased growth over time (Figure 5.1), with (on average) a 40% growth observed between 6 and 24 hours for concentrations below 50µg/ml mCRP. Treatment with 250µg/ml mCRP resulted in a decline in cell number from the initial number of viable unactivated monocytes, as represented by a negative percentage and suggesting cell death had occurred.

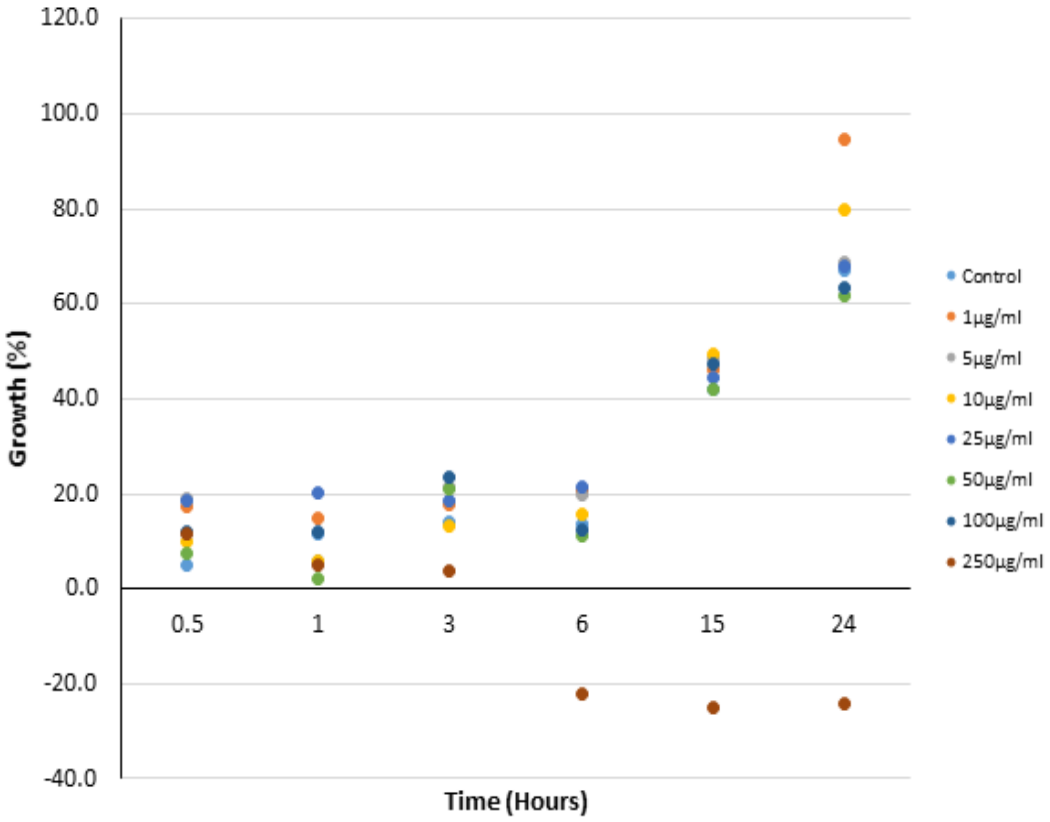



**Figure 5.1 – The effect of mCRP on unactivated monocytes over 24 hours.** Negative percentage represents a decrease in the number of cells compared to the initial viable count used in the assay.

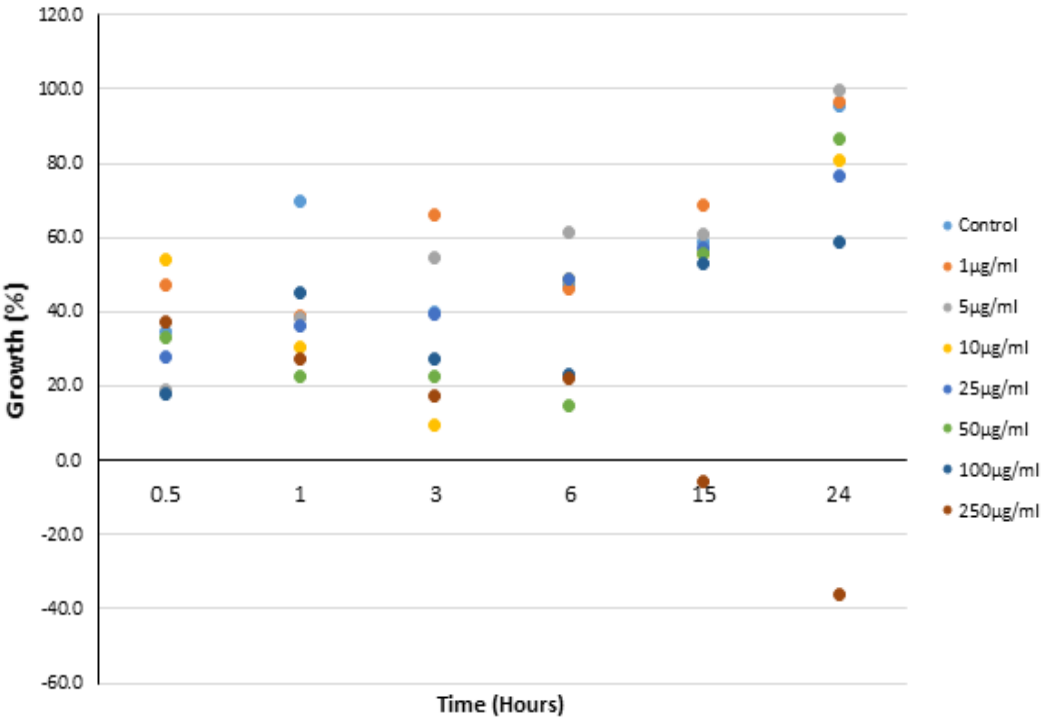



**Figure 5.2 – The effect of mCRP on activated monocytes over 24 hours.** Negative percentage represents a decrease in the number of cells compared to the initial viable count used in the assay.

All concentrations exhibited positive cell growth until the 15 hour time point. The 250µg/ml mCRP treatment diverged from the trend, causing a decrease in viable LPS-activated monocytes below the initial number used to set up the assay, suggesting net cell death at 15 hours and particularly at 24 hours

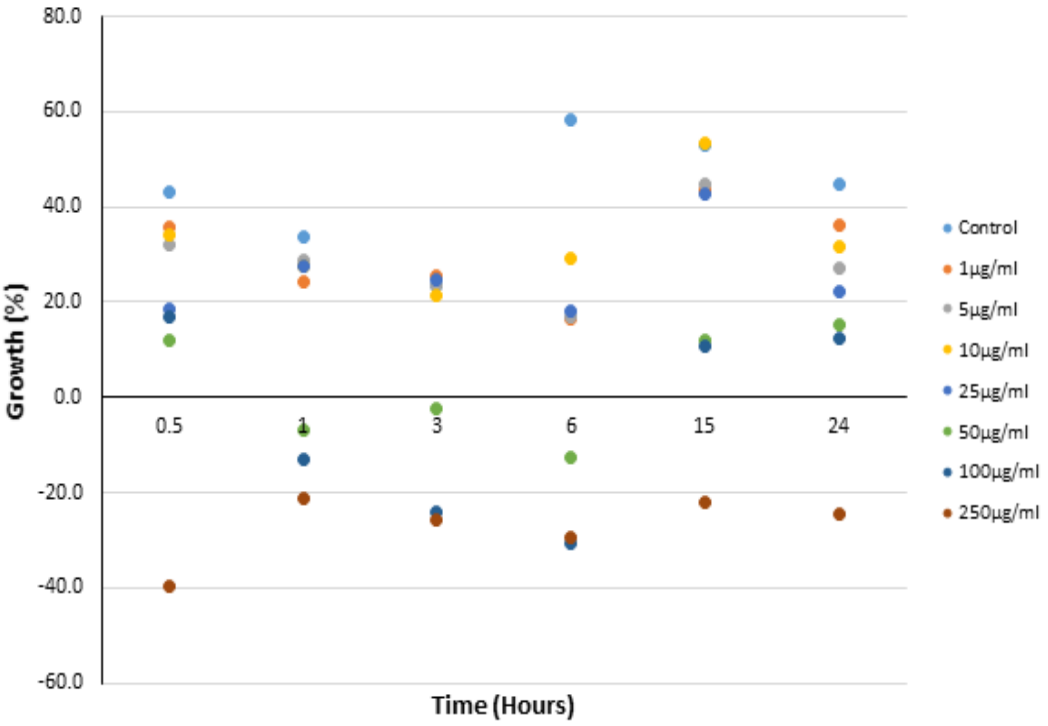



**Figure 5.3 – The effect of mCRP on unactivated macrophages over 24 hours.** Negative percentage represents a decrease in the number of cells compared to the initial viable count used in the assay.

Figure 5.3 shows that 250µg/ml mCRP treatment reduced the growth of unactivated macrophages from the outset, with negative growth indicating net cell death from the initial cell density used to set up the assay. Between 1 hour and 6 hours both the 50µg/ml and 100µg/ml treatments also caused negative cell growth of unactivated macrophages. However positive growth was observed from 15 hours onwards indicating recovery of cell growth at the later time points. All the other treatments with concentration below 50µg/ml retained positive growth across all time points.

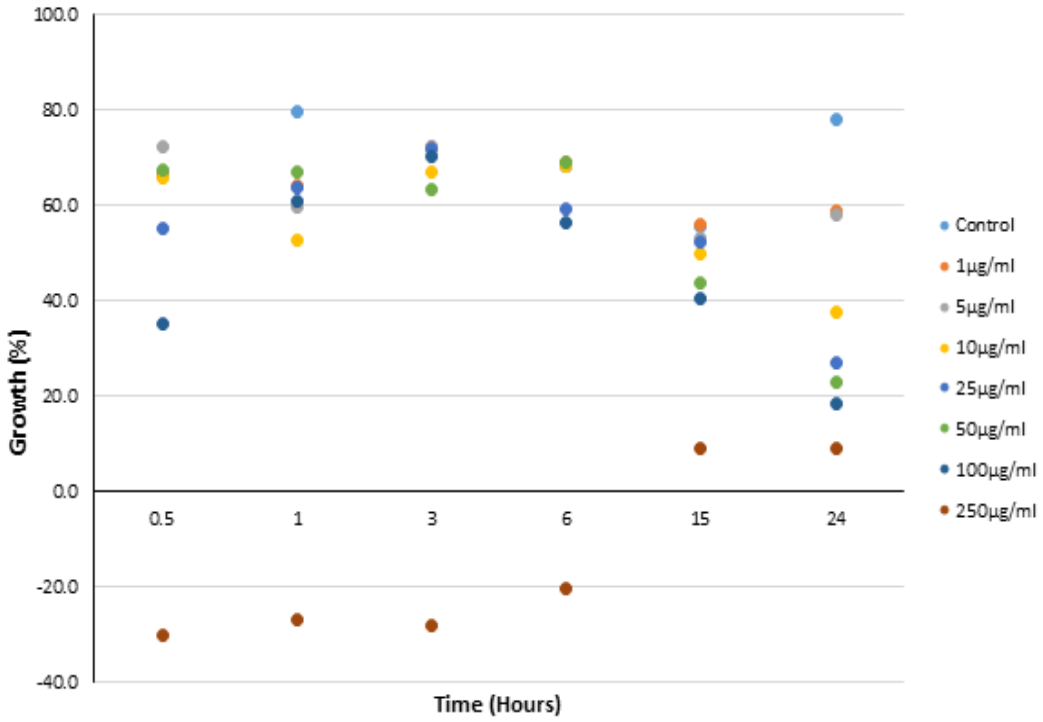



**Figure 5.4 – The effect of mCRP upon activated macrophages over 24 hours.** Negative percentage represents a decrease in the number of cells compared to the initial viable count used in the assay.

Figure 5.4 indicates that treatment with 250µg/ml mCRP resulted in a decrease in activated macrophages from the outset, with negative growth indicating net cell death until and including the 6 hour time point. However at the 15 hour and 24 hour time points there was recovery in cell growth with positive growth observed with 250µg/ml mCRP. The other concentrations of mCRP below 250µg/ml for the most part showed positive but typically declining growth of activated macrophages from the onset to the 24 hour time point.

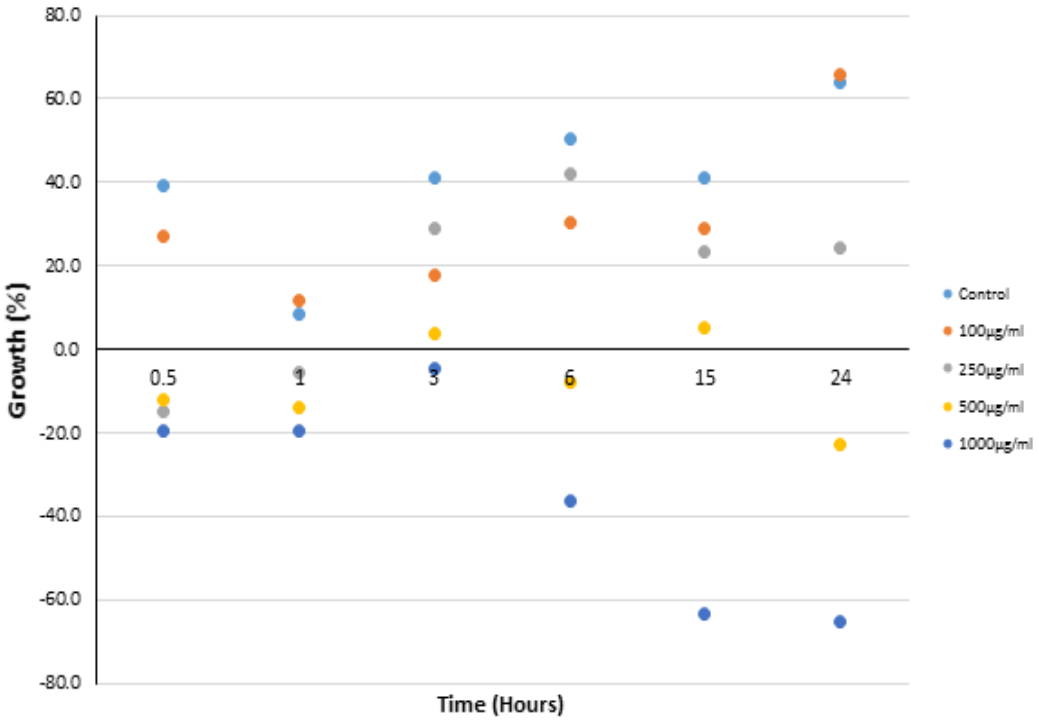



**Figure 5.5 – The effect of nCRP on unactivated monocytes over 24 hours.** Negative percentage represents a decrease in the number of cells compared to the initial viable count used in the assay.

Figure 5.5 shows that when treated with high concentrations (500µg/ml and 1000µg/ml) of nCRP there was a substantial decline in the growth of unactivated monocytes, with 1000µg/ml nCRP causing negative cell growth (net cell death) at all time points. Unactivated monocytes treated with 250µg/ml nCRP showed recovery to positive cell growth after 3 hours and the 100µg/ml nCRP treatment showed positive growth at all time points.

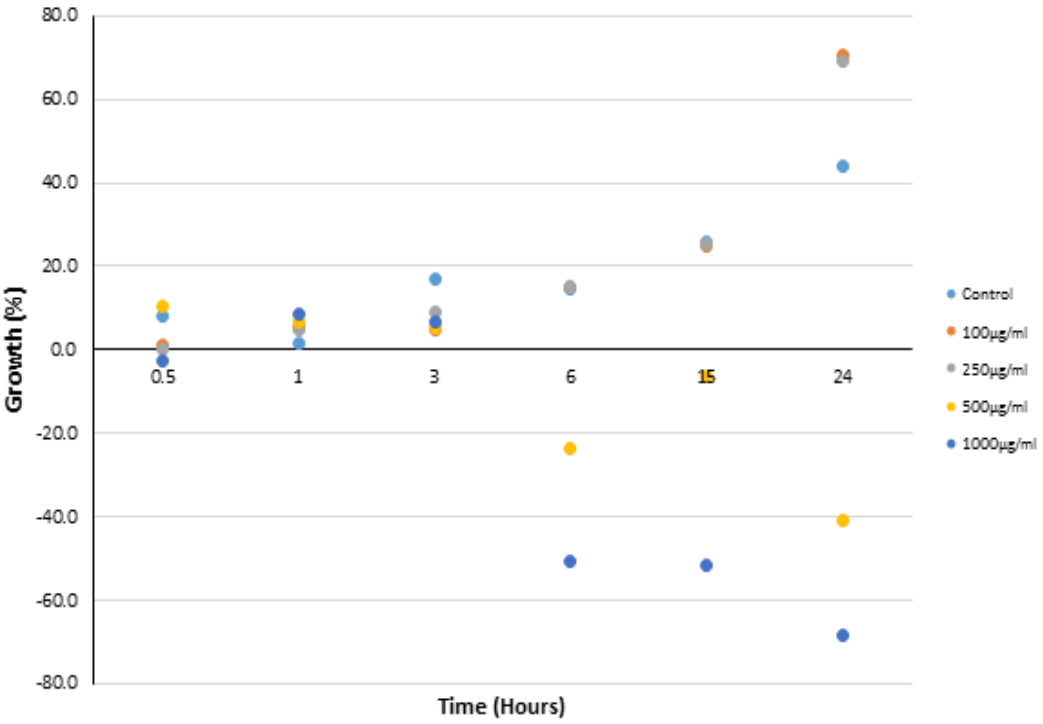



**Figure 5.6 – The effect of nCRP on activated monocytes over 24 hours**. Negative percentage represents a decrease in the number of cells compared to the initial viable count used in the assay.

Figure 5.6 shows that when monocytes were activated with LPS there was positive cell growth from 0.5 hours to 3 hours after which the results diverged. Activated monocytes treated with 500µg/ml and 1000µg/ml nCRP indicated negative growth (net cell death) after 6 hours. The two lower concentrations of nCRP (250µg/ml and 100µg/ml) exhibited over 20% more growth than in the negative control at 24 hours.

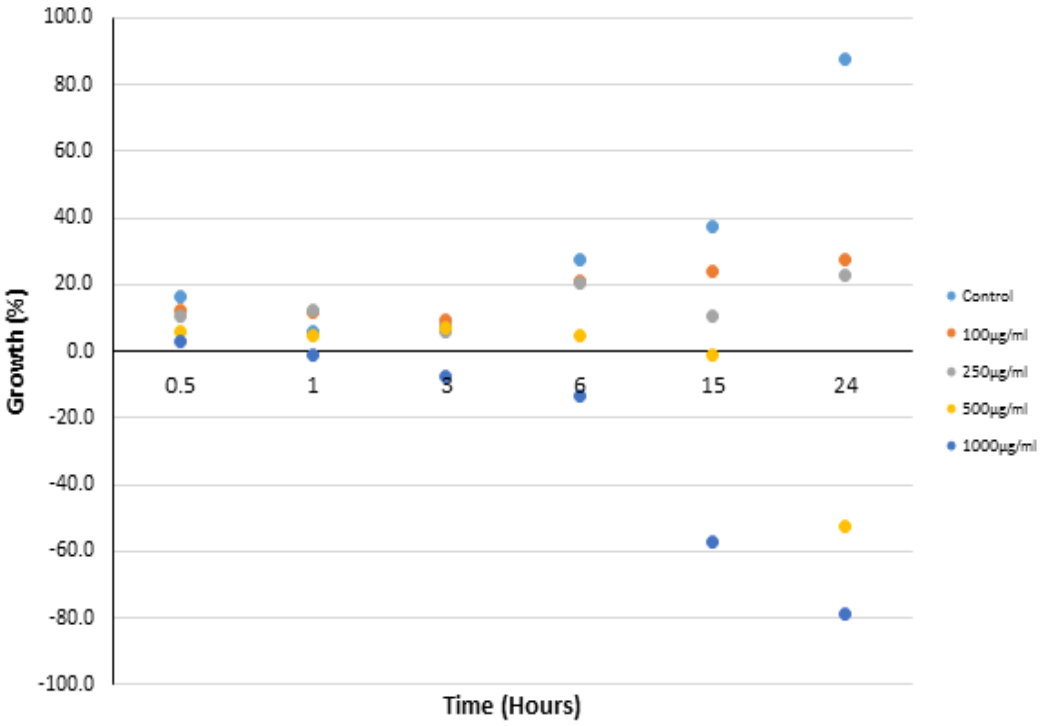



**Figure 5.7 – The effect of nCRP on unactivated macrophages over 24 hours.** Negative percentage represents a decrease in the number of cells compared to the initial viable count used in the assay.

Figure 5.7 shows that nCRP at all concentrations caused positive cell growth in unactivated macrophages from 0.5 hours to 1 hour. However, cell growth following treatment with 1000µg/ml nCRP became negative after 1 hour with nearly 80% of the initial starting count lost by 24 hours. The 500µg/ml treatment had a similar effect but only started to become negative at 15 hours and reaching 50% of the initial cell count at 24 hours. The cells treated with 100µg/ml and 250µg/ml exhibited positive cell growth across all time points but remained below that of the negative control from 6 hours onwards.

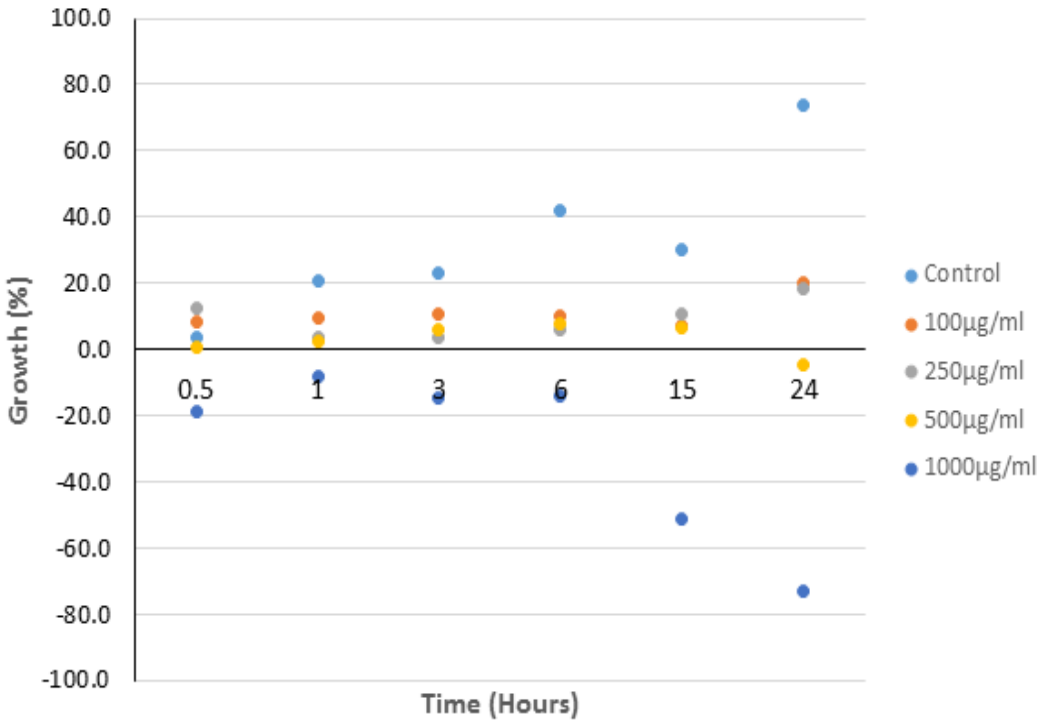



**Figure 5.8 – The effect of nCRP on activated macrophages over 24 hours**. Negative percentage represents a decrease in the number of cells compared to the initial viable count used in the assay.

Figure 5.8 shows that nCRP maintained positive cell growth of activated macrophages at nCRP concentrations at (and below) 250µg/ml, although cell growth was below the negative control after the 0.5 hr time point. Treatment with 1000µg/ml nCRP caused negative growth (net cell death) at all times points, particularly after 15 hours. Treatment with 500µg/ml nCRP caused positive growth of activated macrophages at all time points before 24 hours, after which negative growth (net cell death) occurred.

These figures (Figures 5.1 to 5.8) have identified the general timeline trends but only give some of the information captured by the growth and viability assay. Section 4.7.2 and 4.7.3 provides data on the significant changes in growth and viability at each specific time point.

### 5.7.2 The Effect of mCRP on Growth and Viability of Unactivated Monocytes

Figure 5.9 indicates the effect on growth and viability of unactivated monocytes at each specific time point following treatment with mCRP at several concentrations.

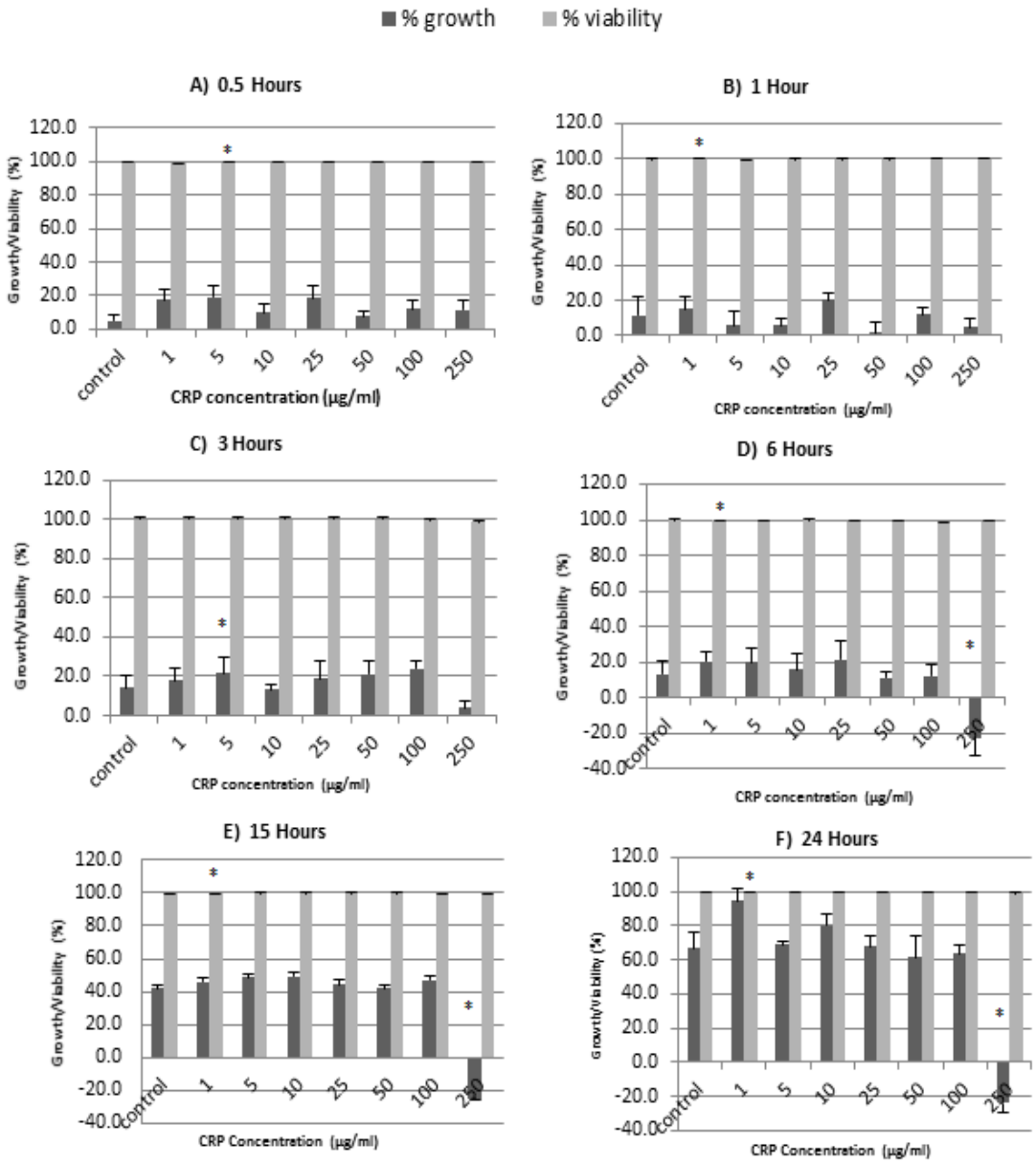



**Figure 5.9 – The effect of various mCRP concentrations on growth and viability of unactivated monocytes at each time point (0.5 hr to 24 hr).** Negative percentage represents a decrease in the number of cells compared to the initial density used to set up the assay. Significant differences ($P<0.05$) compared to the negative control are denoted by *.

From 6 hours onwards, there was a significant difference in cell growth of unactivated monocytes between the 250µg/ml mCRP treatment and the negative control. The assay was set up using $1x10^6$ cells/ml and after 6 hours cell growth of unactivated macrophages became negative, with around a 20% reduction at 24 hours, indicating net cell death compared to initial conditions. However, cell viability remained high, suggesting that either phagocytosis of dead cells was taking place by the remaining viable cells or that non-viable cells were undergoing lysis.

In general, treatment with mCRP (at all concentrations except 250µg/ml) resulted in a growth pattern similar to that of the negative control for the most part with few significant fluctuations observed.

### 5.7.3 The Effect of mCRP on Growth and Viability of Activated Monocytes

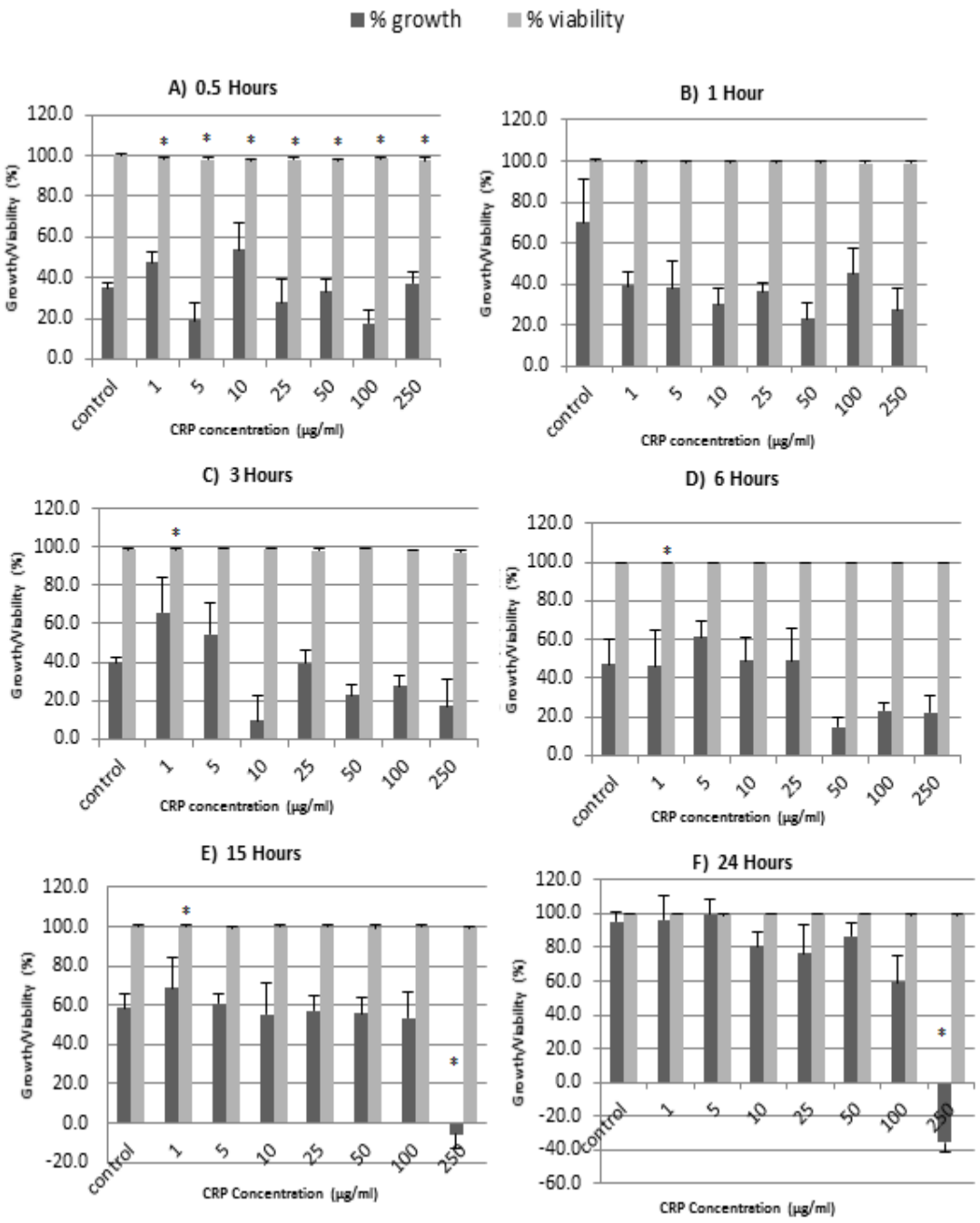



**Figure 5.10 – The effect of various mCRP concentrations on the growth and viability of activated monocytes at each time point (0.5hr to 24 hr).** Negative percentage represents a decrease in the number of cells compared to the initial density used to set up the assay. Significant differences ($P<0.05$) compared to the negative control are

Figure 5.10 indicates the effects of the mCRP isoform upon activated monocytes at a range of concentrations. At 15 hours and 24 hours, treatment with 250μg/ml mCRP showed a significant ($p=0.001$ and $p=0.000$ respectively) decline in cell growth compared to the negative control (as previously seen in Figure 5.9), suggesting net cell death.

### 5.7.4 The Effect of mCRP on Growth and Viability of Unactivated Macrophages.

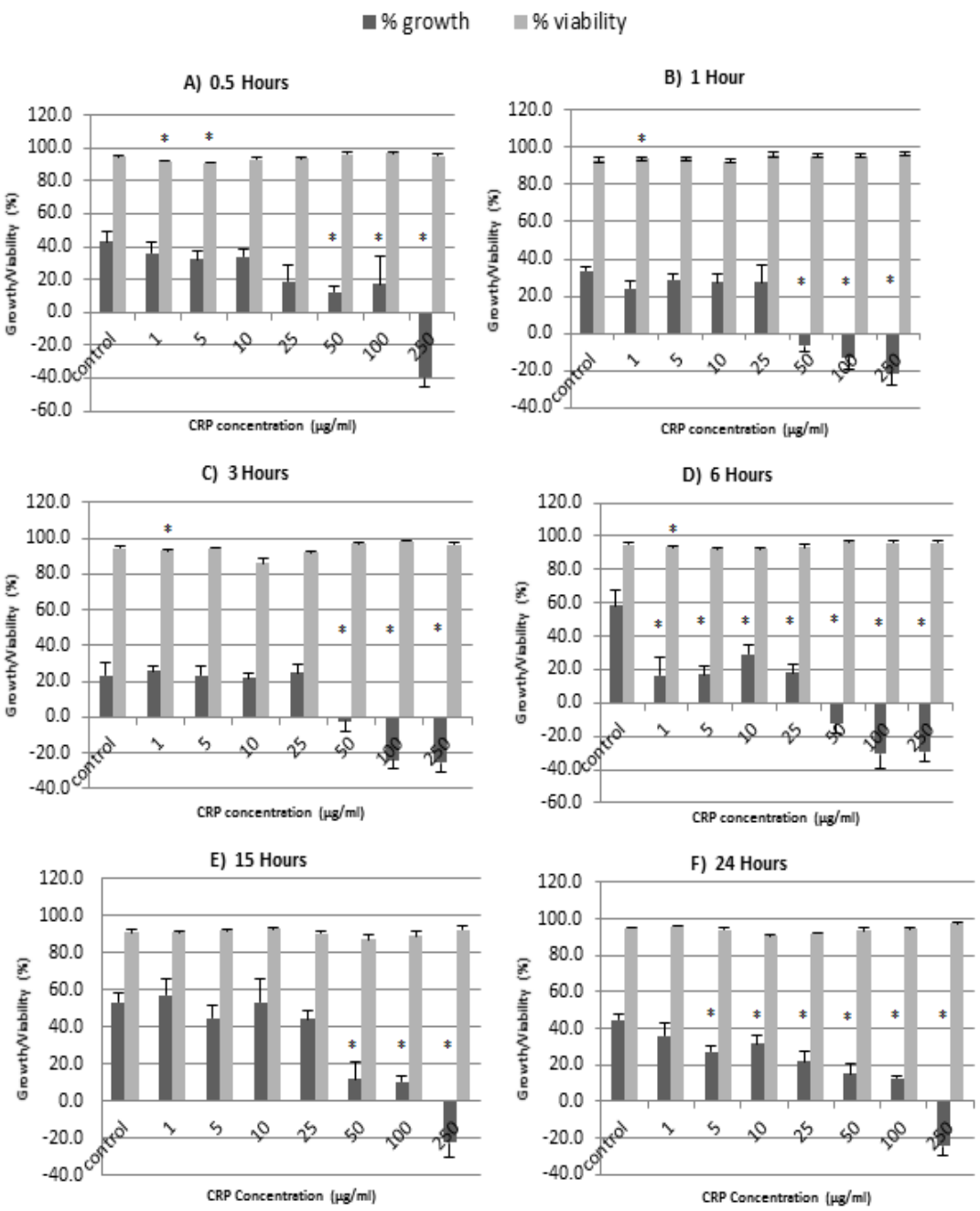



**Figure 5.11 – The effect of various mCRP concentrations on the growth and viability of unactivated macrophages at each time point (0.5 hr to 24 hr).** Negative percentage represents a decrease in the number of cells compared to the initial cell density used to set up the assay. Significant differences ($P<0.05$) compared to the negative control are denoted by *.

Absolute cell viability of unactivated macrophages remained relatively unchanged across all concentrations of mCRP and incubation times.

At 6 and 24 hours, all mCRP concentrations showed a significant reduction in the growth of unactivated macrophages compared to the negative control, with the exception of 1µg/ml at 24 hours. At each time point there was a significant decrease in the growth of unactivated macrophages when treated with 50µg/ml, 100µg/ml and 250µg/ml compared to the negative control. Treatment with 250µg/ml mCRP caused cell growth of unactivated macrophages to remain negative (around 20% lower than the initial count used to set up the assay) at all six time points. For the treatments with 50µg/ml and 100µg/ml mCRP, a significant ($p<0.05$) decline in unactivated macrophages was observed with negative cell growth indicating net cell death after an hour but at 15 hours there was evidence of recovery in cell growth. The amount of cell growth did not recover back to control levels with 50µg/ml and 100µg/ml mCRP but did become positive at 15 hours. . However viability remained high (no significant difference to the negative control) during the whole process suggesting that dead cells were being removed by one or more mechanisms (e.g. phagocytosis and/or cell lysis).

### 5.7.5 The Effect of mCRP on Growth and Viability of Activated Macrophages

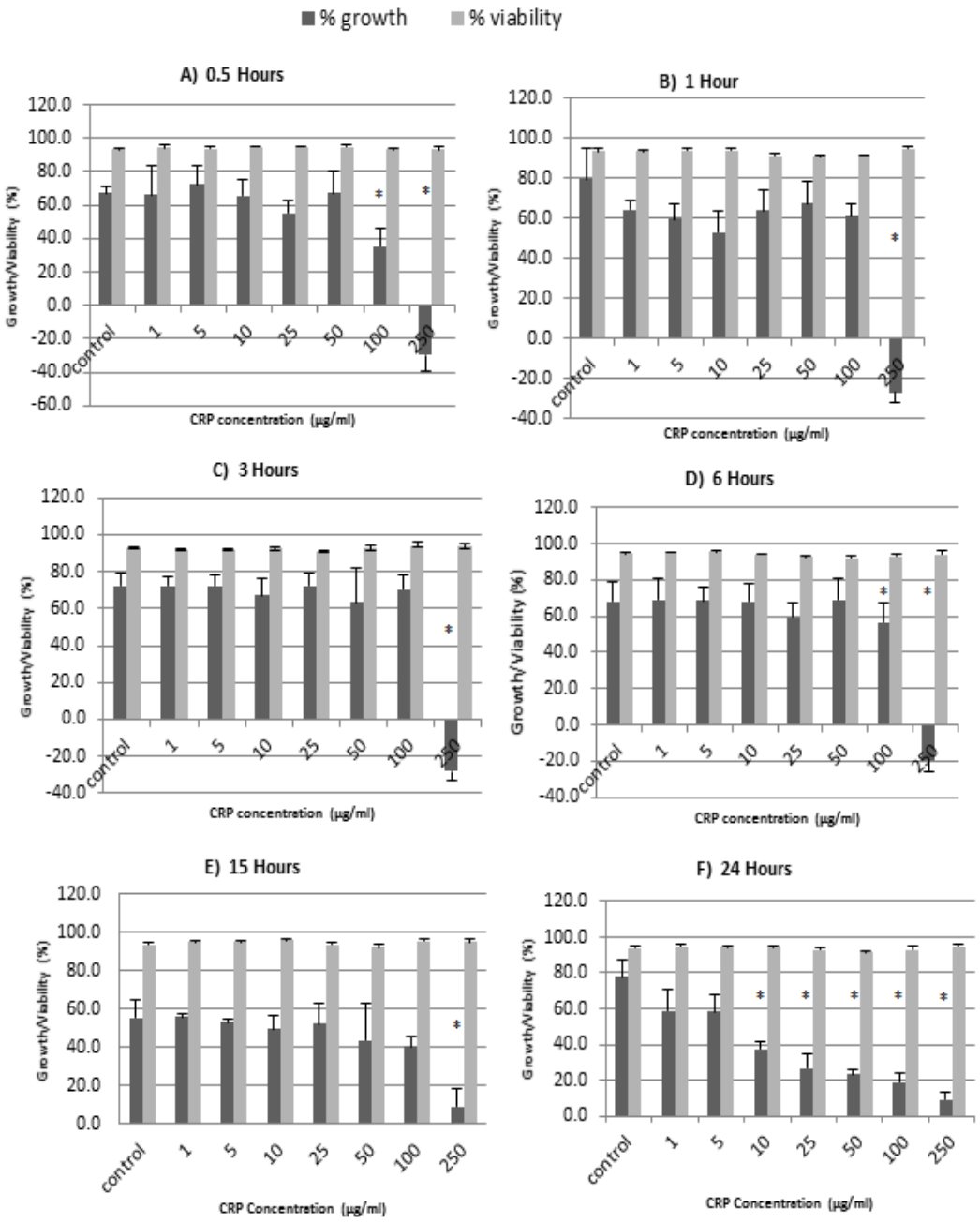



**Figure 5.12 – The effect of various mCRP concentrations on growth and viability of activated macrophages at each time point (0.5 hr to 24 hr).** Negative percentage represents a decrease in the number of cells compared to the initial density used to set up the assay. Significant differences ($P<0.05$) compared to the negative control are denoted by *.

Figure 5.12 indicates the effects of mCRP treatments at several concentrations and incubation times on activated macrophages.

Treatment with 250µg/ml caused a reduction in the growth of activated macrophages that was significantly ($P<0.05$) lower than the negative control. Between 0.5 hours and 6 hours this reduction was around 20% lower than the initial cell control used in the assay, indicating that net cell death had occurred. However at the 15 hour and 24 hour time points there was some recovery with positive growth of around 9% in both cases, although this remained significantly ($P<0.05$) lower than the negative control.

Following 24 hours of treatment with mCRP, there was a significant ($P<0.05$) dose-dependent decrease in the growth of activated macrophages compared to the negative control at concentrations of 10µg/ml mCRP and above. However, the viability of activated macrophages remained high (>90%) in all cases.

### 5.7.6 The Effect of nCRP on Growth and Viability of Unactivated Monocytes

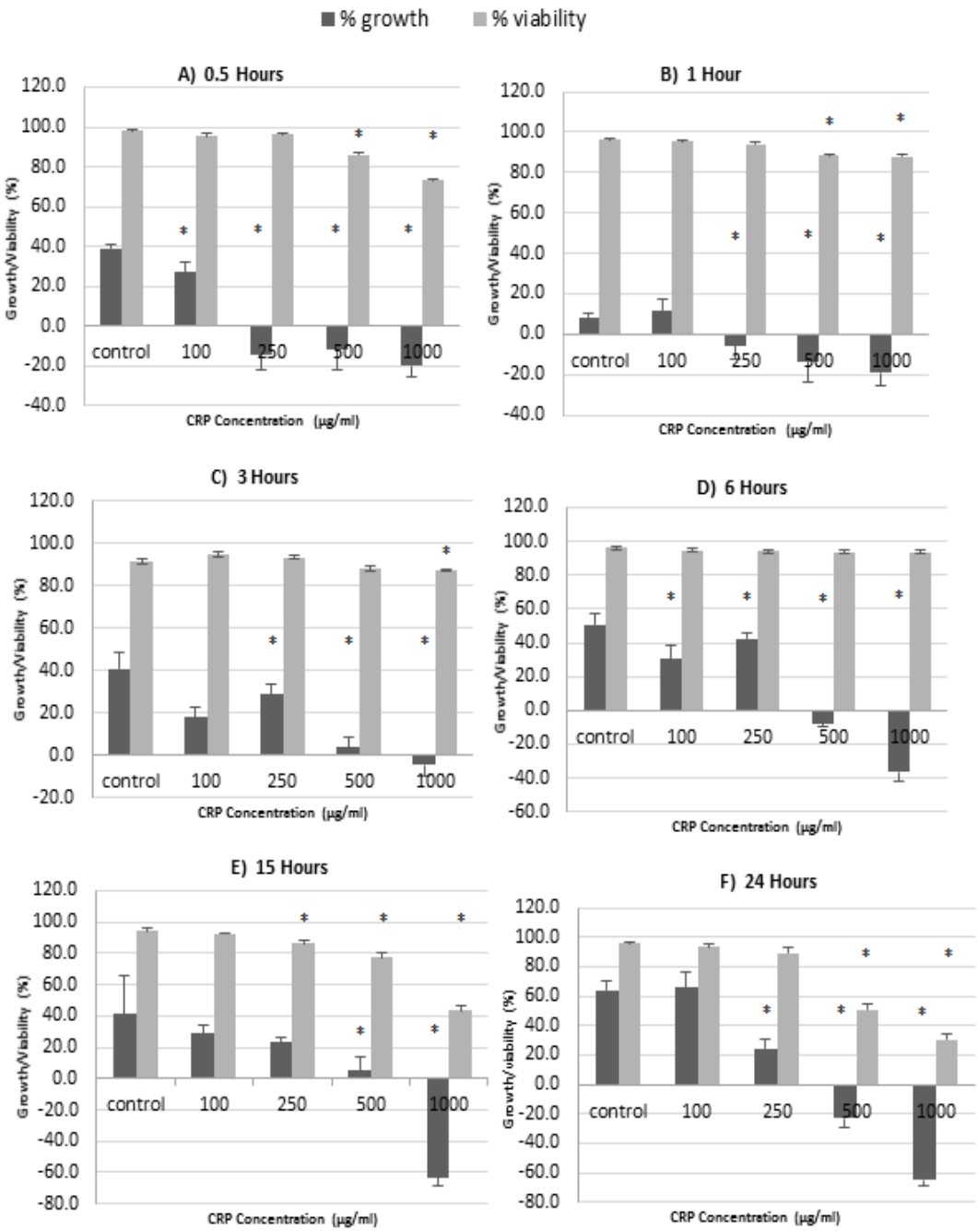



**Figure 5.13 – The effect of various nCRP concentrations on growth and viability of unactivated monocytes at each time point (0.5 hr to 24 hr).** Negative percentage represents a decrease in the number of cells compared to the initial density used to set up the assay. Significant differences ($P<0.05$) compared to the negative control are denoted by *.

Figure 5.13 indicates the effect of nCRP treatments on unactivated monocytes.

From the outset, treatment with 1000µg/ml caused a significant ($P<0.05$) decline in cell growth and a reduction in viability of unactivated monocytes compared to the negative control. Treatment with 500µg/ml also significantly ($P<0.5$) reduced the growth of unactivated monocytes but cell viability was only significantly reduced at 0.5 hour, 1 hours, 15 hours and 24 hours compared to the negative control. In general, treatment with 250µg/ml nCRP also significantly ($P<0.05$) reduced growth of unactivated monocytes compared to the negative control but cell viability remained similar to the negative control (except at 15 hours).

### 5.7.7 The Effect of nCRP on Growth and Viability of Activated Monocytes

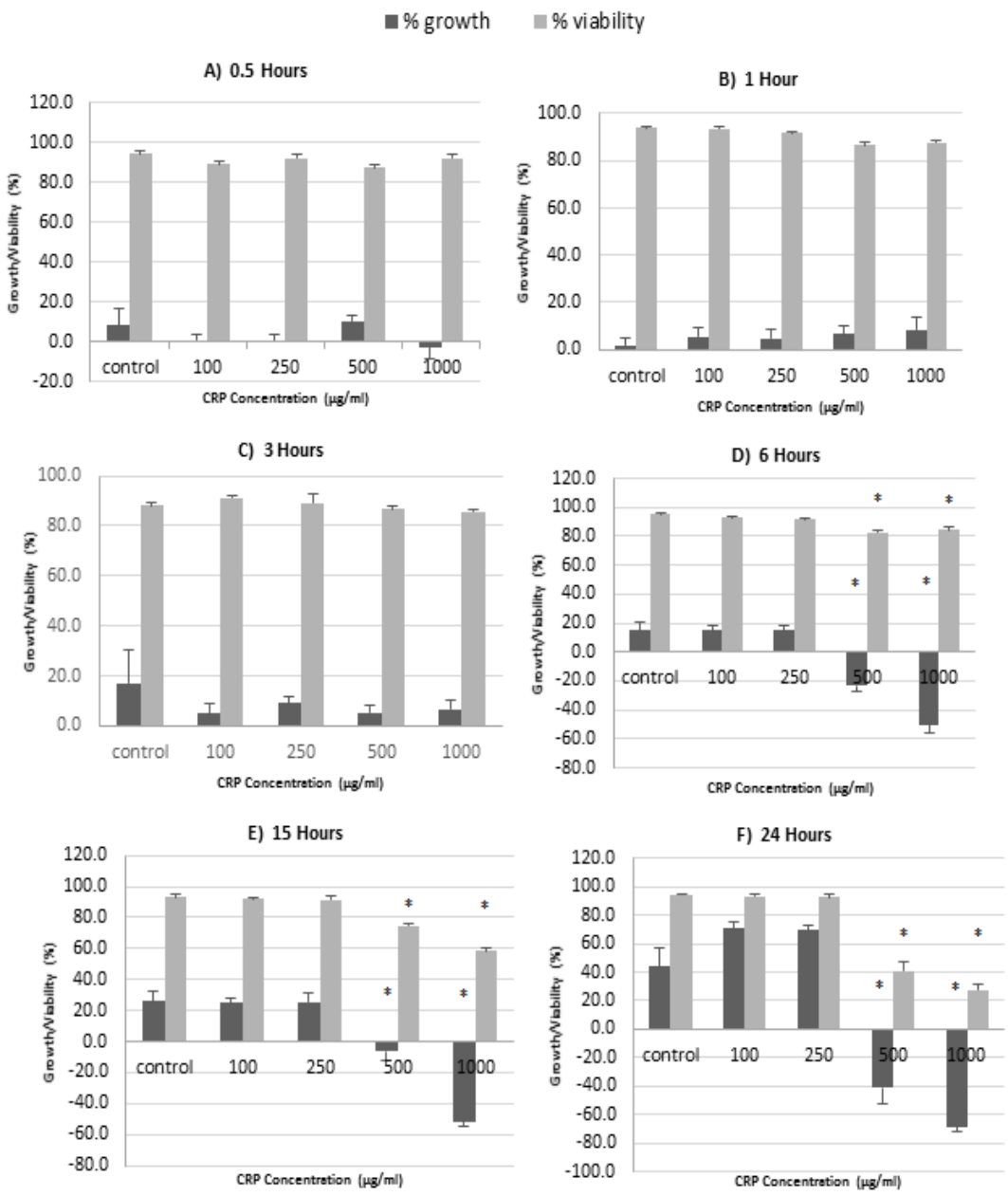



**Figure 5.14 – The effect of various nCRP concentrations on growth and viability of activated monocytes at each time point (0.5 hr to 24 hr).** Negative percentage represents a decrease in the number of cells compared to the initial density used to set up the assay. Significant differences ($P<0.05$) compared to the negative control are denoted by *.

Figure 5.14 indicates the effect nCRP treatments had on activated monocytes. There were no significant changes in either growth or viability compared to the negative control until the 6 hour time point. From 6 hours onwards there was a significant ($P<0.05$) decline in the number of activated monocytes when treated with 500µg/ml or1000µg/ml nCRP, with negative cell growth suggesting net cell death and the higher concentration having the greatest reduction on cell growth. The viability of activated monocytes at 500µg/ml and 1000µg/ml nCRP significantly ($P<0.05$) decreased compared to the negative control from 6 hours onwards, falling to 41.1% and 27.6% respectively at 24 hours.

### 5.7.8 The Effect of nCRP on Growth and Viability of Unactivated Macrophages

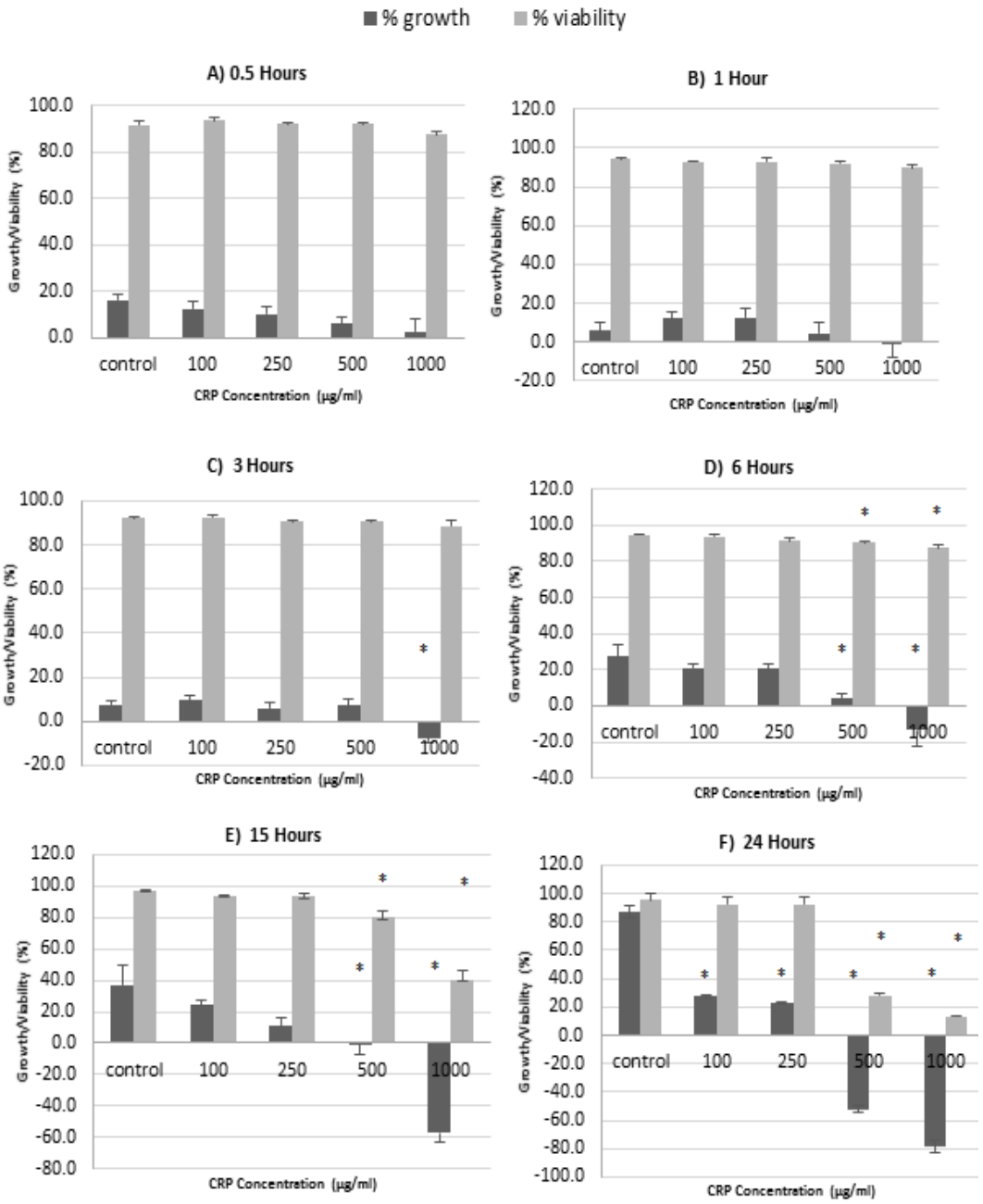



**Figure 5.15 – The effect of various nCRP concentrations on growth and viability of unactivated macrophages at each time point (0.5 hr to 24 hr).** Negative percentage represents a decrease in the number of cells compared to the initial density used to set up the assay. Significant differences ($P<0.05$) compared to the negative control are denoted by *.

Figure 5.15 indicates the effect of nCRP treatments on unactivated macrophages. There were few significant changes in either growth or viability until the 6 hour time point. From 6 hours onwards there was a significant reduction in the number of unactivated macrophages when treated with 500µg/ml and 1000µg/ml with the higher concentration causing negative cell growth (net cell death) from the 3 hour time point onwards. At 24 hours all nCRP concentrations caused a significant ($P<0.05$) reduction in the growth of unactivated macrophages. The viability of unactivated macrophages at 500µg/ml and 1000µg/ml nCRP was significantly ($P<0.05$) reduced from 6 hours onwards, falling to 27.8% and 13.3% respectively at the 24 hour time point.

### 5.7.9 The Effect of nCRP on Growth and Viability of Activated Macrophages

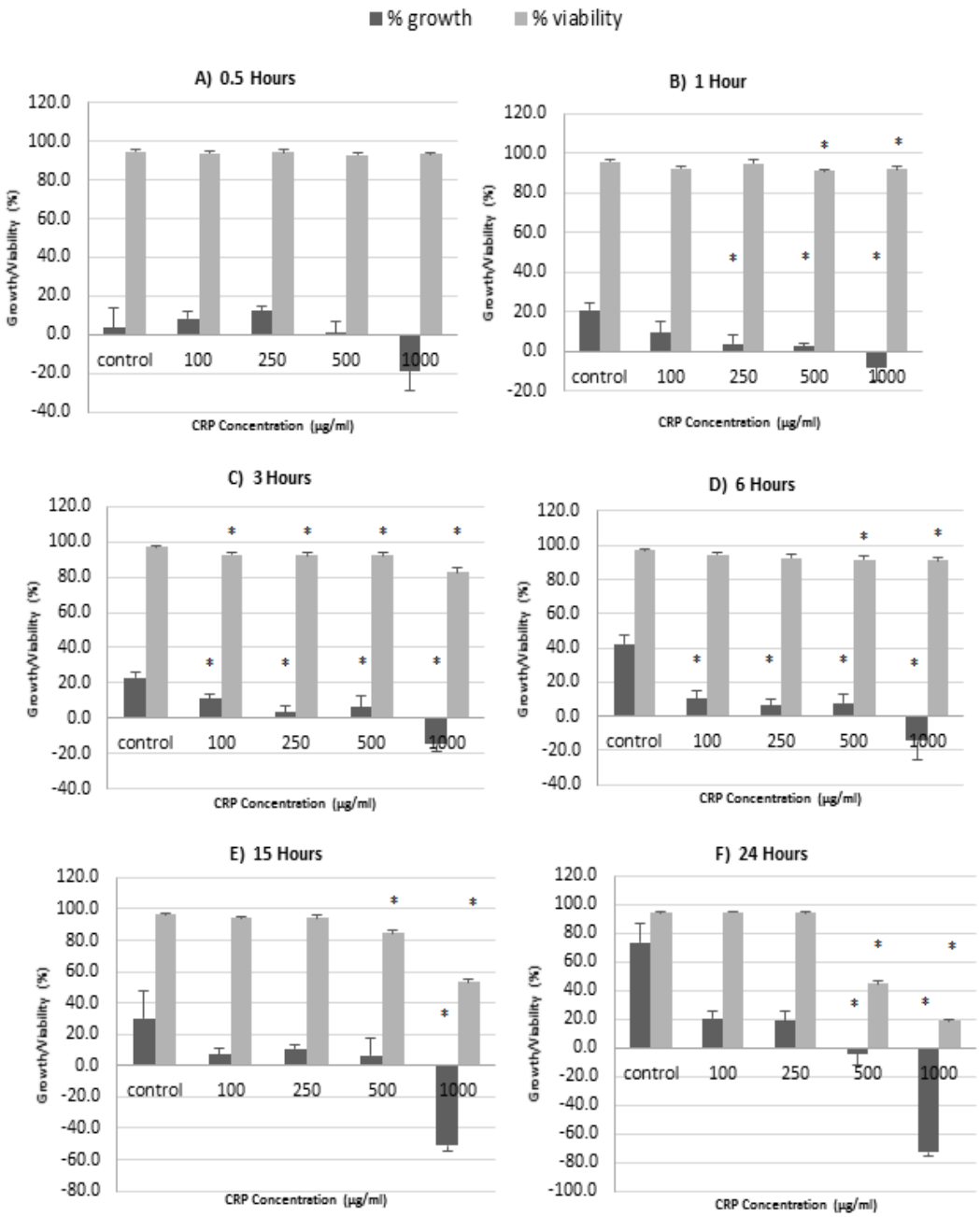



**Figure 5.16 – The effect of various nCRP concentrations on growth and viability of activated macrophages at each time point (0.5 hr to 24 hr).** Negative percentage represents a decrease in the number of cells compared to the initial density used to set up the assay. Significant differences ($P<0.05$) compared to the negative control are denoted by *.

Figure 5.16 indicates the effects of nCRP treatments on activated macrophages. From 1 hour onwards there was a significant reduction in cell viability when treated with 500µg/ml or1000µg/ml, and this trend continued through to the 24 hour time point. In terms of cell growth, 1000µg/ml nCRP treatment caused a significant reduction in the growth of activated macrophages from 1 hour onwards with progressively negative cell growth (net cell death) with increasing incubation time.

### 5.7.10 The Effect of CRP Isoforms on Apoptosis

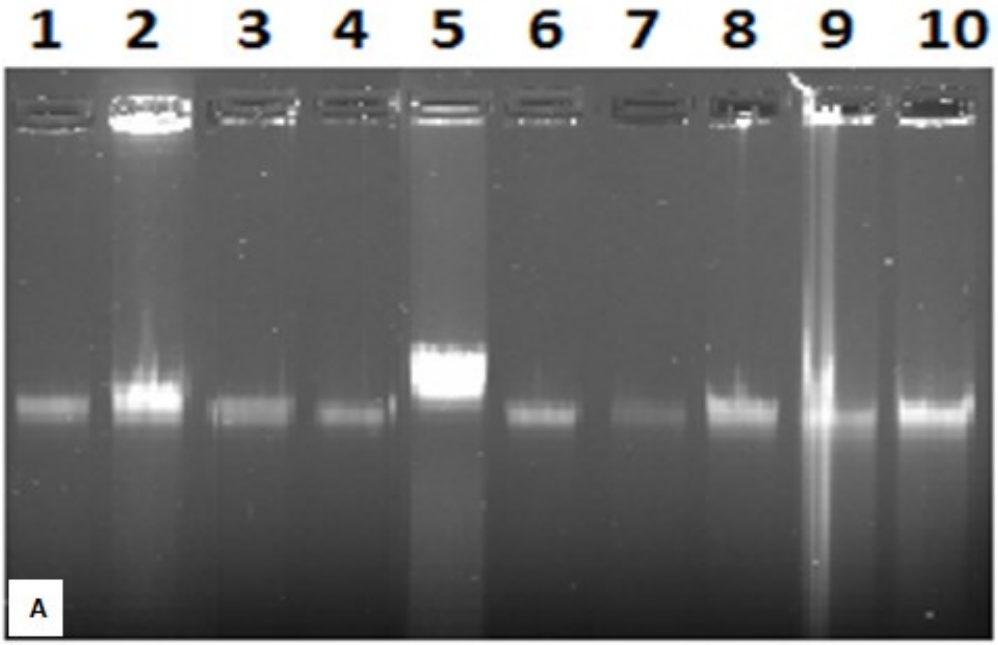
In summary, both the highest concentrations of mCRP and nCRP exhibited negative cell growth in all cell models (phenotypes), suggesting evidence of net cell death. However, in many (but not all) cases cell viability remained relatively high, suggesting that dead cells may have been removed via phagocytosis or cell lysis. Therefore, an apoptosis assay was performed to assess whether the observed cell death was due to apoptosis.

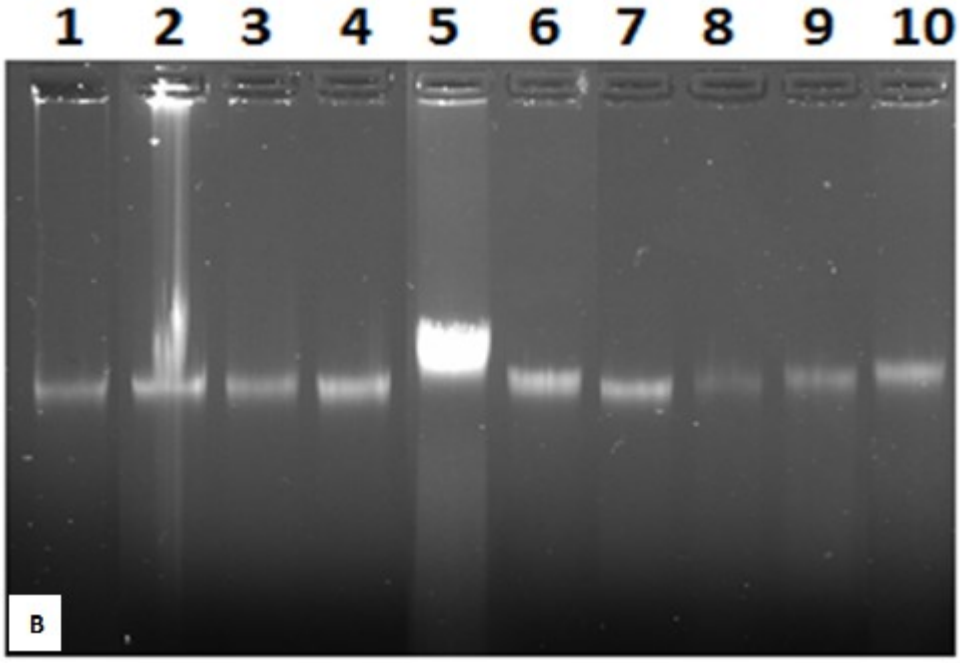
Treatment with 1000µg/ml nCRP provided evidence of cell death via apoptosis in all four cell models (phenotypes). Treatment with 250µg/ml mCRP showed no evidence of apoptosis in any of the cell models (phenotypes), suggesting that cell death following mCRP treatment must have been by some other mechanism(s) such as necrosis. Pharmacological inhibitors were then used to determine whether the nCRP-induced apoptosis could be reversed (Figure 5.17).

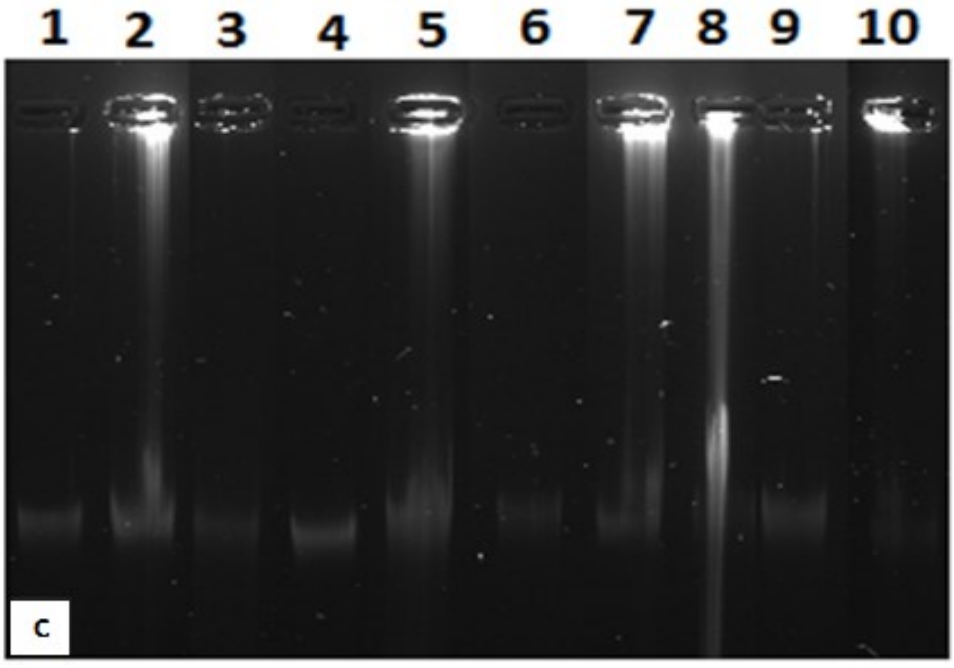
**Figure 5.17 – The Effects of CRP Isoforms on Apoptosis.**

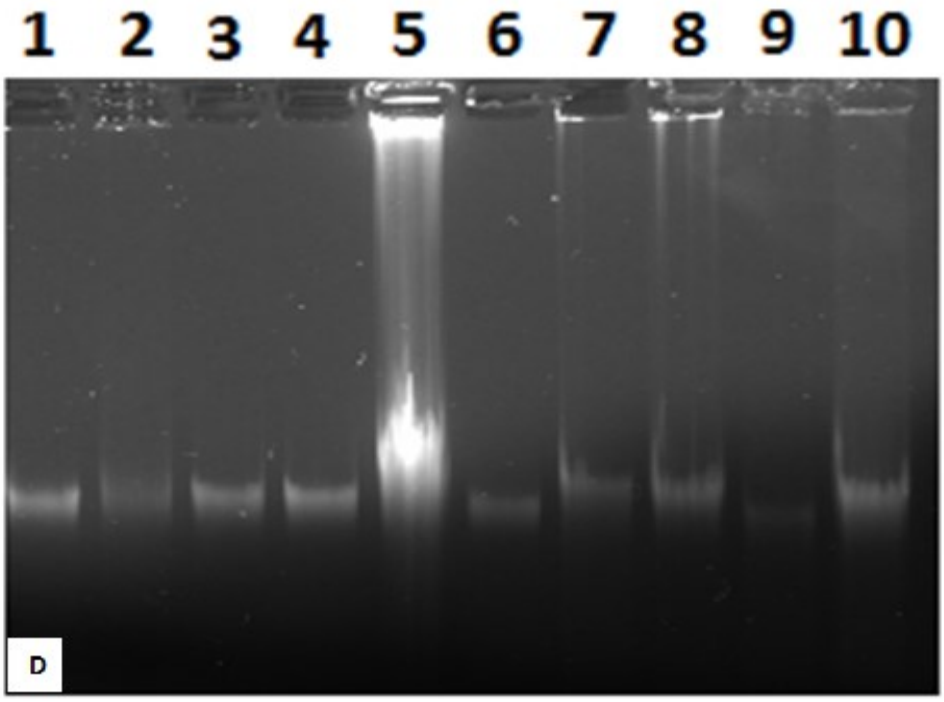
A) Unactivated monocytes  B) Activated monocytes
C) Unactivated macrophages  D) Activated macrophages

1 – Negative Control  2- Positive Control  3-BSA 1000µg/ml  4-BSA 250µg/ml
5-nCRP 1000µg/ml  6- mCRP 250µg/ml
7- nCRP 1000µg/ml with wortmannin  8- nCRP 1000µg/ml with LML
9- nCRP 1000µg/ml with MEK  10- nCRP 1000µg/ml with nystatin




1 2 3 4 5 6 7 8 9 10
B


1 2 3 4 5 6 7 8 9 10
C




The negative control in lane 1 of all four cell models showed no DNA fragmentation (apoptosis) whereas in lane 2 the positive control caused smearing indicating apoptosis had occurred. The two lanes of BSA controls (lanes 3 and 4) showed no apoptosis had occurred. Lane 5 (1000µg/ml nCRP) showed extensive DNA fragmentation (apoptosis). Lane 6 (250µg/ml mCRP) did not show evidence of DNA fragmentation (apoptosis).

Wortmannin reversed the effect of apoptosis in unactivated monocyte cells treated with 1000µg/ml nCRP (Figure 5.17A). All four inhibitors reversed the apoptosis in activated monocytes (Figure 5.17B). In unactivated macrophages, none of the inhibitors reversed the apoptosis (Figure 5.17C). In activated macrophages, only the MAP kinase inhibitor (MEK) reversed the nCRP-induced apoptosis (Figure 5.17D).

## 5.8 Discussion

### 5.8.1 Growth and Viability Assays

The growth and viability assays were performed to analyse the effects of the CRP isoforms on four different states (±LPS activation, ± differentiation) of U937 cells to model different cell stages in the wound healing process.

Undifferentiated U937 cells represent/model circulating monocytes prior to wounding or infection. Differentiated U937 cells represent/model tissue macrophages following wounding. Unactivated and LPS-activated U937 cells represent/model the absence and presence of infection respectively.

Figures 5.1 and 5.2 show that high concentrations (250µg/ml) of mCRP caused negative growth from 6 hours onwards, suggesting net cell death was occurring. However, the growth/viability assay was not able to determine whether necrosis and/or apoptosis were taking place. Indeed, cell viability following treatment with 250µg/ml mCRP remained high (between 90% and 100%) at all time points, suggesting non-viable cells were already removed by cell lysis or phagocytosis.

The activation of the cells seem to have a profound effect by delaying the effects of the treatment with a reduction in the number of cells at 250µg/ml mCRP being seen at 15 hours rather than at 6 hours. This exposure means that the monocytes are undergoing the various processes in wound healing; including the release of cytokines, chemokines and various other instructional factors (Hart, 2002a). Mold *et al* (2002) suggested that CRP causes a moderated cytokine profile in previously LPS-activated cells which in turn reduced the toxic side-effect of LPS after an infection occurs. This process can be suggested that the LPS activation of the cells 'prepares' the cells so that they respond more efficiently to the CRP treatment. This would be a likely suggestion as to why there is this time delay for the effect of mCRP between the unactivated monocytes and the activated monocytes.

Figures 5.5 and 5.6 show a substantial reduction in the number of viable cells with the higher (1000µg/ml) nCRP concentration. A decline in growth was seen from the earliest time point with the unactivated monocytes. However, this was delayed until 6 hours in the LPS activated monocytes. Again, this suggests pre-activation with LPS delays subsequent responses. In both unactivated and activated monocytes there was a significant ($P<0.05$) decrease in viability from 6 hours onwards following treatment with 1000µg/ml nCRP. This was indicative of cell death but the cell viability assay alone could not determine what type of cell death was occurring. The cell viability following treatment with lower concentrations (100µg/ml and 250µg/ml) of nCRP remained above 90% at all time points. This suggests nCRP, which has previously been associated with anti-inflammatory effects (Eisenhardt *et al*, 2012), may reduce the inflammatory cell count by affecting cell survival. This supports evidence that nCRP is the only CRP isoform that induces apoptosis and that apoptosis ceases following loss of its pentameric structure (Khreiss *et al,* 2000).

Figures 5.3 and 5.4 present growth/viability data on differentiated macrophages treated with mCRP, with Figure 5.4 representing LPS-activated macrophages. Figure 5.3 shows that treatment with 250µg/ml mCRP caused a decline in growth of unactivated macrophages from the outset but viability was not affected, supporting evidence that mCRP does not induce apoptosis of cells. Treatment with 50µg/ml and 100µg/ml also showed a decline in the number of unactivated macrophages but positive recovery of growth was observed at and beyond 15 hours. At 24 hours, there was a dose-dependent effect of mCRP on the growth of unactivated macrophages, with increasing mCRP concentration causing a greater reduction in the growth of cells. Viability of unactivated macrophages remained high at all time points and mCRP concentrations, suggesting that a reduced rate of cellular proliferation (rather than increased cell death) was mediating the overall effect of mCRP on net cell growth.

Figure 5.4 shows the effects of mCRP on LPS- activated macrophages. Treatment with 250µg/ml mCRP caused a significant decline in the number of

activated macrophages from 0.5 hours onwards compared to the negative control but in contrast to unactivated macrophages, the cell density recovered from 15 hours onwards. Again, at 24 hours there was a dose-dependent effect of mCRP on the growth of activated macrophages, with increasing mCRP concentration causing a greater reduction in the growth of cells, but the growth was higher than that seen in unactivated macrophages. This suggests pre-activation of macrophages with LPS may negate the inhibition of cell growth induced by mCRP.

Figures 5.7 and 5.8 showed the effect of nCRP on unactivated and activated macrophages. In general, there was a similar effect of nCRP on macrophages to that seen in monocytes. At later time points, higher concentrations of nCRP caused both a significant decline in the number of cells and a decline in macrophage viability. This effect was similar in both unactivated and activated macrophages.

In all four cell states and with both sets of isoform treatments it is interesting that majority of the significant effects are seen at the 24 hour time point since this is when CRP starts to accumulate after an inflammatory signal (Ciubotaru *et al*, 2005).

Most of the significant changes within these data came from high concentration treatments (100µg/ml and 250µg/ml mCRP; 500µg/ml and 1000µg/ml nCRP) and after 6 hours of treatment. As the growth of cells declined over 24 hours this would suggest that cells were not entering into the cell cycle for proliferation and that most likely they would be remaining in the $G_0$ phase where the cells are in a resting state (Scholey *et al*, 2003).

There has been little research conducted into the effect of CRP isoforms upon the proliferation of cells and it can be suggested that CRP does not upregulate any of the cyclin-dependent kinases that are required to pass through the different phases of the cell cycle (van den Heuvel & Harlow, 1993). The results indicated that cell death was likely occurring however these cells were not being replaced via proliferation. This could potentially indicate that the CRP could be inhibiting the

cyclin-dependent kinases that are required for proliferation to occur in order to replace those cells.

Kim & Ryu (2004) suggested that CRP led to the upregulation of p53 which led to a $G_2$/M arrest which ultimately leads to apoptosis. As treatment with nCRP exhibited declined growth and viability, this would support this conclusion, however treatment with mCRP led to a decline in growth without a supporting decline in viability suggesting that this pathway was not being utilised in this case.

Originally lower concentrations of nCRP were tested at similar concentrations to those tested for mCRP (1-100µg/ml). However, lower concentrations of nCRP had no effect upon any of the cell states investigated with U937 cells (data not shown). This is understandable since CRP is usually found at a concentration of about 1µg/ml in the absence of inflammation and can increase up to 1000 fold in circulation following an acute inflammatory response (Gabay & Kushner, 1999). Thus, 1000µg/ml became the upper limit for testing the effects of nCRP in this study, in line with published maximal physiological levels found in circulation.

### 5.8.2 Apoptosis Assay

As mentioned previously both isoforms caused a decline in cell number below that of the initial cell density used to set up the assay. This negative cell growth indicated net cell death had taken place, particularly in the case of nCRP treatments where the viability also significantly reduced. Treatment with mCRP caused negative cell growth without affecting overall cell viability, suggesting dead cells were quickly removed by cell lysis or apoptosis.

The apoptosis assay was run with a positive control of camptothecin to ensure that the electrophoresis gel was detecting fragmented DNA correctly. In all four assays for the different cell states, the negative control showed no DNA fragmentation and neither did controls treated with two concentrations of BSA to mirror those of the CRP isoforms. The assay above showed that addition of a

generic (non CRP) protein did not induce apoptosis in any of cell states tested. The positive control generated DNA fragmentation in all cell states.

Treatment with mCRP did not induce apoptosis in monocytes or macrophages. This is supported by evidence showing mCRP is associated with delayed apoptosis of neutrophils and a prolonged inflammatory response (Khreiss *et* al, 2002). This is interesting since the number of macrophages in chronic wounds has been shown to be excessive (Diegelmann & Evans, 2004) and finding potential mechanisms to reduce the inflammatory response is a therapeutic strategy for the treatment of chronic wounds.

The net cell death observed by the mCRP treatment must have been induced by some other mechanism (e.g. cell lysis/necrosis) rather than apoptosis. Thus, the high cell viability observed following mCRP treatment makes sense since necrosis can occur quickly, removing dead cells through cell lysis and leaving just viable cells.

Khreiss *et al* (2002) suggested a pathway of the inhibition of apoptosis that is utilised by the mCRP isoform. Figure 5.18 below shows this pathway.

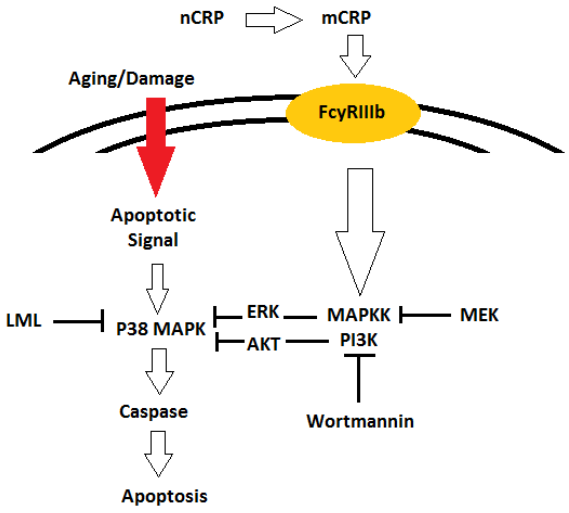



**Figure 5.18 – The method of apoptosis inhibition by monomeric CRP**. Other pathways of inhibition have been added to the diagram. (Modified from *Khreiss et al*, 2002)

This pathway shows that mCRP inhibits apoptosis via the CD16 receptor by activating the MAPK and PI3K pathways to produce ERK and AKT thereby inhibiting the p38 MAPK pathway. This is caused by the prevention of p38 to cleave procaspase-3, preventing the triggering of the caspase cascade and therefore inhibiting apoptosis (McIlwain *et al,* 2015).

This supports why the viability in all of the treatments with mCRP remained high since there was no evidence of apoptosis but other mechanisms of cell death such a necrosis may have occurred. Necrosis leads to the release of intracellular contents and the induction of an inflammatory response, including the release of cytokines. The induction of cytokines by CRP isoforms is assessed in Chapter 7, confirming that mCRP does indeed induce the release of pro-inflammatory cytokines at the same time as causing net cell death.

Another potential mechanism by which mCRP might be inhibiting the apoptosis pathway is by preventing the activation of p53 caused by DNA damage or by preventing the activation of the death receptor Fas, which is expressed in stressed or damaged cells (Elmore, 2007). If this was the case, mCRP may cause the inhibition of the caspase cascade through these other pathways.

Treatment with nCRP was found to fragment the DNA and cause apoptosis of U937 cells, regardless of their cell state. This corresponded to a decline in the number of cells and a decrease in the cell viability. It suggested that nCRP can trigger the apoptosis pathway by binding to CD32 and CD64 receptors on the cell surface to promote the expression of TNF-α and IL-1β. These cytokines trigger the upregulation of p53 and triggering the caspase cascade as a result. This process has been suggested to occur in about 4 hours (Kim & Ryu, 2014) which coincides with the data from the growth/viability assay where most of the significant effects were seen after 6 hours. Blaschke *et al* (2004) found that CRP can induce apoptosis through increased caspase-3 activity. The results of this study indicates that this pathway is potentially being activated when treated with nCRP.

The effect of pharmacological inhibitors would also suggest that nCRP is having an effect on the pathway in Figure 5.18. With unactivated monocytes, only wortmannin reversed the effect of the nCRP. This would suggest that inhibition of apoptosis is occurring through the Akt pathway which inhibits p38 MAPK to prevent apoptosis. This suggests that monocytes undergo apoptosis through this pathway following nCRP treatment. However, the response was not reversed by other inhibitors of this pathway, suggesting that one or more other signalling pathway(s) could also be contributing to the apoptosis seen in the unactivated monocytes.

In the activated monocytes all four inhibitors reverse the effects of apoptosis. Three of the four inhibitors that feature on this pathway would inhibit this process at various stages of the pathway. All three result in the inhibition of the p38-induced cleavage of procaspase-3, resulting in cell survival. The last inhibitor, nystatin, inhibits apoptosis via an alternative pathway. Nystatin is a lipid raft disruptor that causes the formation of pores in the plasma membrane of eukaryotic cells, thereby allowing potassium and other components to cross the membrane. Previously it has been noted that delayed apoptosis was observed in neutrophils treated with nystatin. This was due to the fact that Fas signalling was needed to trigger the activation of capase-8 and therefore the cascade needs an intact lipid raft for this to be successful (Scheel-Toellner *et al,* 2004). This could indicate that there were some physiological changes to the membrane when cells became activated with LPS that would affect the use of the lipid rafts in the activation of apoptosis, however further work would need to be conducted to establish this.

Unactivated macrophages saw no changes with the use of inhibitors. This would suggest that when the cells become differentiated into tissue macrophages, apoptosis is managed via an alternative pathway.

When macrophages were activated with LPS, only the use of the MAPK inhibitor, MEK, brought about a reversal in apoptosis. This would suggest that nCRP induces apoptosis via a different pathway than those in monocytes, and that by inhibiting the MAP/ERK pathway in this case would result in the inhibition of apoptosis. However, the inhibitor LML did not reverse the effect of nCRP, again

suggesting that nCRP may be inducing apoptosis via a different pathway. Further investigations would be needed to confirm the precise pathways that are being utilised by the native CRP isoform to induce apoptosis.

Evidence could also indicate that treatment with nCRP is leading to the increased clearance of cells. The nCRP could be binding to apoptotic cells in and triggering the classical complement pathway (Gershov *et al*, 2000), resulting in protection against the assembly of terminal complement membrane complex and promotes a non-inflammatory clearance of cells (Khreiss *et al,* 2000). This could potentially explain why there is a decline in the growth of the cells but does not explain why apoptosis is only seen in cells that have been treated with only high concentrations of nCRP.

## 5.9 Future Work

These assays only took into consideration four states of inflammatory cell and could be repeated with other inflammatory cells such as neutrophils. Another expansion would be to treat the monocytes and the macrophages with other activators in order to establish their effect (e.g. cytokines) upon the effects of CRP.

The apoptosis assay could also be repeated with numerous other inhibitors to identify specific apoptosis pathways being utilised by nCRP or if there is a combination of pathways working in concert with each other.

## 5.10 Summary

Both CRP isoforms caused a decline in the number of monocytes and macrophages however only treatment with nCRP caused apoptosis to occur. Treatment with mCRP did not cause apoptosis, suggesting the net cell death observed following mCRP may be caused by other mechanisms such as necrosis. Evidence suggests that nCRP causes apoptosis through a variety of pathways.

These growth and viability assays have provided evidence to determine the range of exogenous CRP concentrations to use in the project. The findings indicate that the effect on CRP isoforms on cell number and viability should also be taken into consideration when calculating the effects of the CRP isoforms on other cell properties and assays investigated throughout this study.

# Chapter 6: The Effect of Exogenous C-Reactive protein on Nitric Oxide Production Assay

## 6.1 Introduction

### 6.1.1. Nitric Oxide

Macrophages and other phagocytes utilise many different processes in inflammation. One of these is the generation of reactive oxygen and nitogen species (ROS and RNS). These molecules are used to eradicate material that have been engulfed by the phagocytes. These species are generated in response to inflammatory stimuli such as LPS on bacteria, and results in a process called the respiratoy burst (Neidhart, 1996). These bursts mainly result in the production of ROS by phagocytes however nitric oxide, an RNS, is produced by almost all nucleated cells (Nathan, 1992).

Nitric oxide (NO) is a short lived, pleiotropic free radical regulator which has various biological function in process including vasodilation, neurotransmission, inflammation and macrophage-mediated immunity (Weiming *et al,* 2002). This intercellular signalling molecule is important for the immune system as well as generating free oxygen radicals called peroxynitrites (ONOO-) which can act in a cytotoxic manner causing tissue damage and apoptosis (Bogdan, 2001). NO has been identified as playing a functional role in processes such as leukocyte adhesion and transmigration, cytokine expression as well as proliferation and apoptosis (Clancy *et al*, 1998; Rawlingson, 2003; Cross & Wilson, 2003; Korhonen *et al*, 2005).

Due to the versatility of nitric oxide in its responses it can be classed as having both pro- and anti-inflammatory effects ; however the inhibition of the pathways has shown to beneficial to the treatment of inflammatory disease (Aktan *et al*, 2003). There are several factors that affect the effectiveness of NO. These are the concentration of NO, the rate of reactive nitrogen species formation and the physiological environment. Nitric oxide has properties that allow it to be easily

soluble and diffuse across membrane to conduct its intracellular processes (Coleman, 2001).

During the inflammation process pro-inflammatory cytokines lead to the expression of NO in monocytes and macrophages as well as in neutrophils leading to large amounts being produced beyond normal physiological concentrations (Sharma *et al*, 2007). This amount can also be up to 1000-fold higher than normal (Förstermann *et al,* 1994). Nitric oxide also acts as an inflammatory mediator, a positive feedback molecule which is released by macrophages during phagocytosis in order to recruit more phagocytes to the tissue. However too much NO can lead to tissue destruction as seen in autoimmune diseases, providing evidence for both anti- and pro-inflammatory responses (Pfeilschifter *et al*, 1996).

NO biosynthesis occurs as a by-product during the conversion of L-arginine to L-citrulline including other substrates nicontinamide adenine dinucleotide phosphate (NADPH) as an electron donor and oxygen (Aktan, 2003). This two step oxidative reaction is catalysed by one of four nitric oxide synthases (NOS); endothelial NOS (eNOS), neural NOS (nNOS), inducible NOS (iNOS) and the lesser known mitochondrial NOS (mtNOS) (Giulivi, 2003). These subtypes of synthases depend on the tissue type they are located in; with eNOS and nNOS found in endothliuem. The subtype, iNOS is the only one that is soluble and is not present in resting cells (Aktan *et al*, 2003).

Stimulation of iNOS occurs as a result of immunostimulatory cytokines, bacterial products or infection (Nathan, 1992) and it generates NO in a manner independent of calcium levels (Aktan *et al*, 2003).

The structure of iNOS is classified as having a bi-domain consisting of a c-terminal and an n-terminal. The c-terminal acts a reductase domain that contains the binding site for NADPH and other signalling molecules. The n-terminal acts as an oxygenase domain with the biding site for heme and L-arginine (Aktan *et al*, 2003).

Macrophages express iNOS in the early stages of the wounding process. The production of iNOS in macrophage is mediated by Toll-like receptors and CD14. The

CD14 receptor is a receptor for LPS activation and leads to the activation of the nuclear factor-κβ (NF-κβ) pathway. IFN-γ can also induce the production of iNOS via the JAK-STAT signalling pathway, increasing both iNOS and NO production (Korhonen *et al*, 2005). Inhibition of iNOS significantly impairs reepithelialisation, increases the rate of closure of full thickness wounds and decreases collagen deposition (Park & Barbul, 2004).

The levels of NO produced can be regulated at the transcriptional level depending on the cell type and the type of stimulation that was utilised to activate the pathway. Other pathways can also be affected in this process such as the MAP kinase and PI3 kinase pathways (Aktan *et al*, 2003). There is conflicting evidence if the p38 pathway is involved, with studies showing that there is upregulation, downregulation and no effect on NO production via this pathway (Chan *et al*, 1999; Cho *et al*, 2002). NO can also regulate its own production through the use of both positive and negative feedback loops. The positive feedback utilises the increase in cAMP levels to activate the production of iNOS and increase NO production. The negative feedback loop uses the inhibition of NFκβ to lower NO production (Aktan *et al*, 2003).

### 6.1.2 Nitric Oxide and estrogen

Nitric oxide is regulated by many differeing endogenous factors inlcuding hormones including estrogen (Karpuzoglu & Ahmed, 2006). Studies have shown that estrogen has the ability to increase NO production suggesting a role in the inducation of vasodilation in the cardiovascular system (Nevzati *et al,* 2015) and the implication in the pathogenesis of other disorders (Karpuzoglu & Ahmed, 2006).

Estrogen is known to increase the generation of iNOS and NO in immune cells by upregulating the levels of iNOS mRNA, iNOS protein and NO. This is achieved through the mediation of interferon-gamma (IFN-γ) which is a pro-inflammatory cytokine which is enhanced through estrogen (Karpuzoglu & Ahmed,

2006). These studies have indicated a role for estrogen in inflammation and immunse processes but this has not been fully elucidated.

### 6.1.3 Nitric Oxide and C-Reactive Protein

The role of CRP in nitric oxide production has been identified in a few studies. Several studies have revealed that nCRP inhibits NO production via downregulation of eNOS in cardiovascular endothelial cells, thereby inhibiting angiogenesis *in vitro.* This promotes the pathogenesis of atherosclerotic vascular disease through vasoconstriction, leukocyte adherence and inflammation (Verma *et al*, 2002; Venugopal *et al*, 2002; Devaraj *et al*, 2006; Singh *et al*, 2007). The restrictive factor of this research is that the CRP isoform responsible for this result was not specified but it is assumed that it was the native protein. Another study found that nCRP downregulated eNOS and thus impaired endothelial function in ApoE knockout mice, in a mechanism thought to involve iNOS (Schwedler et al., 2007). This would indicate that the other studies mentioned previously were possibly researching the pentameric protein as well. Eisenhardt *et al* (2009) also stated that CRP upregulated the expression of adhesion molecules and inhibited endothelial nitric oxide synthase expression indicating a role for CRP in the production of NO.

C-reactive protein has the ability to attenuate NO production which is associated with a marked reduction in *in vitro* angiogenesis but it was also shown that both wound cell migration and capillary like tube formation are inhibited by CRP at concentrations known to cause cardiovascular risk (Verma *et al,* 2002).

Eisenhardt *et al* (2009) provided evidence that nCRP suppresses endothelium dependent nitric oxide-mediated dilation by activating the p38 mitogen activated protein kinase (MAP kinase) pathway and NADPH oxidase suggesting multiple pathways could be interacting with this process.

Other studies have indicated that mCRP has the opposite effect with enhanced NO production in neutrophils via upregulation of eNOS (Khreiss *et al*,

2005). This study found that when neutrophils were incubated with mCRP there was a significant increase in the amount of NO that was produced. When reverse transcriptase was performed it showed that there was an amplification of the eNOS mRNA, but not that of iNOS or nNOS. This study also highlighted that there is a calcium ($Ca^{2+}$) mobilisation and activation of calmodulin and PI3 kinase in order for mCRP to induce NO formation (Khreiss *et al,* 2005).

There is very little research that has been conducted into the effect of CRP isoforms on the production of iNOS and NO in either monocytes or macrophages to date.

## 6.2 Aims

The aim of this study was to analyse the effect of the CRP isoforms on the production of nitric oxide in monocytes and macrophages. The second aim was to infer specific signalling pathways being utilised by the C-reactive protein isoforms by using pharmacological inhibitors to block/reverse the effect of CRP isoforms on NO production. The last aim was to investigate the influence of ageing on CRP-mediated NO production by using estrogen supplementation to model the responses during youth.

## 6.3 Objectives

The objectives of this assay were to:

- Determine the effect of the nCRP and mCRP isoforms on the production of NO.
- Investigate if pharmacological inhibitors can reverse CRP-mediated NO production. Investigate the effect of estrogen supplementation on CRP-mediated NO production.

## 6.4 Summary of Procedure

The method of detecting NO in this study was via the Griess assay (Griess, 1879). This assay is based upon the diazotition reaction that uses 2% sulphanilamide and 0.2% *N*-1-napthylethylenediamine dihydrochloride (NED) under 5% phosphoric acid conditions (Giustarini *et al,* 2008). The assay detects nitrites ($NO_2^-$) which are a by-product of NO synthesis, which is stable. The procedure involves a chemical reaction when treated with NED that generates a pink colour as nitrites form a salt. This colour is then detected at a wavelength of 540nm, with a darker pink colour being indicating a higher concentration (Sun *et al,* 2003).

## 6.5 Materials

Human monocyte cell line U937 (Health Protection Agency Culture Collections, Salisbury)

RPMI-1640 Media *(Lonza, Slough)*

Foetal Bovine Serum *(Sigma-Aldrich, Dorset)*

Lipopolysaccharide *(Sigma-Aldrich, Dorset)*

Phorbol 12-myristate 13-acetate *(Applichem, Darmstadt)*

C-reactive Protein, human >95% native, 5mg/ml (*MyBioSource, San Diego)* – Purified as previously stated in Section 2.4.4.

Inhibitors – nystatin prepared as mentioned in section 2.4.9.

Estrogen *(Sigma-Aldrich, Dorset)*

Modified Griess Reagent *(Sigma-Aldrich, Dorset)*

Sodium Nitrite *(Sigma-Aldrich, Dorset)*

## 6.6 Methods

### 6.6.1 Cell Culture

Cells were cultured as previously stated in Section 2.4.1.

### 6.6.2 Activation of Cells

Cells were activated as stated in Section 2.4.2.

### 6.6.3 Differentiation of Cells

Cells were differentiated as stated in Section 2.4.3.

### 6.6.4 CRP Purification

CRP was purified as stated in Section 2.4.4. The samples were then diluted to experimental concentrations using RPMI-1640 media.

### 6.6.5 Estrogen Preparation

Estrogen was prepared at three final concentrations of $1x10^{-7}$M, $1x10^{-8}$M and $1x10^{-9}$M, made up in RPMI-1640 complete media with FBS and penicillin-streptomycin.

### 6.6.6 Griess Reagent Assay

Cells at a concentration of $1x10^{6}$ cells/ml were incubated with physiological concentrations of mCRP (100µg/ml or 250µg/ml) or nCRP (500µg/ml or 1000µg/ml) for 24 hours. NO production was then measured in each treatment group (n=12) by the Griess method (Griess, 1879) with the absorbance of a 1:1 ratio of sample and

Griess reagent read using a Biotech Synergy HT ELISA plate reader at a wavelength of 540nm. Concentrations were calculated against a standard curve based upon a set of standards of sodium nitrite (0µM, 1µM, 10µM, 25µM, 50µM, 75µM and 100µM) and an example if this can be seen in Appendix 5. In the inhibitor assay and ageing model, cells were co-incubated with inhibitors or estrogen during the CRP treatment phase.

### 6.6.7 Inhibitors

The Greiss reagent assay was repeated (Section 6.6.6) with the addition of the inhibitor nystatin, prepared as stated in Section 2.4.9. Some potential inhibitors, wortmannin, MEK and LML, were not used due to known direct effect on iNOS and nitric oxide production (Salh *et al,* 1998; Ajizian *et al*, 1999; Kim *et al,* 2004).

### 6.6.8 Statistical Analysis

Raw data was recalculated for adjust for conditions and cell number. Treatment groups were analysed for normality using a Shapiro-Wilke normality test. Results were statistically compared by t-tests or Mann-Whitney U-test comparisons using SPSS (Version 22) and $P<0.05$ was considered significant in all cases. Graphs were generated using Microsoft Excel 2013.

## 6.7 Results

### 6.7.1 The Effect of Exogenous CRP Isoforms on Nitric Oxide Production.

Figure 6.1 indicates the effect that each isoform has upon NO production in each cell model. Monocytic cells exhibited a significant increase in the production of NO at both concentrations of mCRP when compared to the controls. A

significantly greater increase in NO was seen when the cells were not treated with LPS. Both concentrations of mCRP did not have any significant effect upon NO production in the macrophage and activated macrophage cells.

When treated with the lower concentration of nCRP, only the unactivated monocytes showed no significant (p=0.08) difference in the production of NO. The other three cell states exhibited a significant reduction in the amount of NO produced.

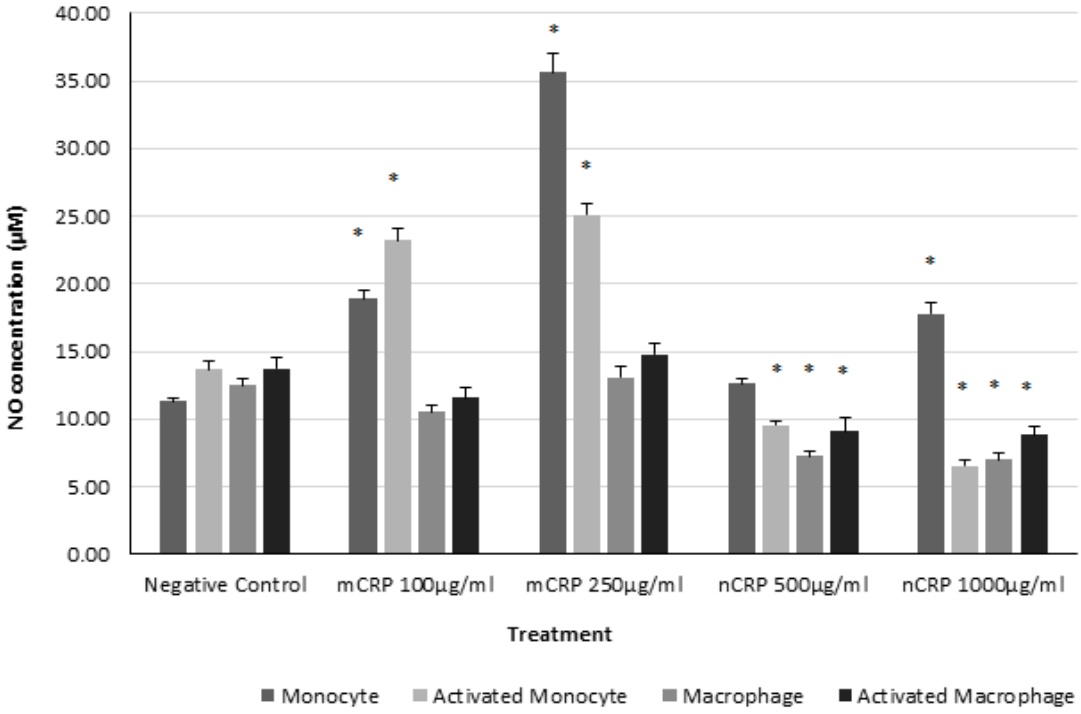



**Figure 6.1 The effect of C-reactive protein isoforms on nitric oxide production in several cell states (monocyte, activated monocyte, macrophage and activated macrophage).** * indicates significant differences (P<0.05) compared with corresponding control.

At the higher concentration of nCRP there was a significant (p=0.036) increase in the production of NO in unactivated monocytes (from 11.29µM to 17.77µM) while the other three cell states exhibited a significant reduction in the production of NO, with the greatest reduction (from 13.66µM to 6.52µM) observed in the activated monocytes.

Samples with significant changes in the production of NO were repeated with the inclusion of pharmacological inhibitors in order to evaluate their ability of reverse the CRP-mediated NO production, thereby indicating potential CRP-mediated pathways.

### 6.7.2 The Effect of Inhibitors on Nitric Oxide Production in C-Reactive Protein Treated Cells

Sample treatments that exhibited a significant change in NO production were repeated with the nystatin inhibitor. Only the nystatin inhibitor was used for this element of the project as the three other inhibitors used in other chapters were known to have a direct effect upon NO production. Figure 6.2 and 6.3 show the successful reversal of NO production by nystatin (non-significant results not shown) for figure clarity.

When unactivated monocytes were treated with 1000μg/ml nCRP there was a significant increase (from 11.29μM to 17.77μM; $p = 0.036$) in the production of NO. When co-treated with the nystatin inhibitor there was a significant ($p=0.001$) reversal in NO production compared to original stimulation of NO produced by 1000μg/ml nCRP. After treatment with nystatin inhibitor, there was no significant ($p=0.112$) difference in production of NO compared to the control, indicating nCRP-induced NO production was fully reversed.

Treatment of activated monocytes with 100 μg/ml mCRP induced a significant increase (from 13.66μM to 23.23μM; $p<0.001$) in the production of NO. Again, nystatin reversed the effect of mCRP-induced NO production, with no significant difference ($p=0.504$) following nystatin treatment compared to the control.

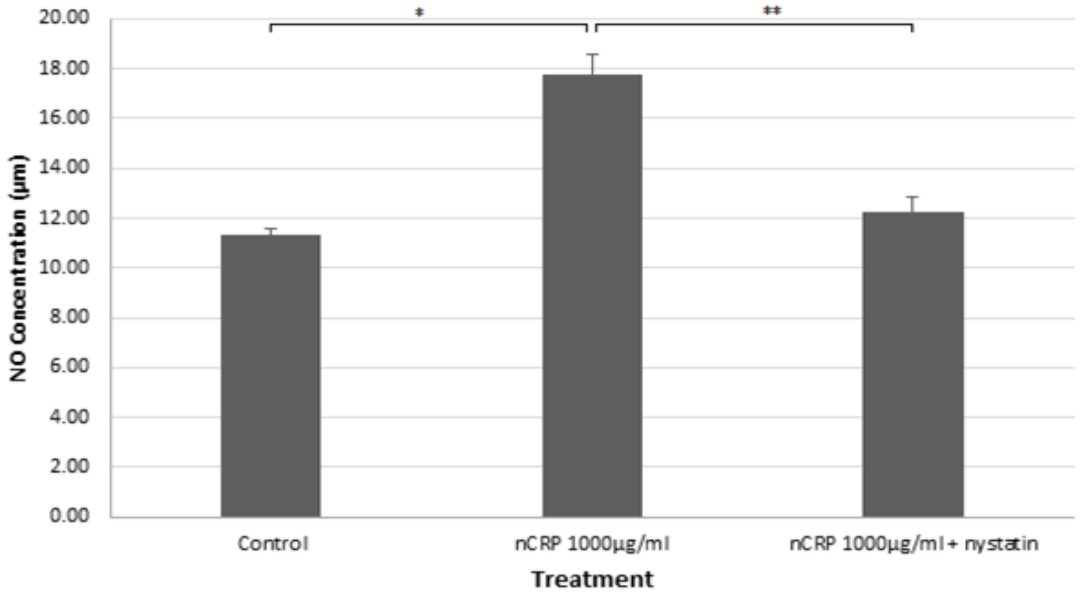



**Figure 6.2 –Reversal of CRP-mediated NO production by nystatin inhibitor in CRP treated unactivated monocyte cells.** * indicates significant (P<0.05) differences compared with the control. ** indicates significant (P<0.05) differences compared with the inhibited CRP treatment.

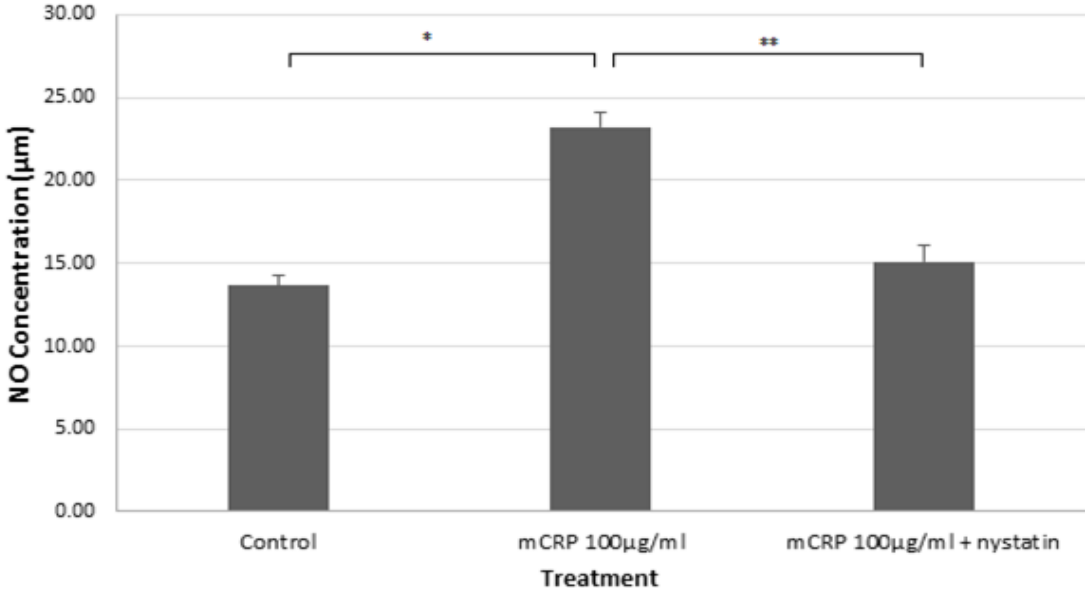



**Figure 6.3 – Reversal of CRP-mediated NO production by nystatin inhibitor in CRP treated activated monocyte cells.** * indicates significant (P<0.05) differences compared with the control. ** indicates significant (P<0.05) differences compared with the inhibited CRP treatment.

### 6.7.3 The Effect of Estrogen on CRP-Induced Nitric Oxide Production

#### 6.7.3.1 Unactivated Monocytes

Treatment with either CRP isoform significantly ($P<0.001$) increased NO production in unactivated monocytes. However, when estrogen was supplemented the concentration of NO significantly reduced (Figure 6.4) in unactivated monocytes at all three estrogen concentrations.

NO significantly increased as the concentration of mCRP increased (Figures 6.4A and 6.4B) in unactivated monocytes. The addition of estrogen significantly reduced ($P<0.01$) the amount of NO produced to a level similar to that of the control (around 10µM).

Estrogen had a concentration dependent effect on NO production in unactivated monocytes treated with nCRP (Figures 6.4C and 6.4D). Both concentrations of nCRP significantly increased the amount of NO to around 12µM. Treatment with $1x10^{-7}$M estrogen produced the greatest (12.61µM to 4.82µM) reduction in NO production in unactivated monocytes. All concentrations of estrogen significantly ($p<0.001$) reduced the amount of NO produced in unactivated monocytes following nCRP treatment.

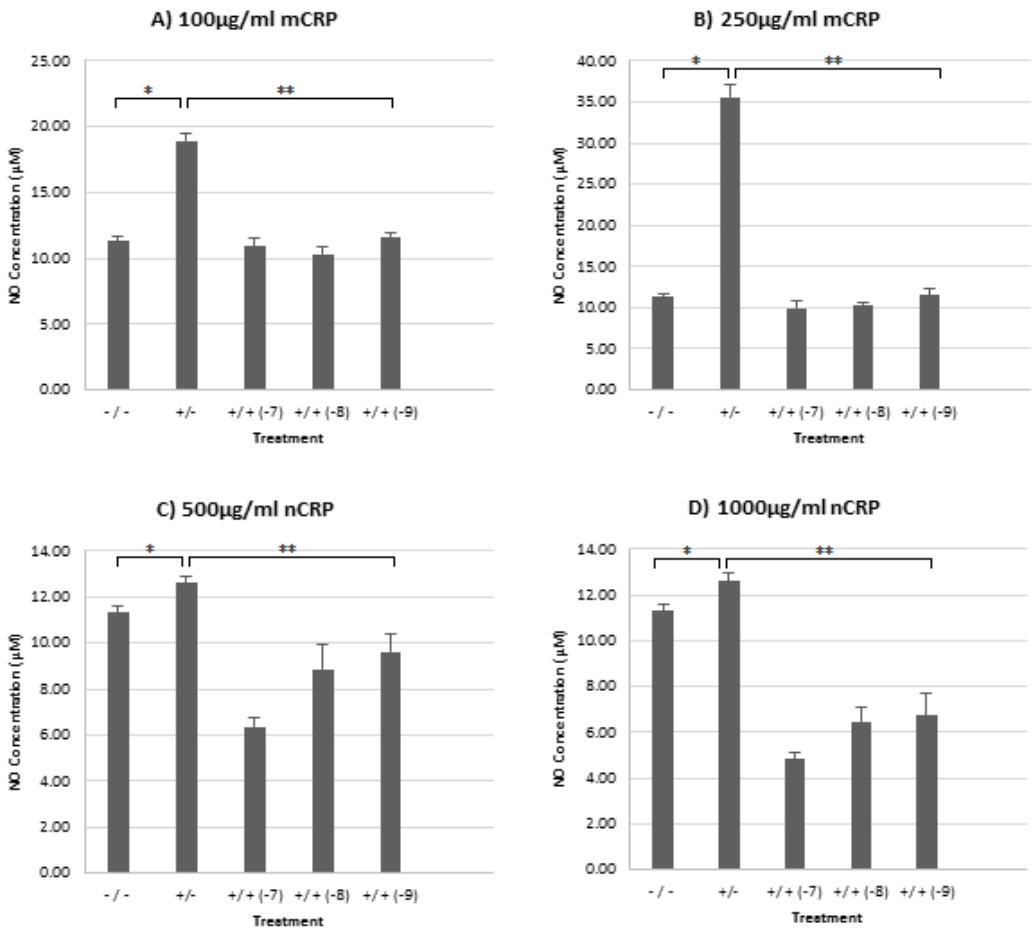



**Figure 6.4 – Nitric oxide production in unactivated monocytes treated with three concentrations of estrogen.** -/- represents the negative control with no CRP and no estrogen supplementation. +/- represents treatment with CRP alone as a positive control. +/+ represents cells that have been treated with both CRP and estrogen. The number in the bracket represent which concentration of estrogen has been used (either $1x10^{-7}$M, $1x10^{-8}$M or $1x10^{-9}$M estrogen). * denotes significant difference between the negative and positive control. ** denotes significant difference to the positive control. Significant difference is $p<0.05$. Errors bars represent SE of the mean (SEM).

#### 6.7.3.2 Activated Monocytes

The concentration of NO produced in activated monocytes shows a similar pattern to the unactivated monocytes in the case of mCRP. With the addition of mCRP the amount of NO increased significantly ($P<0.001$). However, there was a dose dependent significant decrease in NO production in activated monocytes following treatment with nCRP. Estrogen at all of the three concentrations reduced the concentration of NO in activated monocytes. In contrast, treatment with estrogen had no significant effect on NO production in nCRP treated cells, with NO production remaining relatively constant (Figure 6.5).

When mCRP was used as a treatment the amount of NO produced significantly increased ($P<0.01$) as the concentration of mCRP increased (Figures 6.5A and 6.5B). The addition of estrogen significantly reduced the amount of NO produced in activated macrophages to a level similar to the control (around 10µM NO

The data indicated that even though both concentrations of nCRP significantly reduced NO production ($P<0.01$), there was no significant effect of estrogen upon nCRP-mediated NO production in activated monocytes (Figures 6.5C and 6.5D).

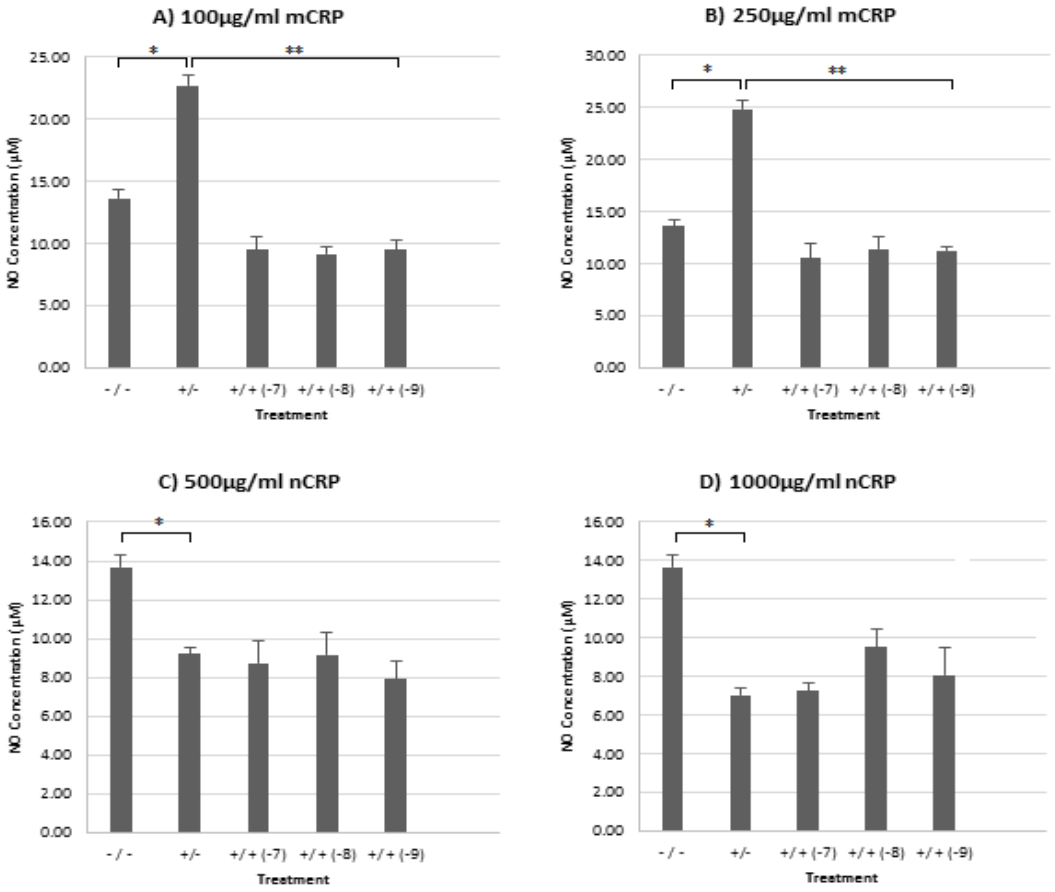



**Figure 6.5 – Nitric oxide production in activated monocytes treated with three concentrations of estrogen.** -/- represents the negative control with no CRP and no estrogen. +/- represents treatment with CRP alone as a positive control. +/+ represents cells that have been treated with both CRP and estrogen. The number in the bracket represent which concentration of estrogen has been used (either $1x10^{-}$M, $1x10^{-8}$M or $1x10^{-9}$M estrogen). * denotes significant difference ($p<0.05$) between the negative and positive control. ** denotes significant difference ($p<0.05$) to the positive control. Errors bars represent SE of the mean (SEM).

#### 6.7.3.3 Unactivated Macrophages

The concentration of NO produced in unactivated macrophages shows that both concentrations of mCRP caused no significant effects upon the production of NO and supplementation with estrogen had no significant effect either (Figures 6.6A and 6.6B).

Both concentrations of nCRP caused a significant reduction in the production of NO compared to the control, approximately halving the NO from around 12.0 μM to around 6.5 μM regardless of the nCRP concentrations used (Figure 6.6C and 6.6D).

All three concentrations of estrogen significantly increased ($P<0.01$) the NO concentration following treatment with 500μg/ml nCRP when compared to the 500μg/ml nCRP treatment alone (Figure 6.6C). However, estrogen at all the concentrations used were unable to reverse the nCRP-induced NO production when 1000μg/ml nCRP was used (Figure 6.6D).

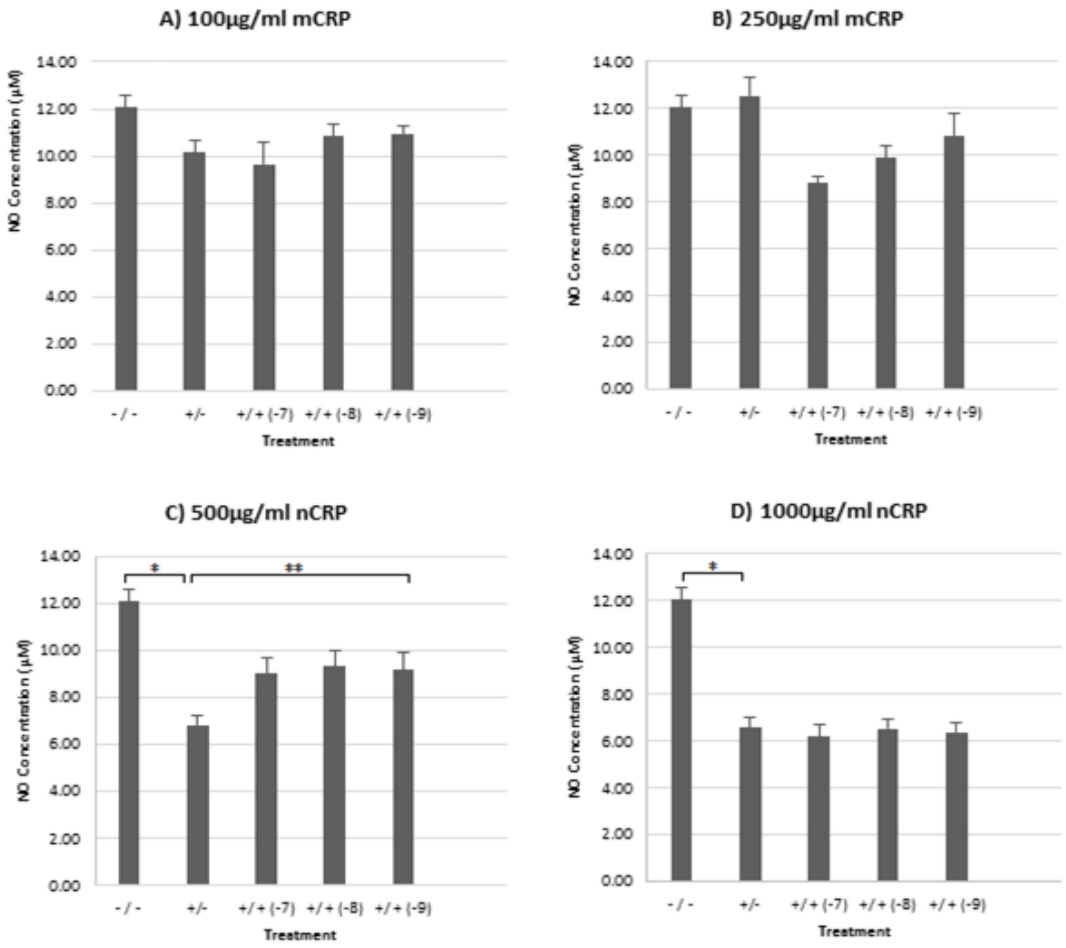



**Figure 6.6 – Nitric oxide production in unactivated macrophages treated with three concentrations of estrogen.** -/- represents the negative control with no CRP and no estrogen. +/- represents treatment with CRP alone as a positive control. +/+ represents cells that have been treated with both CRP and estrogen. The number in the bracket represent which concentration of estrogen has been used (either $1x10^{-7}$M, $1x10^{-8}$M or $1x10^{-9}$M estrogen). * denotes significant ($p<0.05$) difference between the negative and positive control. ** denotes significant ($p<0.05$) difference to the positive control. Errors bars represent SE of the mean (SEM).

#### 6.7.3.4 Activated Macrophages

Both concentrations of mCRP had no significant effect upon the production of NO in activated macrophages and the addition of estrogen had no significant effect on the production of NO (Figures 6.7A and 6.7B).

Both concentrations of nCRP significantly ($p<0.001$ and $p<0.001$) reduced the amount of NO produced by activated macrophages and supplementation with estrogen had no significant effect on this nCRP-induced NO production (Figures 6.7C and 6.7D).

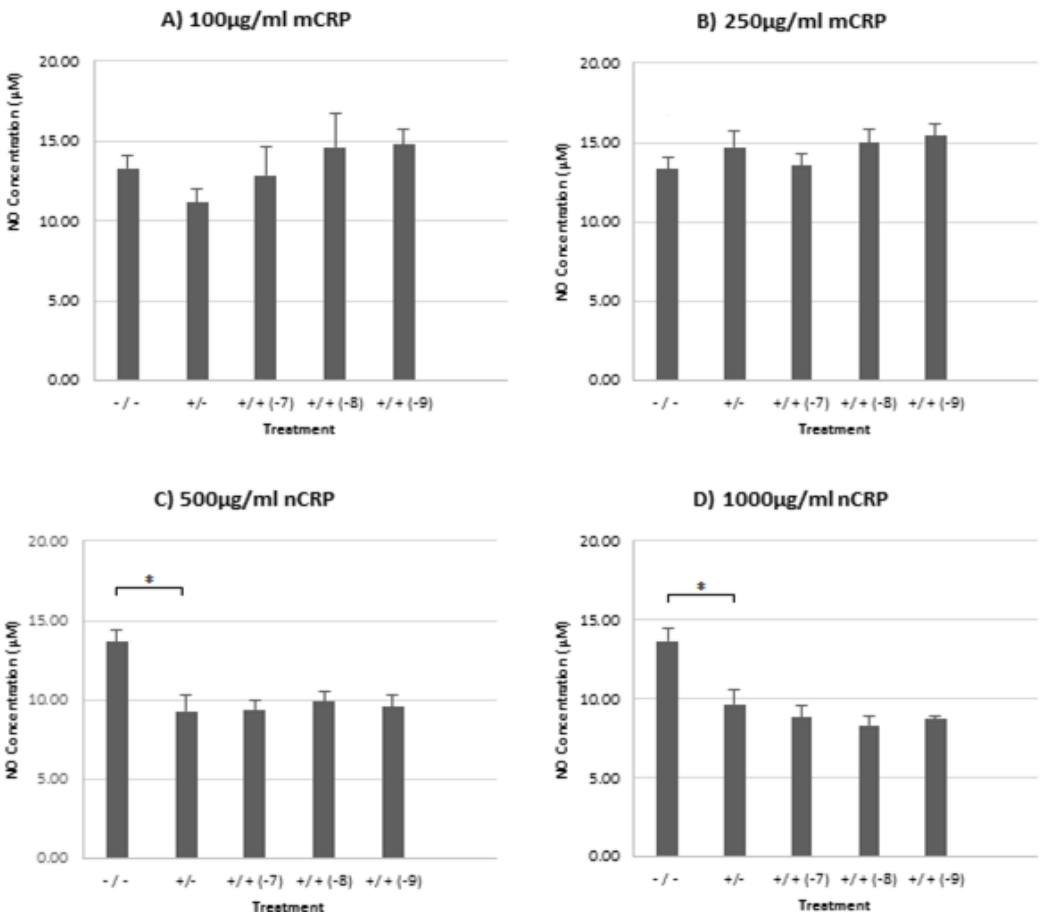



**Figure 6.7 – Nitric oxide production in activated macrophages treated with three concentrations of estrogen.** -/- represents the negative control with no CRP and no estrogen. +/- represents treatment with CRP alone as a positive control. +/+ represents cells that have been treated with both CRP and estrogen. The number in the bracket represent which concentration of estrogen has been used (either $1x10^{-7}$M, $1x10^{-8}$M or $1x10^{-9}$M estrogen). * denotes significant ($p<0.05$) difference between the negative and positive control. ** denotes significant ($p<0.05$) difference to the positive control. Errors bars represent SE of the mean (SEM).

## 6.8 Discussion

### 6.8.1 The Effect of Exogenous CRP Isoforms on Nitric Oxide Production.

The mCRP isoform stimulated NO production in monocyte-like cells but had no effect on NO levels in macrophage-like cells, regardless of activation by LPS (bacterial endotoxin). This suggests mCRP may stimulate NO production in both the presence and absence of infection, supporting evidence from Khreiss *et al (*2005).

In stark contrast, the nCRP isoform reduced NO production in macrophage-like cells both in the presence and absence of endotoxin activation. Furthermore, in monocytes activated by bacterial endotoxin nCRP reduced NO levels in a concentration-dependent manner. These findings support the growing evidence that mCRP promotes inflammation whilst nCRP often opposes the actions of mCRP and reduces the overall inflammatory response (Ciubotaru *et al*, 2005; Eisenhardt *et al*, 2009).

The response to each CRP isoform appeared to be dependent on the differentiation state of cells and whether pre-activation with bacterial endotoxin had taken place. The findings indicated nCRP may have an inhibitory effect on NO production in monocytes following activation by bacterial endotoxin, and in macrophages with or without prior LPS-activation.

In contrast, mCRP had a stimulatory effect on NO production in monocytes with or without prior activation by bacterial endotoxin but this effect was lost once monocytes differentiated into macrophages. In the absence of infection, the findings indicate both nCRP and mCRP may increase NO production in unactivated monocytes but the stimulation by nCRP is relatively modest compared to that produced by mCRP.

The relative proportion of each CRP isoform *in vivo* could possibly influence the inflammatory response generated, with the formation of mCRP from the dissociation of nCRP promoting inflammation. Elevated levels of NO are known to induce the production of pro-inflammatory cytokines (Kobayashi, 2010) including

IL-6 (Hur *et al*, 2004) and tumour necrosis factor alpha (TNF-α) in both monocytes and macrophages (Wang *et al,* 1997*)* and neutrophils (Dervort *et al*, 1994). The release of these cytokines can then potentially stimulate iNOS further to produce more NO (Nathan, 1992) There is also evidence of NO increasing phagocyte function in terms of bacterial killing and phagocytosis (Tumer *et al*, 2007).

### 6.8.2 The effect of inhibitors upon the production of nitric oxide in CRP treated cells

Nystatin was the only inhibitor that was applied in this study. The other inhibitors could not be used due to their ability to have a direct effect upon the NO production (Salh *et al,* 1998; Ajizian *et al*, 1999; Kim *et al*, 2004) and, therefore may have provided misleading results.

Nystatin showed inhibitory effects in only two cell treatments; with 1000µg/ml nCRP in unactivated monocytes and with 100µg/ml mCRP treatment in activated monocytes. This suggests that nCRP may interact with lipid rafts in monocytes in the absence of infection whereas mCRP may associate with lipid rafts in monocytes in the presence of infection. In both unactivated and activated macrophages, nystatin had no significant effect on the production of NO. This suggests that either the nystatin concentration was too low to detect effects or once monocytes become mature macrophages the disruption of lipid rafts by nystatin has no effect on CRP isoform function in relation to NO production. Findings show that mCRP had no effect on NO production in macrophages, in the presence or absence of infection, so nystatin inhibition would be irrelevant in that case. In terms of nCRP that reduced NO production in macrophages, it would suggest either the inhibitor concentration was too low to have effect or that this isoform was not associated with lipid rafts in mature macrophages and therefore nystatin would have no effect on nCRP-induced NO production. Data from the inhibitor assay can only provide tentative evidence to include/confirm involvement of particular processes/pathways but cannot provide evidence to exclude them

(since an increase in inhibitor concentration may show effects not seen at the concentration used). Therefore, no conclusions can be drawn about the mechanisms by which nCRP may reduce NO production in macrophages.

Nystatin is an inhibitor of the lipid-raft-caveolae endocytosis pathway (Ivanov, 2008) as well as being able to form barrel like pores in the membrane which leads to potassium leakage (Chen *et al,* 2011). These lipid rafts are enriched in cholesterol and glycosphingolipids which are involved in many trafficking pathways (Chen *et al,* 2011). The mCRP isoform is known to associate with lipid rafts in the cell membranes (Ji, *et al.,* 2009). When activated monocytes are treated with higher concentrations of mCRP in particular, this is likely to lead to a more pro-inflammatory effect as the concentration of NO increases. As cell membranes become disrupted by the nystatin, NO production is reduced and this may be at least in part due to disruption of the interaction of mCRP with lipid rafts. .

The effect of the lower concentration of mCRP on NO production in activated monocytes was reversed with the addition of nystatin whereas at higher concentrations of mCRP the reversal was not detected. This was most likely due to the inhibitor concentration being too low to fully reverse the effects of high concentrations of mCRP. Future studies should investigate a range of inhibitor concentrations but this was beyond the scope of this current study due to time and cost restrictions.

The 100µg/ml mCRP treatment caused the concentration of NO to significantly increase in activated monocytes, while treatment with the nystatin significantly decreased NO production back to control levels. The mCRP is known to associate with the cell membrane of the inflammatory cells and as nystatin works by disrupting the lipid-rafts in the cell membrane (Scheel-Toellner *et al,* 2004). If the lipid rafts of cell membranes are disrupted by nystatin then mCRP may be prevented from inducing NO production. No production in macrophages treated with nCRP was not significantly affected by the addition of the nystatin suggesting that either the inhibitor concentration was too low to see effects or that the nCRP isoform may not associate with lipid rafts in mature macrophages. Therefore, the

inhibitor assay provides evidence to suggest mCRP associates with lipid rafts in monocytes in the presence and absence of infection and that NO production is probably mediated at least in part by lipid raft associated mCRP. Beyond this, the inhibitor assay does not provide any evidence relating to nCRP or other cell states.

### 6.8.3 The Effect of Estrogen Upon the Production of Nitric Oxide in CRP Treated Cells

From the data it seems that physiological concentrations of estrogen had opposing effects on CRP-induced NO production in the various cells states. Previous studies have shown that estrogen can have suppressive and enhancing effects upon iNOS and NO production (Karpuzoglu & Ahmed, 2006).

#### 6.8.3.1. Monocytes

Evidence from the data shows that when the monocytes have not been previously activated with bacterial LPS there is an increase in NO production, suggesting pro-inflammatory activity. Estrogen significantly reduced the NO production in CRP-treated cells, indicating that estrogen is behaving in an anti-inflammatory way in the presence of CRP, typical of its properties during wound healing during youth.

When monocytes were pre-activated using LPS, only treatment with mCRP significantly increased NO production. This mCRP-induced NO production in LPS-activated cells was reversed with estrogen treatment. In contrast, treatment with nCRP significantly decreased NO production with no effect observed when supplemented with physiological concentrations of estrogen. Evidence has shown that mCRP has a more pro-inflammatory effect during inflammation (Thiele *et al,* 2014) and therefore will increase the amount of NO. In monocytes, estrogen can suppress inflammation by inhibiting the IL-6 inflammatory response which leads to the inhibition of NF-κβ activity (Pelekanou *et al,* 2016). Estrogen also has the ability

to block pro-inflammatory effects of inflammation triggered by LPS -activation which is an effect mirrored here in LPS-activated monocytes following mCRP treatment (Vegeto *et al,* 2000; Vegeto *et al,* 2001).

#### 6.8.3.2 Macrophages

In both the unactivated and LPS-activated macrophages, treatment with mCRP (with or without estrogen supplementation) had no effect upon NO production. Treatment with both nCRP concentrations caused a significant decrease in NO production, indicative of a more anti-inflammatory effect (Thiele *et al,* 2014).

The nCRP-induced reduction of NO in macrophages was only reversed by estrogen in unactivated macrophages treated with 500µg/ml nCRP (physiological concentrations of estrogen were unable to reverse the effect of very high 1000µg/ml concentrations of nCRP). In concordance with this finding, Karpuzoglu & Ahmed (2006) found that unactivated macrophages exposed to estrogen show increased iNOS expression and therefore NO production. This indicates a pro-inflammatory effect of estrogen in unactivated macrophages. Evidence from the literature agrees with this finding since estrogen has been shown to be a potent inducer of IFN-γ (Fox *et al*, 1991), which is secreted by tissue macrophages (Duque & Descoteaux, 2015). This increase in IFN-γ leads to a subsequent increase in the production of nitric oxide (Karpuzoglu & Ahmed, 2006).

## 6.9 Future Work

The findings of this study can be utilised as a basis to investigate which particular nitric oxide synthases (NOS) are generating the NO production. Evidence would suggest that iNOS would be the synthase involved and this could be confirmed by western or dot blots on cell lysate samples to analyse changes in iNOS production using an antibody raised against iNOS. Such an investigation could

assess the effect of CRP isoform concentrations on iNOS levels, followed by work with the pharmacological inhibitors and estrogen supplementation.

The relationship between CRP isoforms and NO levels could be investigated *in vivo* using animal models of wound healing. Using mouse models of acute and age-related impaired healing, it would be possible to determine the effect of CRP isoforms on NO production in relation to ageing processes.

## 6.10 Summary

In conclusion, the findings suggest that both mCRP and nCRP may be potential targets for mediating NO levels in human monocytes and macrophages. NO levels were differentially altered by each CRP isoform in a concentration-dependent manner and responses were dependent on the differentiation state of the cells (monocyte versus macrophage-like cells) and whether there was pre-stimulation with bacterial endotoxin.

In the presence or absence of LPS activation, nCRP had an inhibitory effect on NO production in macrophage-like cells whereas mCRP had a stimulatory effect on NO synthesis in monocyte-like cells. The nCRP isoform had bi-directional effects in monocyte-like cells, reducing NO production following LPS-activation but stimulating NO in unactivated monocyte–like cells. The mCRP isoform had no effect on NO production in macrophage-like cells , regardless of whether pre-stimulation with bacterial endotoxin took place.

This study supports growing evidence that mCRP generally promotes the inflammatory response whilst nCRP often has opposing anti-inflammatory properties. This highlights potential therapeutic strategies could be developed for the treatment of chronic inflammatory conditions that promote the stability of nCRP and/or prevent the formation of mCRP from the dissociation of nCRP.

# Chapter 7 – The Effect of C-Reactive Protein Isoforms on Pro-inflammatory Cytokine Production

## 7.1 Introduction

Cytokines are a group of small proteins that induce cell signalling pathways in order to trigger the function of a number of other cells (Zhang & An, 2007). Cytokines include chemokines, interferons, interleukins and tumour necrosis factors that provide a broad range of activation in cells such as macrophages, lymphocytes, endothelial cells and fibroblasts (Zhang & An, 2007).

Cytokines act through receptors on the surfaces of cells and play major roles in the immune process as well as allowing communication between the cells (Dinarello, 2000). Cytokines can act in an autocrine action, acting upon the cells that have secreted them; or in a paracrine action, acting upon nearby cells; or in an endocrine action, acting upon distant cells (Zhang & An, 2007).

There are a number of pro-inflammatory cytokines (Dinarello, 2000) and this study investigated four that are known to play a key role in CRP-mediated inflammatory processes.

### 7.1.1 Interleukin-6 (IL-6) and C-Reactive Protein

Interleukin-6 (IL-6) is a pro-inflammatory cytokine which is encoded by the IL-6 gene. IL-6 is a major regulator of the acute phase response and is secreted by T cells and macrophages during this response. Its main roles are focused in the host's ability to fight infection (Zhang & An, 2007). Even though it is a pro-inflammatory cytokine it does exert anti-inflammatory effects through TNF-α and IL-1 (Scheller *et al,* 2011).

IL-6 is a soluble mediator of the acute phase response and is one of few cytokines that are capable of crossing the blood-brain barrier (Heinrich *et al*, 1990). IL-6 is secreted in response to pathogen-associated molecular patterns (PAMPs) on

pathogens or damage-associated molecular patterns (DAMPs) which are found on cell components in dead or damaged cells. These molecules bind to pattern recognition receptor (PRRs) which are present in the cell surface. These receptors include toll-like receptors (TLRs) and c-type lectin receptors (CLR) as well as many others. These receptors are present on cell surfaces and when activated through binding induce the intracellular signalling cascade which gives rise to cytokine production (Scheller *et al,* 2011).

The IL-6 receptor is expressed mainly on the immune effector cells such as T and B cells, monocytes, macrophages and neutrophils however they are also expressed on other non-immune cells such as the hepatocytes and pancreatic islet cells (Tanaka & Kishimoto, 2014). The receptor is comprised mainly of the gp130 glycoprotein (Kishimoto *et al,* 1995) and allows the IL-6/IL6-R complexes to associate with gp130 and leads to signal initiation (Scheller *et al,* 2011). These signals then lead to activation of several pathways including the JAK/STAT, ERK and PI3 kinase pathways (Scheller *et al,* 2011).

IL-6 is synthesised in the initial stages of inflammation and then travels in the bloodstream to the liver. During this process, the IL-6 induces a number of acute phase proteins including CRP (Tanaka *et al*, 2014). At the same time IL-6 can also reduce the production of fibronectin, albumin and transferrin as well as the promotion of $CD4^+$ T helper cells which initiates the linking of innate and acquired immunity (Tanaka *et al,* 2014).

Previous research has shown that CRP plays a role in the inflammation processes (Du Clos, 2000) and that there is a correlation between the increasing levels of IL-6 during inflammation and the increasing levels of CRP (Szalai *et al*, 1998), with IL-6 inducing the CRP gene (Weinhold *et al*, 1997).

There is little evidence that the two isoforms of CRP have varying effects upon IL-6 production and there has been no record of CRP inducing the production of IL-6 in a feedback loop during the inflammatory process. Instead, published research considers the production of CRP by IL-6 and generally fails to mention

which isoform of CRP is generated by IL-6. In most cases, it can be deduced that the CRP isoform being measured is the native isoform due to the antibodies being used to detect CRP and the fact that nCRP is the isoform known to be synthesised in the hepatocytes (Boras *et al,* 2014), where most research to date has been conducted.

### 7.1.2 Interleukin-8 (IL-8) and C-Reactive Protein

Interleukin-8 (IL-8) is a cytokine that is produced by macrophages and is a member of the CXC family (Koch *et al,* 1992). The IL-8 gene encodes a protein translation that is 99 amino acids long (Harada *et al,* 1994). IL-8 is a potent chemoattractant with neutrophils and a subset of T-lymphocytes being the main targets for this (Koch *et al,* 1992). Another role is to stimulate the release of granules from neutrophils by a process called degranulation. These granules contain a range of antimicrobial properties that can help combat infection (Palomino & Marti, 2015).

It is also suggested that IL-8 can induce the rearrangement of the cytoskeleton (Kibayashi *et al,* 2005). This is due to changes in intracellular calcium levels activing integrins. There is also evidence showing that IL-8 can also induce the exocytosis of granule proteins and activate the respiratory burst (Palomino & Marti, 2015).

It is known that IL-8 is overexpressed in chronic inflammatory diseases and during sceptic shock (Bickel, 1993). It is suggested that this is due to the neutrophil being the primary target as it expressed both CXCR1 and CXCR2. As neutrophils are the primary cells to arrive at the site of inflammation this leads to the recruitment of phagocytic cells. The process of phagocytosis allows for the release of chemotactic mediators which then recruit other leukocytes to the affected tissue (Palomino & Marti, 2015).

C-reactive protein has also been implicated in the expression patterns of IL-8. Kibayashi *et al* (2005) indicated that CRP plays a role in atherosclerosis via IL-8 production. The production of IL-8 was enhanced significantly when the cells were

treated with CRP and the expression of IL-8 mRNA increased in a CRP dose dependent manner. This effect was also inhibited by using MAPK inhibitors such as SB203580. Kibayashi *et al* (2005) concluded that CRP plays a role in atherosclerosis via the production of IL-8 and the activation of the ERK, p38 MAPK and JNK pathways.

The effect of the different isoforms upon IL-8 production has also been investigated. Khreiss *et al* (2004) showed that nCRP had no detectable effect upon the production of IL-8, however mCRP did. Treatment with mCRP increased IL-8 production as well as increasing gene expression, thus promoting pro-inflammatory activity through a p38 MAPK-dependent mechanism. When treated with anti-CD16 there was inhibition of mCRP stimulated nitric oxide formation and IL-8 release (Khreiss *et al, 2004).*

Wigmore *et al* (1997) indicated that IL-8 can work at a local level and induce CRP production in the hepatocytes, indicating that other cytokines may also be involved in the activation of the CRP pathways.

### 7.1.3 Monocyte Chemoattractant Protein-1 (MCP-1) and C-Reactive Protein

Monocyte chemoattractant protein-1 (MCP-1) is a cytokine that plays a role in the regulation of migration and infiltration of monocytes and macrophages. MCP-1 is also known as CCL2 and is a member of the C-C chemokine family. It is located on chromosome 17 at position q11.2 and is composed of 76 amino acids (Deshmane *et al, 2009*). It is released by a number of cell types in response to events such as oxidative stress, cytokine release and growth factor release (Yadav *et al*, 2010). Receptors for MCP-1 are expressed on a number of cells and human MCP is known to bind to at least two receptors (Deshmane *et al,* 2009). The production of MCP-1 can be induced by IL-4, IL-1, TNF-α, bacterial LPS and IFN-γ (Yadav *et al*, 2010).

There is also increasing evidence that MCP-1 influences T-cell immunity by enhancing the secretion of interleukin-4 (IL-4) by T cells as well as having a role in

the migration of leukocytes (Deshmane *et al,* 2009). This in turn has a regulatory function on the monocytes and macrophages which are is major source of MCP-1 (Yadav *et al*, 2010). MCP-1 is known recruit monocytes to the vessel wall (Han *et al,* 2004) as well as the ability to cause the firm arrest of rolling monocytes on endothelial monolayers which express E-selectin (Pasceri *et al,* 2001). This means there is an implied role in spreading and shape change of monocytes attached to the endothelium (Pasceri *et al,* 2001).

MCP-1 exerts its effect through binding to G-protein coupled receptors on the surface of leukocytes. This then activates a number of reactions ultimately resulting in the formation of inositol triphosphate. This then allows for intracellular calcium release and protein kinase-c (PKC) activation (Yadav *et al*, 2010). PKC activation is required for processes such as gene expression, protein secretion, cell proliferation and the inflammatory response (Poole *et al,* 2004).

Multiple pathways have been implicated in MCP-1 signal transduction, including the MAP kinase pathways ERK1 and ERK2, p38 MAP kinase pathway and the PI3 kinase pathways (Pasceri *et al,* 2001). However, the full extent of how these pathways are involved is not fully understood.

It has been suggested that CRP can influence the production and function of MCP-1. CRP can stimulate endothelial cells to express MCP-1 as well as being a monocyte chemoattractant for monocytes itself (Yeh & Willerson, 2003). CRP can promote monocyte chemotactic activity in response to MCP-1 via upregulation of the monocyte chemotaxis receptor CCR2 (Han *et al,* 2004). Han *et al* (2004) suggested that CRP can induce the production of MCP-1 by endothelial cells. This study suggested that elevated CRP levels promoted the accumulation of monocytes in the atherogenic arterial wall via MCP-1 by promoting the activity of chemokines and cytokines (Han *et al,* 2004).

When vascular smooth muscle cells were exposed to increasing levels of CRP, MCP-1 mRNA substantially increased within 2h, remaining elevated for at least 24 hours after (Hattori *et al*, 2003). Incubation with mCRP increased the secretion

of MCP-1 leading to pro-inflammatory activity through a p38 MAPK-dependent mechanism whereas nCRP had no detectable effect (Khreiss *et al,* 2004).

### 7.1.4 Tumour Necrosis Factor-α (TNF-α) and C-Reactive Protein

Tumour Necrosis Factor-α (TNF-α) is a component of the acute phase response of inflammation. Also known as cachectin, this signalling molecule is mainly produced by monocytes and macrophages but can be produced by numerous other immune cells such as neutrophils, natural killer cells and eosinophils (Zelová & Hošek, 2013). The main stimulant of TNF-α production is lipopolysaccharide (LPS) however other pathological conditions can stimulate production such as trauma infection, burns and heart failure to name a few examples (Zelová & Hošek, 2013).

TNF-α has been implicated in a wide range of inflammatory activities and is not usually detectable in a healthy host however levels become elevated in a number of inflammatory and infectious conditions (Zelová & Hošek, 2013). Evidence shows that it is involved in processes such as cell proliferation, differentiation and apoptosis.

TNF-α has shown to have many biological functions including vasodilation, by the stimulation of iNOS in macrophages and leukocytes (Sanders *et al,* 2001); involvement in oedema by augmenting the permeability of vessels during inflammation (Chappell *et al,* 2009); facilitating the adhesion of leukocytes to inflamed tissues (Chandrasekharan *et al,* 2007) and contributing to the generation of fever (Steinman, 2010).

TNF-α is responsible for a number of signalling events within cells which usually lead to the apoptotic and necrotic pathways while also providing resistance to infection and cancer. TNF-α is produced in two forms; a soluble form and a membrane bound form (Schall *et al,* 1990); and is produced as needed (Zhou *et al*, 2002). TNF-α exerts it main abilities by binding to one of two receptors, TNFR-1 (CD120a) or TNFR-2 (CD120b). TNFR-1 is expressed in all human cell types where as

TNFR-2 is expressed mainly on endothelial and immune cells (Fuchs *et al,* 1992; Santee & Owen-Schaub, 1996).

Studies have shown that there is a correlation between the level of TNF-α produced and the concentration of CRP in inflammation. TNF-α induces a dose dependent secretion of CRP in hepatocytes which also corresponds to an increase in CRP mRNA (Calabró *et al*, 2003). However when CRP levels become elevated in atheroma, this leads to the induction of IL-1β, IL-6, and TNF-α release by macrophages indicating that CRP may have a direct effect upon both TNF-α and IL-6 release (Han *et al,* 2004).

Research has shown that there is a relationship between TNF-α and IL-6 levels in inflammation (Popa *et al,* 2007). Therefore, TNF-α and CRP may correlate in a very similar way to IL-6 and CRP. TNF-α is also a known activator of CRP, alongside IL-6 (Zhang *et al,* 1996). However, there is some evidence to show that there could potentially be an inhibitory effect of CRP upon TNF-α production, suggesting there could be a negative-feedback mechanism whereby elevating levels of CRP inhibits further stimulation of CRP by inhibiting TNF-α production (Inatsu *et al,* 2009).

## 7.2 Aims

The aim of this study is to observe the effects of the exogenous CRP isoforms on the production of four pro-inflammatory cytokines (IL-6, IL-8, MCP-1 and TNF-α) in a model of ageing. A further aim was to elucidate some of the potential mechanisms/pathways involved in CRP-mediated cytokine production by using pharmacological inhibitors.

## 7.3 Objectives

The fundamental objectives for this assay are:

- To determine the effect of exogenous nCRP on the production of four pro-inflammatory cytokines.
- To determine the effect of exogenous mCRP on the production of four pro-inflammatory cytokines
- To determine the effect of estrogen on CRP-induced production of these four pro-inflammatory cytokines in a model of age-related impaired healing. To elucidate potential mechanisms/pathways that may be mediating the CRP-mediated production of cytokines.

## 7.4 Summary of Procedure

This assay utilised the Milliplex Map Kit which is a magnetic bead-based assay. The assay is based on Luminex xMAP technology that uses internally colour-coded microspheres with two fluorescent dyes which can be set to specific concentrations in each bead. The beads correspond to a specific antibody designed to capture a particular antigen. The analyte was captured by the bead and a biotinylated detection antibody was added, followed by Streptavidin-PE conjugate which is a reporter molecule that coats the surface of the bead and completes the detection reaction. The plate was read through a Luminex analyser, which is based on flow cytometry technology to detect the beads and measure fluorescent reporter signals for each specific cytokine. This assay was used to detect and measure multiple cytokines at the same time from the same sample.

## 7.5 Materials

Human monocyte cell line U937 *(Health Protection Agency Culture Collections, Salisbury)*

RPMI-1640 Media *(Lonza, Slough)*

Foetal Bovine Serum *(Sigma-Aldrich, Dorset)*

Lipopolysaccharide *(Sigma-Aldrich, Dorset)*

Phorbol 12-myristate 13-acetate *(Applichem, Darmstadt)*

C-reactive Protein, human >95% native, 5mg/ml (*MyBioSource, San Diego)* – Purified as previously stated in Section 2.4.4.

Inhibitors – prepared as mentioned in section 2.4.9.

Estrogen *(Sigma-Aldrich, Dorset)*

Milliplex Map Kit – Human Cytokine/Chemokine Magentic Bead Panel *(Merck Millipore, Hertfordshire)*

Anti-human IL-6 beads

Anti-human IL-8 beads

Anti-human MCP-1 beads

Anti-human TNF-α beads

Human Cytokine/Chemokine Standard

Human Cytokine Quality Controls 1 & 2

Assay Buffer

Wash buffer

Human Cytokine Detection Antibodies

Streptavidin-Phycoerythrin

Bead Diluent

Sheath Fluid *(Millipore, Hertfordshire)*

## 7.6 Methods

### 7.6.1 Cell Culture

Cells were cultured as previously described in section 2.4.1.

### 7.6.2 Activation of Cells

Cells were activated as described in Section 2.4.2.

### 7.6.3 Differentiation of Cells

Cells were differentiated as described in Section 2.4.3.

### 7.6.4 CRP Purification

CRP was purified as described in Section 2.4.4. The samples were then diluted to experimental concentrations using RPMI-1640 media. CRP concentrations were set to 250µg/ml mCRP and 1000µg/ml nCRP.

### 7.6.5 Inhibitors

The assay was repeated with the addition of pharmacological inhibitors (wortmannin, LML, MEK and nystatin) prepared as described in Section 2.4.9.

### 7.6.6 Estrogen Preparation

Estrogen was prepared to a final concentration of $1x10^{-7}$M, made up in RPMI-1640 complete media, with FBS and penicillin-streptomycin.

### 7.6.7 Sample Preparation

U937 cells, set to $1x10^{6}$, were prepared to obtain the relevant cell status before being treated with the CRP isoforms, with or without estrogen supplementation, for 24 hours. A negative control was generated with BSA in place of CRP. Cell samples were then loaded into the Milliplex Assay plate for further analysis (See section 6.6.8). If samples exhibited a significant change in cytokine levels compared to the negative control the experiment was repeated with addition of inhibitors.

### 7.6.8 Milliplex Assay

The beads in the assay were mixed with bead diluent after sonication for 30 seconds. Quality controls 1 and 2 provided in the assay kit were reconstituted with deionized water and mixed. The human cytokine standard was reconstituted with deionized water to 10,000pg/ml followed by serial dilution with assay buffer to make 2,000pg/ml, 400pg/ml, 80pg/ml, 16pg/ml and 3.2pg/ml standards according to the manufacturer's guidance. The wells of the assay plate were incubated at room temperature for 10 minutes with 200µl wash buffer to prime the wells. The wash buffer was removed and 25µl of each of the standards and controls were added to relevant wells. The assay buffer was added to appropriate wells as an assay background control. This was followed by 25µl of assay buffer to every well with cell media being added to control and standard wells. Samples as prepared in Section 6.6.7 were then added to relevant triplicate wells at a volume of 25µl. The beads were mixed again and 25µl was added to every well prior to incubating the plate overnight at $4^{o}$C. The plate was then washed twice with 200µl wash buffer

using a magnetic plate washer. Detection antibodies were added to the plate, at a volume of 25µl prior to incubation for 1 hour at room temperature with agitation. This was followed by the addition of 25µl Streptavidin-Phycoerythrin to every well before being incubated for 30 minutes at room temperature with agitation. The plate was washed again with the magnetic plate washer followed by the addition of 150µl sheath fluid to every well. The plate was agitated for 5 minutes before being read by the Luminex 200™ using xPONENT® software using 100µl and reading 50 beads per bead set.

### 7.6.9 Statistical Analysis

Data (n=6) for each treatment was normalised to determine cytokine levels per million cells, before being analysed with SPSS Statistical Software (Version 22). A Shapiro-Wilke normality test was first conducted followed by t-test or Mann-Whitney U tests dependent upon if the data were parametric or nonparametric. Graphs were generated using Microsoft Excel 2013.

## 7.7 Results

### 7.7.1 Model of Age-Related Impaired Healing

#### 7.7.1.1 The Effect of the CRP Isoforms on Interleukin-6 Concentration

Overall, treatment with 1000µg/ml nCRP did not have any significant effect on cytokine production in any cell type (Figure 7.1). Treatment with 250µg/ml mCRP caused a significant ($p<0.05$) increase in IL-6 production in both activated monocytes and unactivated macrophages (Figure 7.1B and 7.1C).

Further investigations with inhibitors indicated that no inhibitor could reverse the effects of the CRP-induced IL-6 in unactivated macrophages (Figure 7.1C). However treatment with wortmannin and LML significantly reversed the CRP-induced IL-6 production in the activated monocytes (Figure 7.1B).

It was noted that there was a massive increase in IL-6 production within the LPS-activated macrophages with levels increasing from around 2.5pg/ml in the unactivated macrophages to around 23,000pg/ml after LPS-activation (Figure 7.1D), suggesting IL-6 levels increase substantially following infection.

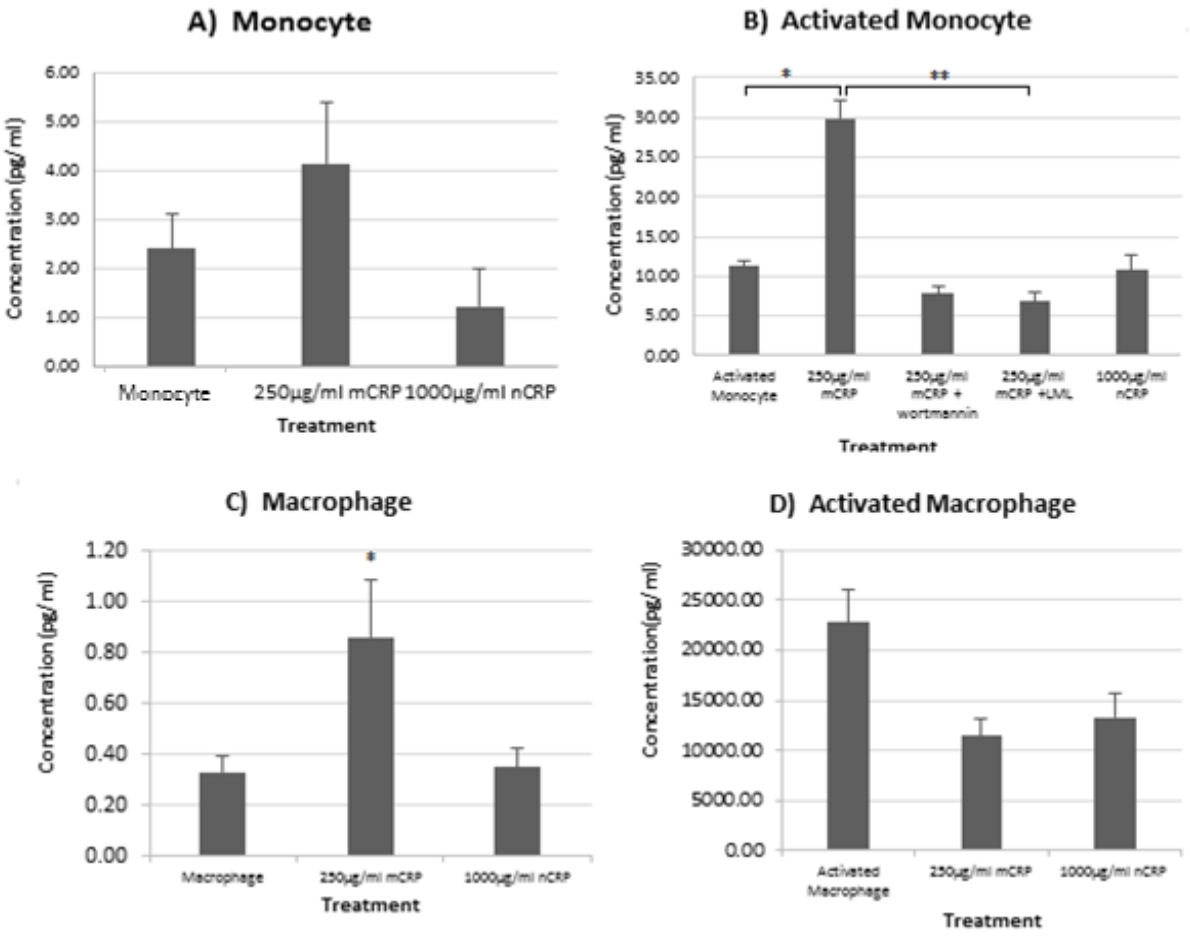



**Figure 7.1 – The effect of the CRP isoforms on IL-6 concentration.** A) Monocyte cells B) LPS activated monocyte cells. C) Macrophage cells. D) LPS activated macrophage cells. Error bars representative of the standard error of the mean (SEM). BSA-treated controls are the first bars labelled monocyte, activated monocyte, macrophage or activated macrophage. * denotes significance against the control ($P<0.05$). ** denotes significance against the CRP-mediated IL-6 production. ($P<0.05$). n= 6 in all cases.

#### 7.7.1.2 The Effect of the CRP Isoforms on Interleukin-8 Concentration

Figure 7.2A shows that treatment with both CRP isoforms caused a significant ($p<0.05$) reduction in the production of IL-8 in the unactivated monocytes, with nystatin only able to reverse the mCRP-induced reduction of IL-8. IL-8 production in the unactivated monocytes was considerably less than in the other cells types.

In the LPS-activated monocyte treatment with 250µg/ml mCRP significantly increased IL-8 production ($p<0.05$), with nystatin being able to significantly reverse this effect (Figure 7.2B). Treatment with 1000µg/ml nCRP significantly decreased ($p<0.05$) IL-8 production with LML and nystatin being able to reverse this effect (Figure 7.2B).

In unactivated macrophages, treatment with nCRP had no significant effect upon IL-8 production (Figure 7.2C). However there was a significant increase seen in the LPS-activated macrophages (Figure 7.2D). No inhibitors were able to significantly reverse this effect.

Treatment with mCRP had the opposite effect, causing a significant increase in IL-8 concentration in unactivated macrophages (Figure 7.2C) but no significant effect in the LPS-activated macrophages (Figure 7.2D).

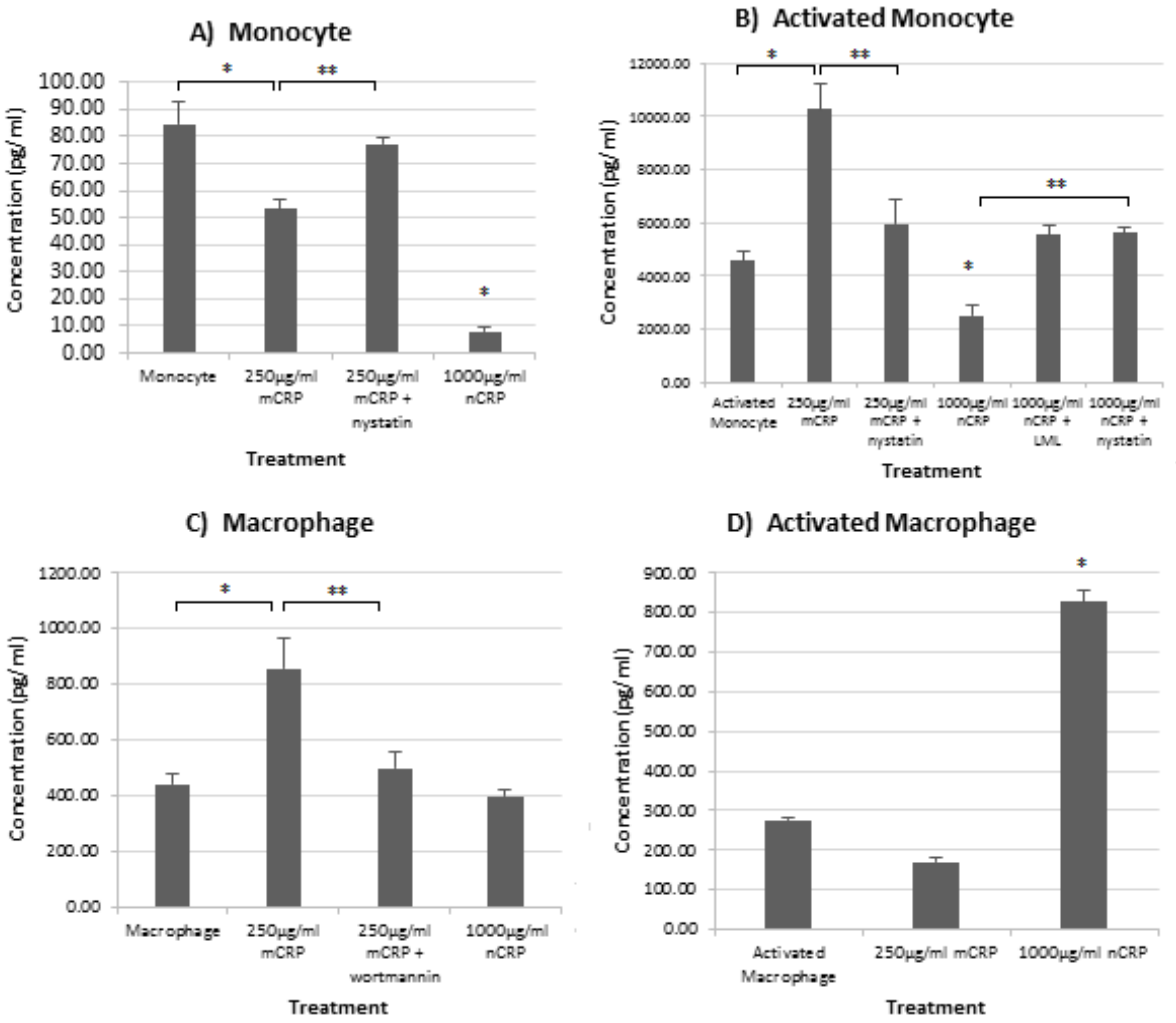



**Figure 7.2 – The effect of the CRP isoforms on IL-8 concentration.** A) Monocyte cells B) LPS activated monocyte cells. C) Macrophage cells. D) LPS activated macrophage cells. Error bars representative of the standard error of the mean (SEM). BSA-treated controls are the first bars labelled monocyte, activated monocyte, macrophage or activated macrophage. * denotes significance against the control ($P<0.05$). ** denotes significance against the CRP-mediated IL-8 production. ($P<0.05$). n= 6 in all cases.

#### 7.7.1.3 The Effect of the CRP isoforms on Monocyte Chemoattractant Protein-1 Concentration

In unactivated monocytes, only treatment with 1000µg/ml nCRP caused a significant decrease in MCP-1 concentration, with all inhibitors unable to reverse the effect (Figure 7.3A).

In LPS-activated monocytes, treatment with both CRP isoforms caused a significant increase of MCP-1 concentration, with the treatment with mCRP causing higher MCP-1 production than treatment with nCRP (Figure 7.3B). All inhibitors were unable to reverse any of the effects seen by the CRP treatments.

In both unactivated and activated macrophages, treatment with nCRP caused no significant changes in MCP-1 whereas treatment with mCRP caused a significant decrease in MCP-1 concentration (Figures 7.3C and 7.3D). The four inhibitors were unable to reverse any of the effects seen after the CRP isoform treatments.

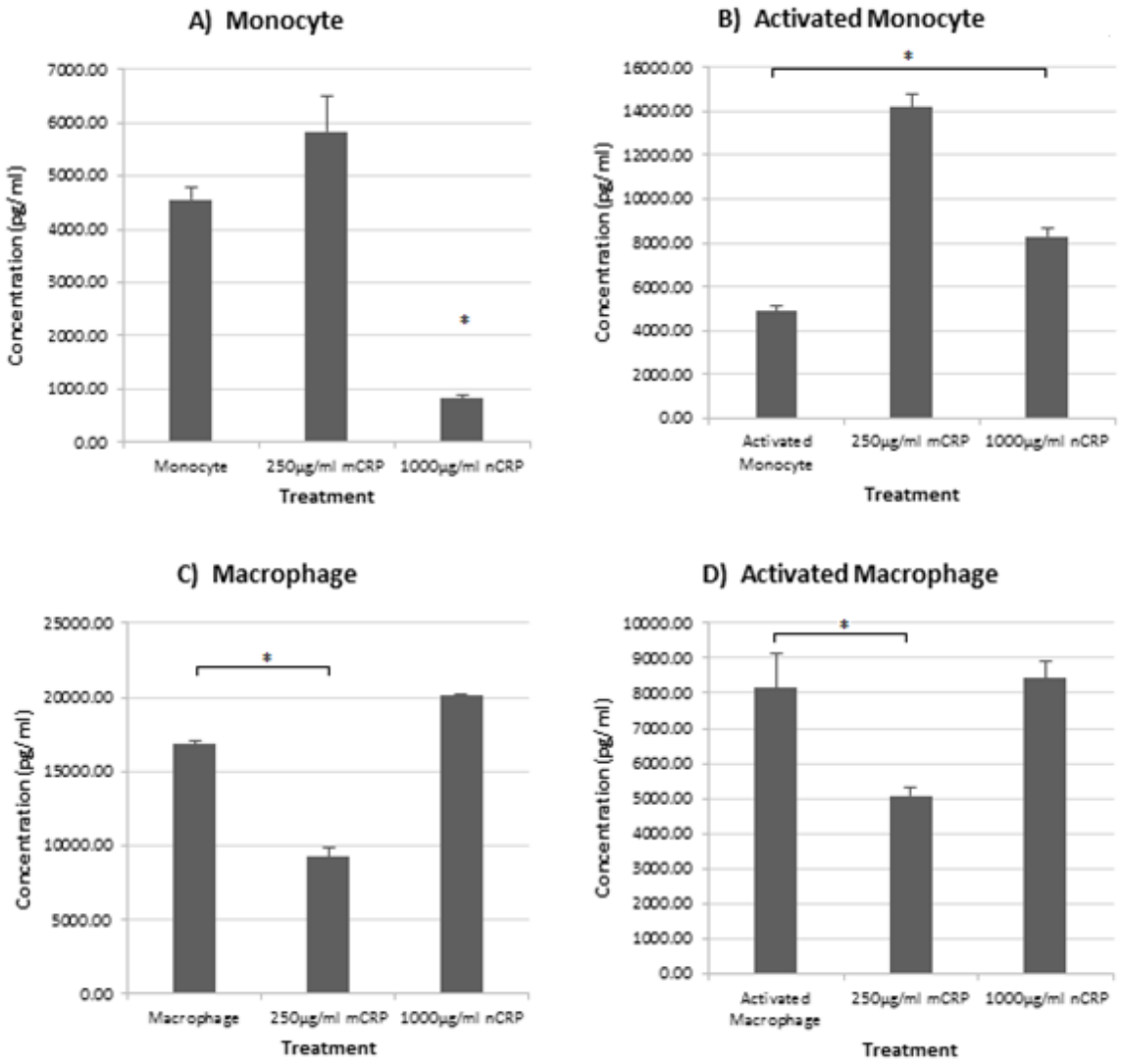



**Figure 7.3 – The effect of the CRP isoforms on MCP-1 concentration.** A) Monocyte cells B) LPS activated monocyte cells. C) Macrophage cells. D) LPS activated macrophage cells. Error bars representative of the standard error of the mean (SEM). BSA-treated controls are the first bars labelled monocyte, activated monocyte, macrophage or activated macrophage. * denotes significance against the control ($P<0.05$). ** denotes significance against the CRP-mediated MCP-1 production. ($P<0.05$). n= 6 in all cases.

#### 7.7.1.4 The Effect of the CRP Isoforms on Tumour Necrosis Factor-α Concentration

Treatment with both CRP isoforms caused a significant decrease ($p<0.05$) in TNF-α concentration in unactivated monocytes (Figure 7.4A) as well as in LPS-activated monocytes (Figure 7.4B). Inhibitors could not reverse the effects of the CRP in the unactivated monocytes or the treatment of the activated monocytes treated with 1000µg/ml nCRP. However, all four of the inhibitors could reverse the effect of the 250µg/ml mCRP treatment in the LPS-activated monocytes (Figure 7.4B) with nystatin having the greatest effect.

In the unactivated macrophages, only nCRP caused a significant decrease ($p<0.05$) in the concentration of TNF-α with wortmannin and nystatin reversing the effects (Figure 7.4C). In activated macrophages, only treatment with mCRP caused a significant decrease in TNF-α concentration with no reversal was observed using inhibitors (Figure 7.4D).

Again, levels produced by activated macrophages far exceeded those seen in the other cell types with levels increasing from around 18pg/ml in the unactivated macrophages to 1600pg/ml in the LPS-activated macrophages.

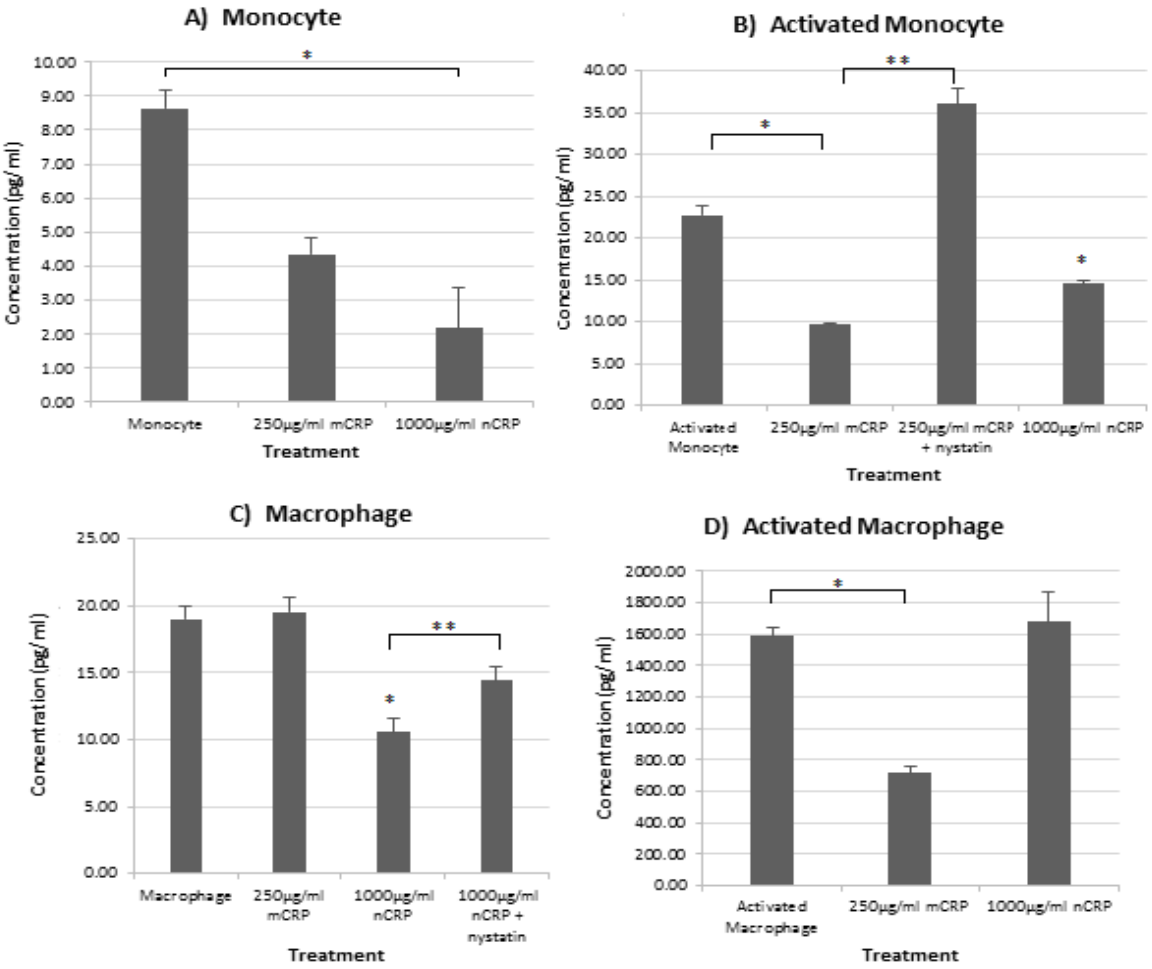



**Figure 7.4 – The effect of the CRP isoforms on TNF-α concentration.** A) Monocyte cells B) LPS activated monocyte cells. C) Macrophage cells. D) LPS activated macrophage cells. Error bars representative of the standard error of the mean (SEM). BSA-treated controls are the first bars labelled monocyte, activated monocyte, macrophage or activated macrophage. * denotes significance against the control ($P<0.05$). ** denotes significance against the CRP-mediated TNF-α production. ($P<0.05$). n= 6 in all cases.

### 7.7.2 Model of Acute Healing in Youth

#### 7.7.2.1 The Effect of Estrogen on CRP-Mediated Levels of Interleukin-6.

In unactivated monocytes, both CRP isoforms caused a significant increase ($p<0.05$) in the concentration of IL-6 (Figure 7.5A). This increase in IL-6 was only seen following mCRP treatment in LPS-activated monocytes (Figure 7.5B).

In unactivated macrophages, treatment with mCRP significantly increased IL-6 concentration whereas the nCRP treatment significantly decreased the IL-6 concentration (Figure 7.5C). In the activated macrophages, both treatment with nCRP and mCRP significantly decreased IL-6 concentration, with mCRP inducing a greater reduction in IL- than nCRP (Figure 7.5D).

A substantial increase in IL-6 production was noted in unactivated macrophages when compared to the other cell types (Figure 7.5C).

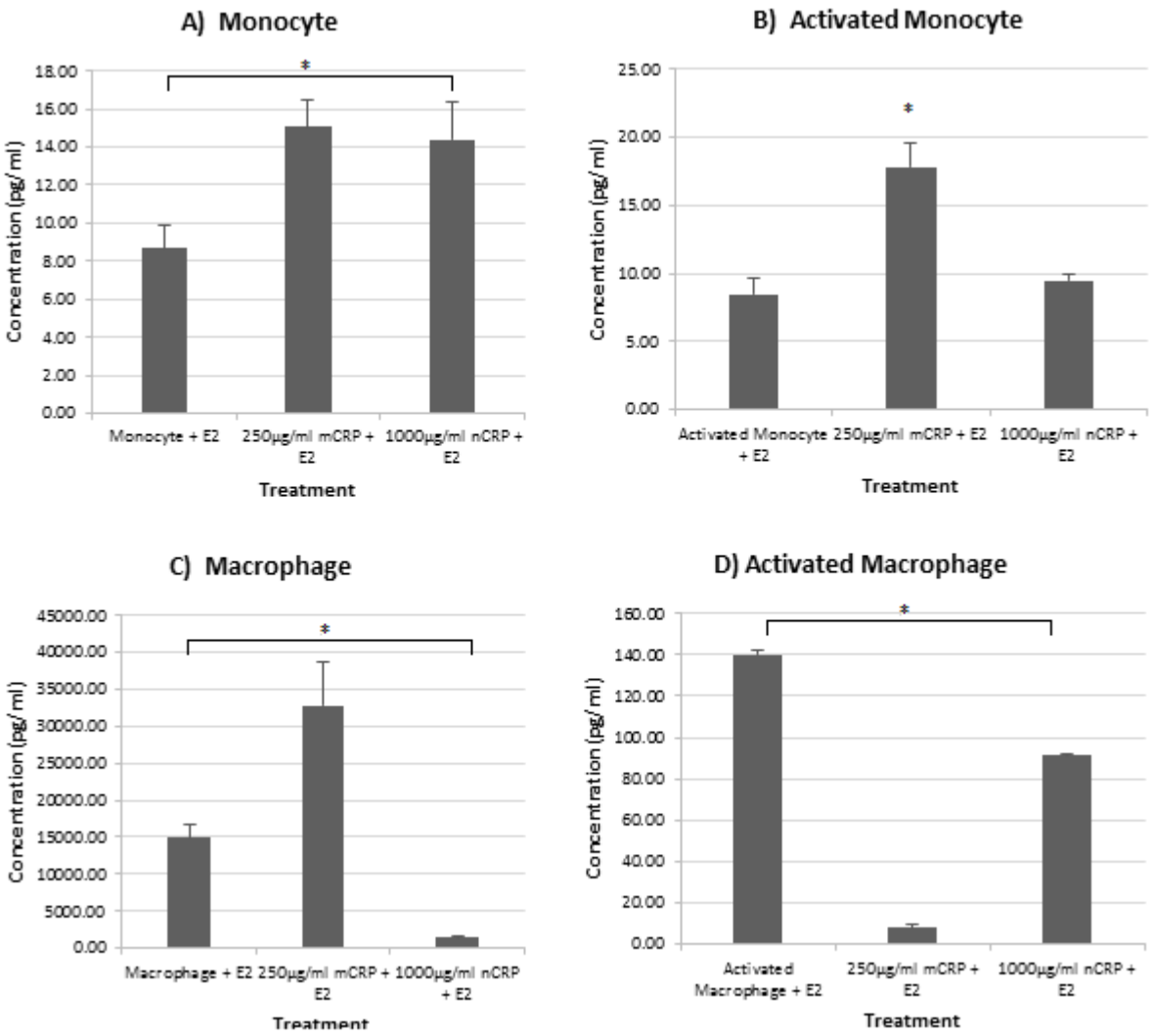



**Figure 7.5 – The effect of the CRP isoforms and estrogen on IL-6 concentration.** A) Monocyte cells B) LPS activated monocyte cells. C) Macrophage cells. D) LPS activated macrophage cells. Error bars representative of the standard error of the mean (SEM). * denotes significant differences ($P<0.05$). n=6 in all cases.

*E2 – Addition of Estrogen*

#### 7.7.2.2 The Effect of Estrogen on CRP-Mediated Levels of Interleukin-8

In both unactivated and LPS-activated monocytes, there was a significant increase ($p<0.05$) in IL-8 concentration when treated with either mCRP or nCRP. Treatment with mCRP caused a higher concentration of IL-8 when compared to the nCRP treatment (Figures 7.6A and 7.6B).

In unactivated macrophages, treatment with mCRP significantly increased IL-8 concentration (Figure 7.6C). However, in the LPS-activated macrophages mCRP significantly decreased IL-8 concentration (Figure 7.6D). Treatment with nCRP had no significant effect upon IL-8 concentration in macrophages, regardless of activation.

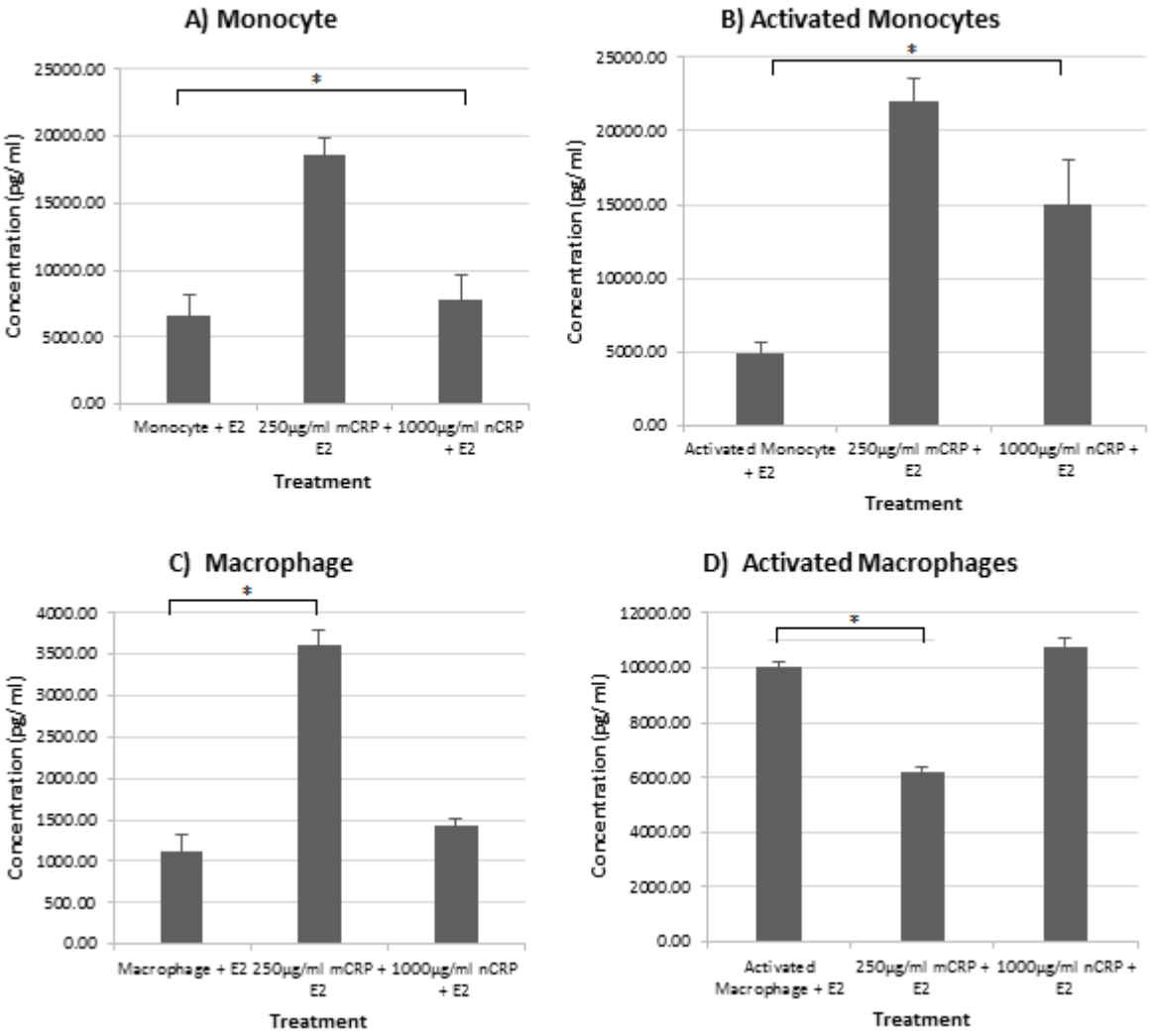



**Figure 7.6 – The effect of the CRP isoforms and estrogen on IL-8 concentration.** A) Monocyte cells B) LPS activated monocyte cells. C) Macrophage cells. D) LPS activated macrophage cells. Error bars representative of the standard error of the mean (SEM). * denotes significant differences ($P<0.05$). n=6 in all cases.

*E2 – Addition of Estrogen*

### 7.7.2.3 The Effect of Estrogen on CRP-Mediated Levels of Monocyte Chemoattractant Protein-1

Treatment with nCRP did not yield any significant changes in MCP-1 levels in any of the cell states.

Treatment with 250μg/ml mCRP caused a significant increase ($p<0.05$) in MCP-1 concentration in unactivated monocytes (Figure 7.7A), LPS-activated monocytes (Figure 7.7B) and unactivated macrophages (Figure 7.7C). In the LPS-activated macrophages there was a significant decrease ($p<0.05$) in the concentration of MCP-1 (Figure 7.7D).

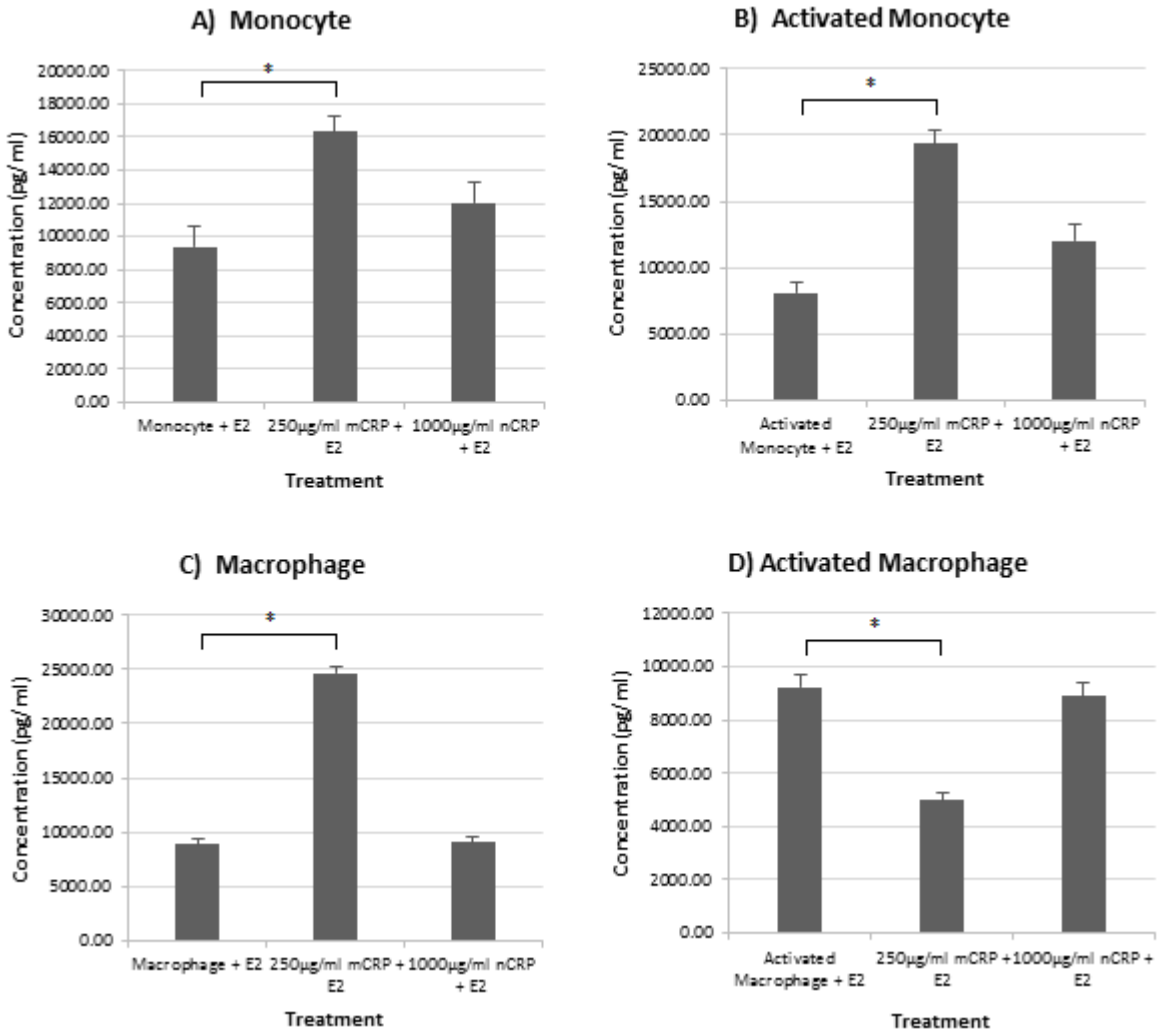



**Figure 7.7 – The effect of the CRP isoforms and estrogen on MCP-1 concentration.** A) Monocyte cells B) LPS activated monocyte cells. C) Macrophage cells. D) LPS activated macrophage cells. Error bars representative of the standard error of the mean (SEM). * denotes significant differences ($P<0.05$). n=6 in all cases.

*E2 – Addition of Estrogen*

#### 7.7.2.4 The Effect of Estrogen on CRP-Mediated Levels of Tumour Necrosis Factor-α

Treatment of unactivated monocytes with either CRP isoform did not produce any significant changes to TNF-α concentration following estrogen supplementation (Figure 7.8A). However, in LPS activated monocytes, both isoforms caused a significant increase ($p<0.05$) in the concentration of TNF-α (Figure 6.8B) following estrogen supplementation.

In unactivated macrophages, only treatment with 250µg/ml mCRP caused a significant increase ($p<0.05$) in TNF-α concentration following estrogen supplementation, with nCRP having no significant effect on levels of TNF-α (Figure 7.8C). In contrast, when macrophages were activated with LPS, both isoforms caused a significant decrease ($p<0.05$) in the concentration of TNF-α following estrogen supplementation (Figure 7.8D).

It was noted that unactivated and activated macrophages produced higher levels of TNF-α when compared to monocytic cells following estrogen supplementation. Levels of TNF-α were higher in the unactivated macrophages than in LPS-activated macrophages following estrogen supplementation.

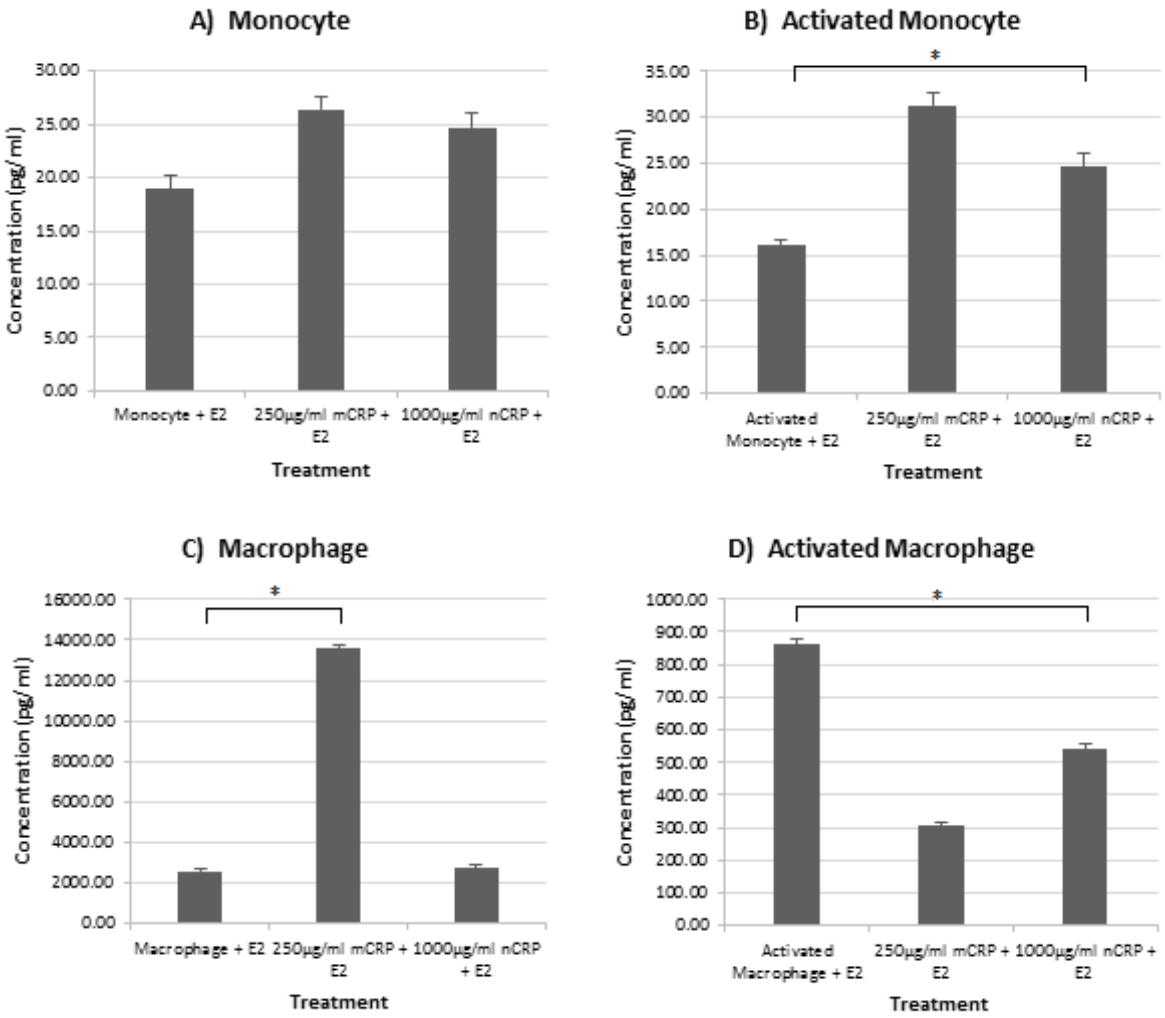



**Figure 7.8 – The effect of the CRP isoforms and estrogen on TNF-α concentration.** A) Monocyte cells B) LPS activated monocyte cells. C) Macrophage cells. D) LPS activated macrophage cells. Error bars representative of the standard error of the mean (SEM). * denotes significant differences ($P<0.05$). n=6 in all cases.

*E2 – Addition of Estrogen*

### 7.7.3 Comparison of models of healing

#### 7.7.3.1 Comparison of IL-6 Production in Models of Young and Age-Related Impaired Healing

Supplementation of estrogen to treatments was used to model the inflammatory response during acute healing found in youth. Treatments in the absence of estrogen supplementation were used to model age-related impaired healing.

Supplementation of monocytes with estrogen leads to a significant increase ($p<0.05$) in the concentration of IL-6 in unactivated monocytes (Figure 7.9A). In activated monocytes, estrogen supplementation only caused a significant decrease following treatment with mCRP, with other treatments not showing any difference in IL-6 levels (Figure 7.9B).

In the unactivated macrophages there was a significant ($p<0.05$) increase in the concentration of IL-6 in all treatments following estrogen supplementation (Figure 7.9C). However this effect was reversed in the LPS-activated macrophages where the addition of estrogen significantly decreased ($p<0.05$) IL-6 concentration following treatment with mCRP or nCRP (Figure 7.9D).

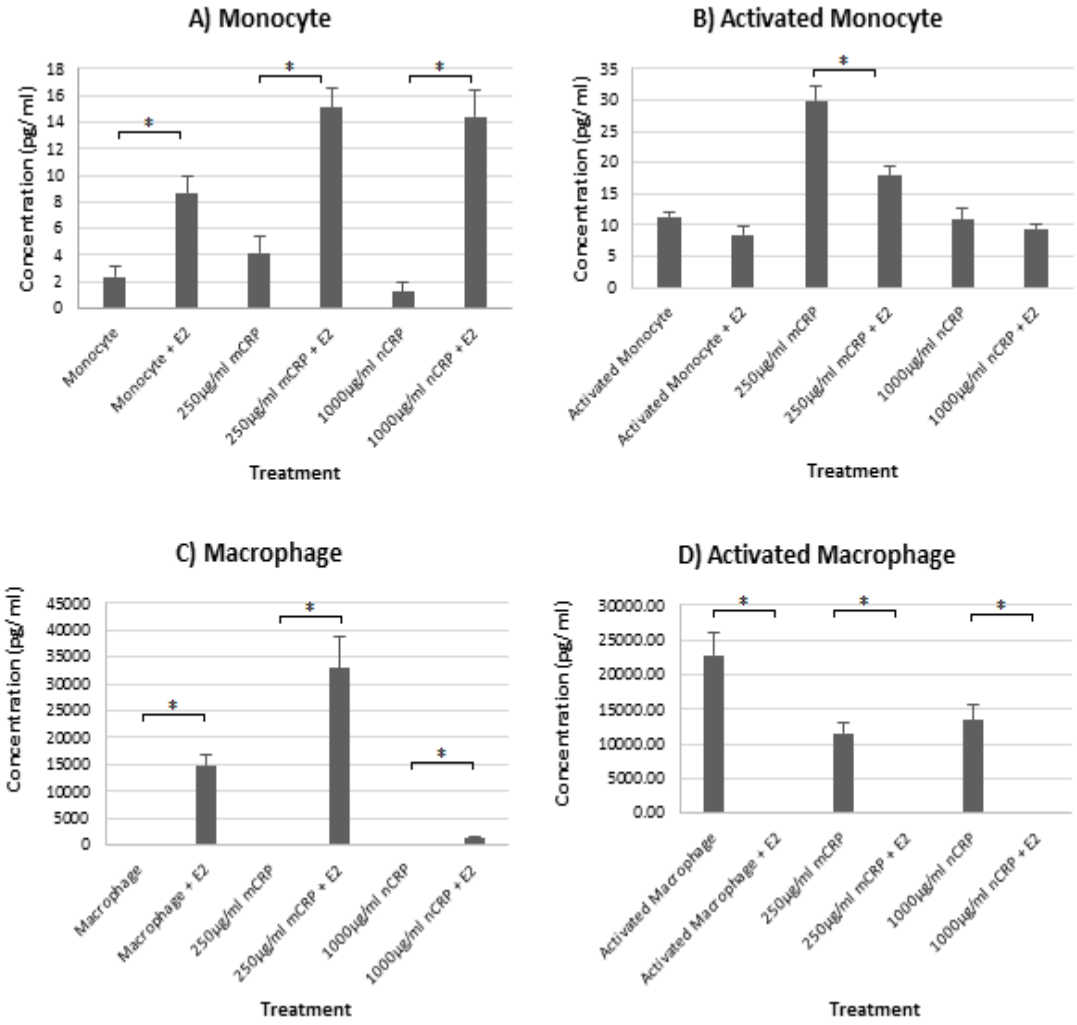



**Figure 7.9 – The difference in IL-6 profiles between models of young and age-related impaired model.** A) Monocyte cells B) LPS activated monocyte cells. C) Macrophage cells. D) LPS activated macrophage cells. Error bars representative of the standard error of the mean (SEM). BSA-treated controls are the first bars labelled monocyte, activated monocyte, macrophage or activated macrophage. * denotes significant differences ($P<0.05$). n=6 in all cases.

#### 7.7.3.2 Comparison of IL-8 Production in Models of Young and Age-Related Impaired Healing

Supplementation with estrogen in unactivated monocytes caused a significant increase ($p<0.05$) in IL-8 within all CRP treatments (Figure 7.10A). When the cells were LPS activated both CRP isoform caused a significant increase ($p<0.05$) in IL-8 concentration following estrogen supplementation when compared to the absence of estrogen (Figure 7.10B).

Supplementation with estrogen in unactivated and LPS-activated macrophages led to a significant increase ($p<0.05$) in the production of IL-8 for all of the CRP treatments (Figure 7.10C and 7.10D)

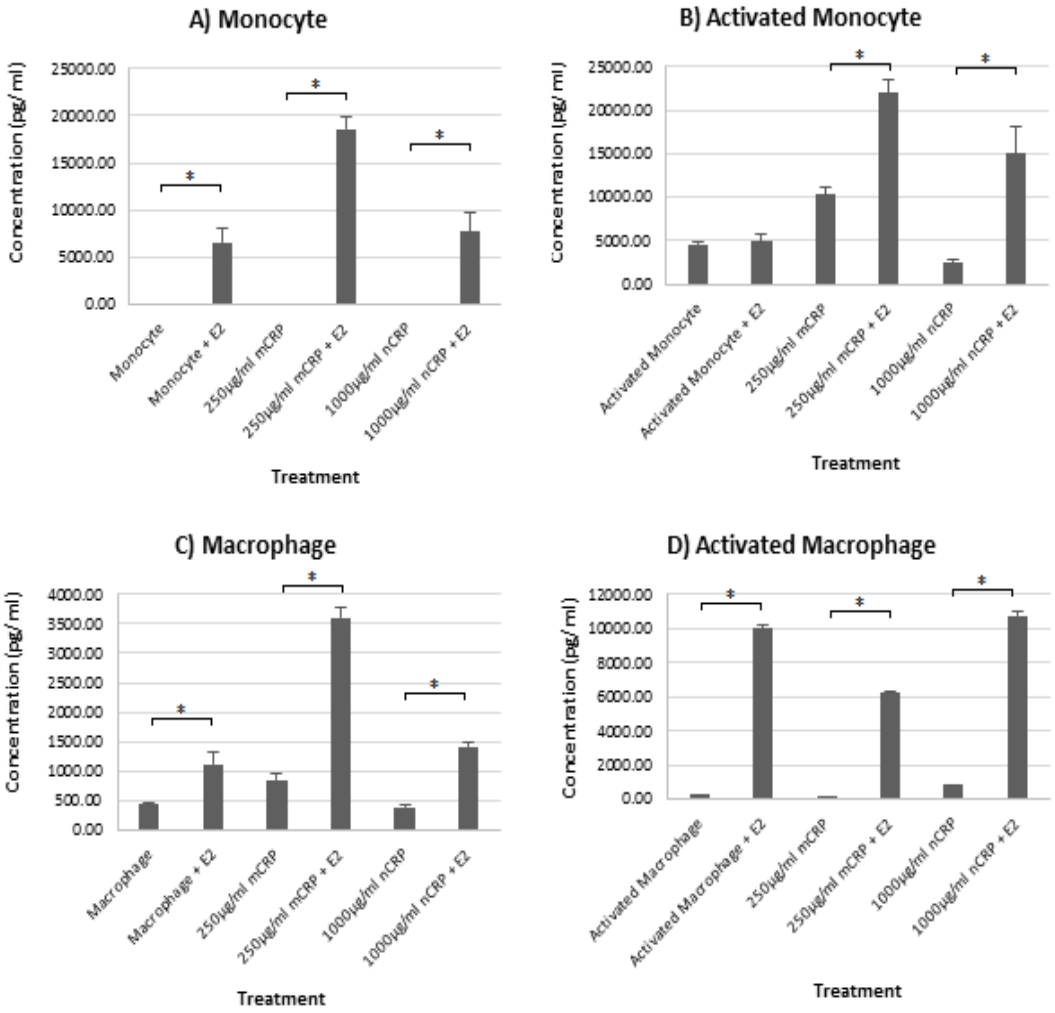



**Figure 7.10 – The difference in IL-8 profiles between healthy model and age-impaired model.** A) Monocyte cells B) LPS activated monocyte cells. C) Macrophage cells. D) LPS activated macrophage cells. Error bars representative of the standard error of the mean (SEM). BSA-treated controls are the first bars labelled monocyte, activated monocyte, macrophage or activated macrophage. * denotes significant differences ($P<0.05$). n=6 in all cases.

#### 7.7.3.3 Comparison of MCP-1 Production in Models of Young and Age-Related Impaired Healing

Estrogen supplementation in unactivated and LPS-activated monocytes caused a significant increase ($p<0.05$) in the concentrations of MCP-1 for all CRP treatments compared to levels found in the absence of estrogen. (Figure 7.11A and 7.11B).

Estrogen supplementation significantly decreased ($p<0.05$) the MCP-1 concentration in unactivated macrophages following treatment with nCRP compared to levels found in the absence of estrogen. However, estrogen supplementation caused a significant increase ($p<0.05$) in MCP-1 concentration following treatment with mCRP compared to levels found in the absence of estrogen. (Figure 7.11C).

There was no significant difference in the concentration of MCP-1 between activated macrophages supplemented with estrogen and those in the absence of estrogen following treatment with either CRP isoform (Figure 7.11D).

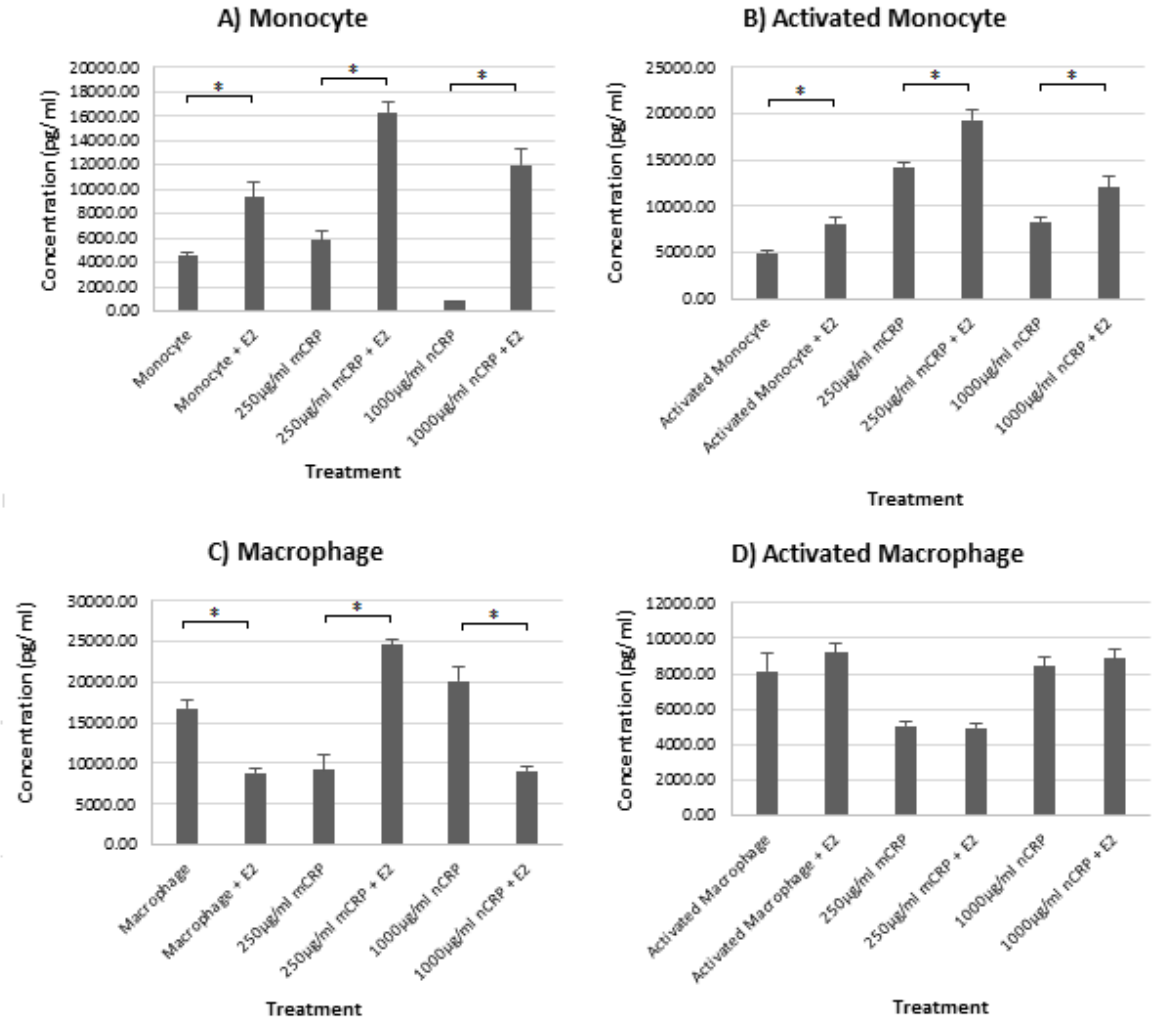



**Figure 7.11 – The difference in MCP-1 profiles between healthy model and age-impaired model.** A) Monocyte cells B) LPS activated monocyte cells. C) Macrophage cells. D) LPS activated macrophage cells. Error bars representative of the standard error of the mean (SEM). BSA-treated controls are the first bars labelled monocyte, activated monocyte, macrophage or activated macrophage. * denotes significant differences ($P<0.05$). n= 6 in all cases.

#### 7.7.3.4 Comparison of TNF-α Production in Models of Young and Age-Related Impaired Healing

In unactivated monocytes, supplementation with estrogen caused a significant increase ($p<0.05$) in the TNF-α concentration compared to levels found in the absence of estrogen following treatment with all CRP isoforms (Figure 7.12A).

Estrogen supplementation caused a significant decrease ($p<0.05$) in TNF-α concentration in LPS-activated monocytes compared to levels in the absence of estrogen. However, in contrast estrogen supplementation in LPS-activated monocytes caused a significant increase ($p<0.05$) in TNF-α concentration (Figure 7.12B) following treatment with CRP isoforms compared to levels in the absence of estrogen.

Estrogen supplementation in unactivated macrophages caused a significant increase ($p<0.05$) in TNF-α following treatment with CRP isoforms compared to levels in the absence of estrogen (Figure 7.12C). Estrogen supplementation in LPS-activated macrophages caused a significant decrease ($p<0.05$) in TNF-α concentration following treatment with CRP isoforms compared to levels in the absence of estrogen (Figure 7.12D).

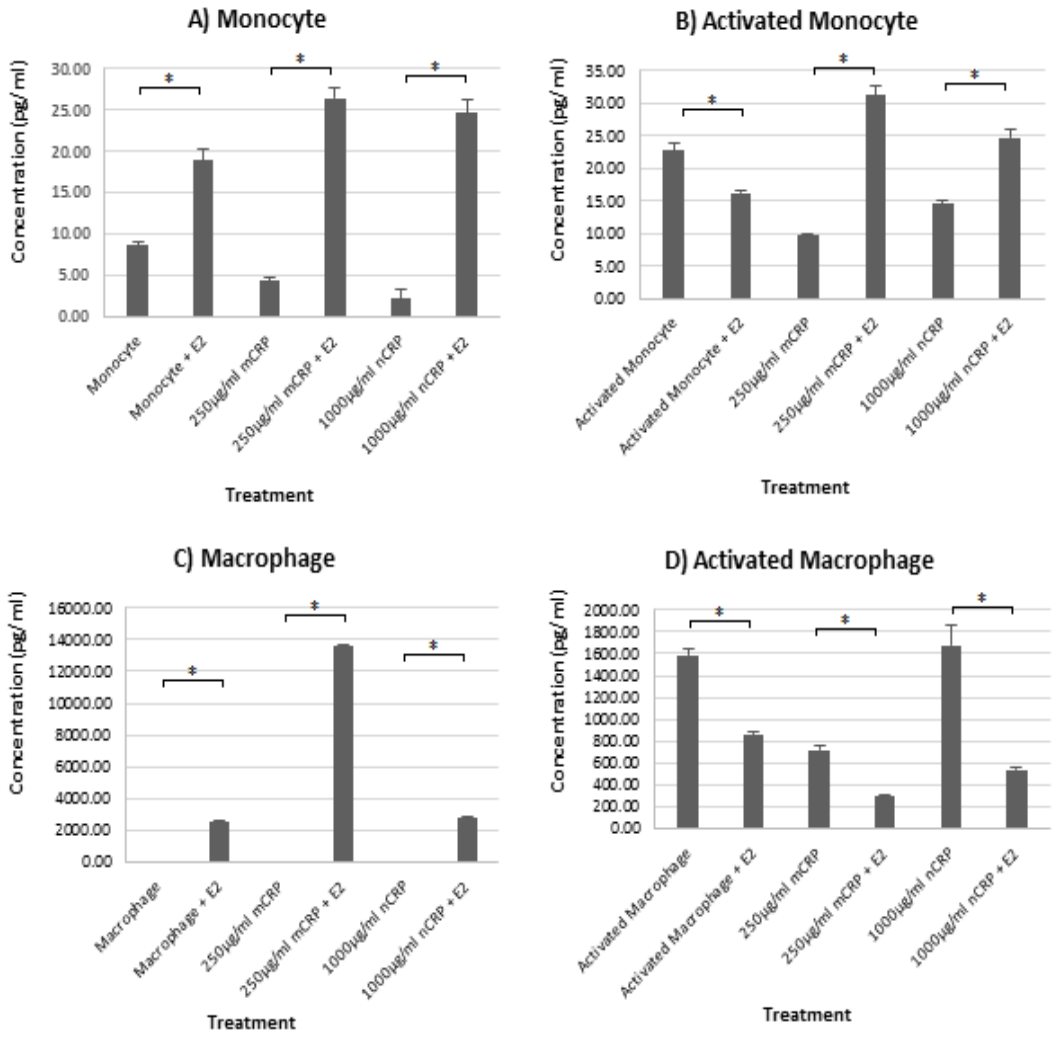



**Figure 7.12 – The difference in TNF-α profiles between healthy model and age-impaired model.** A) Monocyte cells B) LPS activated monocyte cells. C) Macrophage cells. D) LPS activated macrophage cells. Error bars representative of the standard error of the mean (SEM). BSA-treated controls are the first bars labelled monocyte, activated monocyte, macrophage or activated macrophage. * denotes significant differences ($P<0.05$). n=6 in all cases.

## 7.8 Discussion

### 7.8.1 Interleukin-6 Production

Figures 7.1, 7.5 and 7.9 all provide evidence to show the effect of CRP on interleukin-6 (IL-6) levels in both a young and age-related impaired models of healing. IL-6 is a known stimulator of CRP in hepatocytes (Weinhold *et al*, 1997); however, the data presented here indicates that CRP could also alter IL-6 levels in a reciprocal manner. In both models of healing, in general, treating monocytes (both unactivated and LPS-activated) and unactivated macrophage cells with 250µg/ml mCRP increased IL-6 levels. However, mCRP caused a significant decrease in the production of IL-6 in LPS-activated macrophages in both models of healing. It is known that IL-6 levels increase during periods of inflammation (Scheller *et al,* 2011); however the findings of this study indicate that mCRP can increase IL-6 levels in monocytes (both unactivated and LPS-activated) and unactivated macrophages .

In contrast, treatment of LPS-activated macrophages with mCRP caused a significant decrease in the amount of IL-6 being produced in both models of healing. These cells were already in a heightened inflammatory response due to the LPS-activation and therefore mCRP could be inhibiting its own production in a feedback loop by reducing IL-6, thereby bringing about a resolution of inflammation rather than acting in a typical pro-inflammatory manner (Thiele *et al,* 2014).

In the age-related impaired model of healing, treatment with nCRP did not have any effect upon IL-6 levels in any of the cell states. In the model of acute healing in young adults, the addition of nCRP caused an increase in IL-6 production in the unactivated monocytes, but a decrease in IL-6 in both unactivated and LPS-activated macrophages. Taking into consideration that nCRP is considered to be more anti-inflammatory in nature (Thiele *et al,* 2014), this would indicate that the reduction of IL-6 in tissue macrophages afforded by nCRP during youth (estrogen supplementation) may be lost with increasing age (absence of estrogen). The literature supports this theory since IL-6 levels are elevated in age-related healing compared to the healing seen in young adults (Ogura *et al,* 2008). Estrogen seems

to a key mediator of IL-6. Estrogen caused an increase in IL-6 levels in both unactivated and LPS-activated monocytes. However, in LPS-activated macrophages, estrogen caused a significant decrease in IL-6 levels. This comparison shows that estrogen can act in a dual manner, being both pro-inflammatory and anti-inflammatory depending on the cell status. This is supported by Chakrabarti *et al* (2008) who showed estrogen can promote or inhibit inflammation in different tissues at the same time. In cells indicative of tissue macrophages typically found in wounds (activated macrophages) estrogen was anti-inflammatory in nature, reducing IL-6 levels. In cells indicative of circulating monocytes (unactivated and activated monocytes), estrogen was pro-inflammatory in nature, stimulating IL-6 levels. Vegeto *et al* (2002) showed estrogen has a protective role in tissue inflammation, inhibiting the synthesis of pro-inflammatory cytokines such as IL-6. In line with this, estrogen has been shown to promote the infiltration of immature monocytes from circulation but this pro-inflammatory effect is reversed in mature tissue macrophages, causing the down-regulation of inflammation at wound sites (Ashcroft et al, 2003). Macrophages are recruited to sites of inflammation and local levels of cytokines in tissues are controlled accordingly without affecting circulating levels (Corcoran *et al,* 2010). In the model of age-related impaired healing, there were only a few cases where the addition of an inhibitor significantly reduced the effect of the CRP treatment on IL-6 levels. In activated monocytes, the mCRP-induced IL-6 production was significantly reduced with the addition of either wortmannin or LML (Figure 7.1B).

The addition of wortmannin indicates that mCRP is at least in part interacting with the PI3K pathway, of which IL-6 is a main activator (Heinrich *et al,* 2003). Inhibition of the PI3K pathway would suggest that either the feedback loop of IL-6 has been disrupted or that mCRP is contributing to the increasing levels of IL-6 through this pathway. The p38 pathway is utilised to activate the IL-6 gene in order to lead to the production of IL-6. By inhibiting p38 using LML this pathway is blocked, suggesting that the mCRP could potentially be activating IL-6 production via p38 activation (Craig *et al,* 2000). Both the PI3K and p38 pathways utilise nuclear

factor-κβ (NF-κβ) to induce the production of IL-6 (Heinrich *et al,* 2003; Craig *et al,* 2000). This suggests that in LPS-activated monocytes, mCRP could potentially be stimulating the production of NF-κβ in order to produce higher levels of IL-6, and this process may be blocked by either wortmannin or LML.

### 7.8.2 Interleukin-8 Production

Figures 7.2, 7.6 and 7.10 all provide evidence on the effects of CRP isoforms on interlekin-8 levels in models of young and age-related impaired healing.

Estrogen supplementation in the model of acute healing in young adults led to a significant increase in IL-8 production following mCRP treatment in three of four cell states. Indeed, estrogen is known to generally increase the levels of IL-8 so this is not unexpected (Bengtsson et al, 2004). However, similar to IL-6, levels of IL-8 significantly decreased in LPS-activated macrophages supplemented with estrogen, suggesting it is mechanism to bring about the resolution of inflammation by mature macrophages in young adults. In the age-related impaired model of healing a significant decrease in IL-8 was seen in unactivated monocytes and significant increases in IL-8 were seen in activated monocytes and unactivated macrophages following mCRP treatment. The increases in IL-8 are concordance with research showing mCRP increases IL-8 production, thus leading to the promotion of pro-inflammatory activity (Khreiss *et al*, 2004).

Wigmore *et al* (1997) indicated that IL-8 can work at a local level and induce CRP production. Thus the induction of IL-8 by mCRP detected in this study may contribute to a positive feedback mechanism, causing further production of CRP via the stimulation of IL-8, if the CRP generated by IL-8 includes the mCRP isoform. In line with this, Kibayashi *et al* (2005) showed CRP plays a role in atherosclerosis through the activation of CRP and that mCRP generally causes IL-8 levels to rise. Significant increases in IL-8 in unactivated macrophages following mCRP treatment could also be partly due to the fact that IL-8 is mainly produced by macrophages (Koch *et al,* 1992).

Previous studies suggested that treatment with nCRP produces no significant effect on the production of IL-8 (Khreiss *et al,* 2004). However this study has shown that nCRP can increase IL-8 production in both unactivated and activated monocytes in the model of healing in young adults, indicating a pro-inflammatory role for nCRP in circulation. In the age-related impaired model of healing, treatment with nCRP led to a significant decrease in IL-8 production in unactivated and LPS-activated monocytes and a significant increase in LPS-activated macrophages. The study conducted by Khreiss *et al* (2004) did not specifically look at macrophages and therefore could be the reason why they discovered no detectable effects.

The use of inhibitors to reverse significant results in the age-related impaired healing model showed multiple inhibitors could reverse CRP-mediated IL-8 levels. Nystatin reversed the mCRP-mediated reduction in IL-8 in unactivated monocytes (Figure 7.2A) and the mCRP-mediated increase in IL-8 in LPS-activated monocytes (Figure 7.2B). In addition, LML and nystatin reversed the nCRP-mediated reduction in IL-8 in LPS-activated monocytes (Figure 7.2B). Wortmannin reversed the mCRP-induced increase in IL-8 in unactivated macrophages (Figure 7.2C).

Kibayashi *et al* (2005) provided evidence that IL-8 production could be inhibited using MAPK inhibitors such as SB203580 (LML) in a study of atherosclerosis. However, LML treatment did not reverse the CRP-mediated changes in IL-8 observed in this study, suggesting the mechanisms mediating IL-8 levels may be dependent on cell type and state.

The inhibitor, nystatin, had a significant effect upon CRP-mediated IL-8 levels. Nystatin is an inhibitor of the lipid-raft-caveolae endocytosis pathway (Ivanov, 2008) as well as being able to form barrel like pores in the membrane which leads to potassium leakage (Chen et al, 2011). It is known that mCRP can associate with the cell membrane via lipid rafts (Ji *et al,* 2009) which could lead to the changes observed in mCRP treated cells. When nystatin reversed nCRP-mediated changes in IL-8, it could in part be due to nCRP molecules dissociating into mCRP.

The fact that wortmannin had some effect on IL-8 levels is interesting. The activation of IL-8 does not usually occur through the PI3K pathway but usually through activation of the ERK, p38 MAPK and JNK pathways (Kibayashi *et al,* 2005). However, it has been suggested that IL-8 activation can occur through the activation of PI3K having downstream effects ultimately leading to the activation of IL-8 (Takami *et al*, 2002). Thus, mCRP might be affecting IL-8 through the PI3K pathway in unactivated macrophages via some downstream inhibition of the IL-8 production.

### 7.8.3 Monocyte Chemoattractant Protein-1 Production

Figures 7.3, 7.7 and 7.11 provide evidence on the effect of CRP isoforms on monocyte chemoattractant protein-1 levels in both models of healthy and age-related impaired healing.

In the age-related impaired model, the addition of mCRP caused a significant increase in MCP-1 production in unactivated and LPS-activated monocytes but a significant decrease in both unactivated and LPS-activated macrophages. In the model of acute healing in young adults, both unactivated and LPS-activated monocytes and unactivated macrophages had significantly increased MCP-1 levels whereas LPS-activated macrophages showed significantly less MCP-1 production following treatment with mCRP.

This is supported by the literature since estrogen has been shown to promote the infiltration of immature monocytes from circulation via production of MCP-1 but this pro-inflammatory effect is reversed in mature tissue macrophages, causing the down-regulation of inflammation at wound sites (Ashcroft et al, 2003). In both the young and age-related impaired models of healing, the monocytes produced more MCP-1 following mCRP treatment, which fits with evidence that MCP-1 is principally produced in monocytes (Deshmane *et al, 2009*) and mCRP is pro-inflammatory in general (Khreiss *et al, 2004)*. It is known that CRP can also activate monocytes in the arterial wall, leading to the promotion of atherosclerosis

through the release of MCP-1 (Han et al, 2004). Macrophages exhibited a profile of CRP isoforms that was indicative of a reduced inflammatory response compared to the profile of CRP isoforms in monocytes. This is evidence that CRP increases MCP-1 levels in atherosclerosis (Han *et al,* 2004).

In the age-related impaired model of healing, unactivated macrophages produced higher levels of MCP-1 following mCRP treatment, which was not seen following estrogen supplementation in the model of healing in young adults. This suggests that the reduction of estrogen in the age-related impaired model of healing, results in a more profound (excessive) pro-inflammatory response, with higher levels of MCP-1 leading to the further recruitment of monocytes. In both the young and age-related models of healing however MCP-1 levels were significantly reduced in LPS-activated macrophages, indicating a more dampened inflammatory response in activated tissue macrophages, potentially leading to a resolution of the inflammatory response.

Treatment with nCRP yielded no significant findings in the model of acute healing in the young. However in the age-related impaired healing model treatment with nCRP only had an effect on monocytes, significantly decreasing MCP-1 levels in unactivated monocytes but significantly increasing MCP-1 levels in the LPS-activated monocytes. This response would lead to reduced monocyte recruitment from circulation in the absence of infection and increased monocyte recruitment in the presence of infection, suggesting the anti-inflammatory nature of nCRP (Khreiss *et al,* 2004) relates to situations where infection is absent. This involvement of monocytes also concurs with literature showing MCP-1 is produced predominantly in monocytes (Deshmane *et al, 2009*). The fact nCRP only regulated MCP-1 in the age-related impaired model of healing suggests that the loss of estrogen during ageing changes the profile of MCP-1 production by monocytes, leading to a more pronounced inflammatory response in the presence of infection.

In summary, supplementation of estrogen led to significantly higher levels of MCP-1 in the monocytes following treatment with mCRP but no effect was observed following nCRP treatment. The addition of estrogen is known to increase

the levels of MCP-1 production in the monocytes, therefore this change was not unexpected (Bengtsson *et al*, 2004). In contrast, supplementation of estrogen reduced MCP-1 levels in activated macrophages following treatment with mCRP and no effect was observed following nCRP treatment. Estrogen has been shown previously to reduce MCP-1 in mature tissue macrophages of wounds (Ashcroft *et al*, 2003).

Pervin *et al* (1998) stated that treatment with estrogen supressed MCP-1 expression in macrophages in atherosclerosis. Ashcroft et al (2003) also showed that estrogen suppresses MCP-1 expression by mature tissue macrophages, thus inhibiting further infiltration of immature monocytes into murine wounds. These publications agree with the lower levels of MCP-1 found in the estrogen-treated macrophages in this study.

### 7.8.4 Tumour Necrosis Factor-α Production

Data from Figures 7.4, 7.8 and 7.12 all provide evidence on the effect of CRP on tumour necrosis factor-α (TNF-$\alpha$) levels in both a model of acute wound healing in young adults and a model of age-related impaired healing.

In the model of age-related impaired healing, in general both CRP isoforms caused a significant decrease in TNF-α levels. In the model of acute wounding in the young there was a significant increase in TNF-α levels in LPS-activated monocytes but not unactivated monocytes following treatment with both CRP isoforms. This concurs with the literature since LPS is the main stimulator of TNF-α production (Zelová & Hošek, 2013) and suggests TNF-$\alpha$ levels in monocytes are likely to be higher in the presence of infection. TNF-$\alpha$ levels in activated macrophages were significantly reduced following treatment with either CRP isoform.

When the estrogen was removed in the age-impaired model, the effects of the CRP isoforms caused significant decreases in the TNF-α levels. This can possibly be explained through the fact that CRP can bind and crosslink FcγR1 receptors on cells, which in turn induces the production of interleukin-10 (IL-10). This leads to

the cells becoming immunosuppressive and thus turning off the production of interleukin-12 (IL-12) and TNF-α (Marnell *et al*, 2005). The indication is that the presence of estrogen can supress this process, explaining why the production of TNF-α in the healthy model increases.

Treatment with the nystatin inhibitor reversed the mCRP-mediated reduction of TNF-α levels in LPS-activated monocytes (Figure 7.4B). Treatment of nystatin reversed the nCRP-mediated reduction of TNF-α in unactivated macrophages (Figure 7.4C). The other inhibitors (wortmannin, LML and MEK) were not used in this case since they are known to also directly reduce TNF-α levels (Smith *et al,* 2007; Campbell *et al*, 2004), similar to the response observed with the CRP isoforms. The inhibitor, nystatin, also reversed the effects on TNF-α levels in both LPS-activated monocytes and unactivated macrophages. As mCRP can associate with the cell membrane via lipid rafts (Ji *et al,* 2009), nystatin may disrupt the inhibition of TNF-α in LPS-activated monocytes following treatment with mCRP. Nystatin reversal of nCRP-mediated reduction of TNF-α suggests that nCRP might become dissociated in unactivated macrophages to form mCRP that then interacts with lipid rafts in the membrane.

Supplementation with estrogen has shown that estrogen is having a dual function in regards to cell type. In unactivated monocytes and unactivated macrophages, estrogen is behaving in a pro-inflammatory manner leading to higher amounts of TNF-α. However, in LPS-activated monocytes and LPS-activated macrophages, estrogen significantly decreased TNF-α levels. There have been many studies that have shown that estrogen has a dual-modality, increasing or decreasing inflammation depending on cell type/status (Chakrabarti *et al*, 2008). The reduction of TNF-α by estrogen is achieved through the inhibition of the Hun NH(2)-terminal kinase (JNK) (Srivastava *et al,* 1999).

## 7.9 Future Work

Due to time and financial constraints, this study investigated levels of only four cytokines in inflammatory cells. Several other cytokines that are also involved in the actions and function of CRP would also be good candidate proteins to investigate in future work. Three of the main cytokines that could be analysed are interleukin-1β (IL-1β), interleukin-10 (IL-10) and interleukin-12 (IL-12). CRP can interact with FcγRI receptors to upregulate the production of cytokines; including IL-1, especially IL-1β which can also stimulate production of CRP; and IL-10 which is an inhibitor of IL-12 (Du Clos & Mold, 2004). IL-12 production is upregulated during periods of pronounced inflammation induced by LPS (Du Clos & Mold, 2004). IL-1β has been shown to act in a similar way to IL-6 and TNF-α, stimulating the production of CRP in the hepatocytes (Berger *et al,* 2002).

## 7.10 Summary

In general, the two CRP isoforms had varying effects upon the production of these four pro-inflammatory cytokines, dependent on the cell type and status. Comparison of the model of acute healing in the young and the model of age-related impaired healing, indicates that estrogen plays a substantial role in how these CRP isoforms function during the inflammatory response.

# Chapter 8: The effect of C-reactive protein isoforms on the recovery of MRSA colonies from an *in vitro* phagocytosis assay

## 8.1 Introduction

### 8.1.1 C-Reactive Protein and Pathogens

C-reactive protein is a marker for inflammation and its levels increase during bacterial infection (Healy & Freedman, 2006). Kingsley and Jones (2008) stated that during infection CRP rises in response to monocytic mediators such as IL-1 and IL-6 and that it has a stable decay rate. It is thought that most of the interaction between CRP and the immune response to pathogens lies within the binding of CRP to phosphocholine (PCh) and the activation of the classical complement pathway (Mortensen, 2001). There is much evidence that CRP can confer some protection against pathogenic infections. It was first documented by Mold *et al* (1981) who found CRP provides mice with protection against infection with the gram-positive pathogen *Streptococcus pneumoniae* by binding to a PC determinant of the pathogen cell wall and activating the complement pathway. Mice pre-treated with 200µg CRP before being infected showed an increase in percentage survival across all pathogen doses tested. The study concluded that the ability of CRP to protect against infection lies in its ability to bind to pneumococcal polysaccharide C in the bacterial cell wall (Mold *et al,* 1981).

Szalai *et al* (2000) also showed that CRP can confer protective benefits against *Salmonella enterica* serovar Typhimurium; a gram-negative pathogen which provides a model of typhoid fever in mice. Using transgenic mice expressing human CRP the study found that CRP offered protection against a low dose of Typhimurium and increased resistance to a fatal infection with a low dose of Typhimurium. CRP can bind both PC and phosphoethanolamine (PEt) but Typhimurium has PEt, yet does not bind to CRP *in vitro* and therefore should not be opsonised. Szalai (2000)

concluded that CRP increases the early clearance of IV-injected bacteria from the blood and reduces dissemination of bacteria to the liver and spleen during the initial stages of infection, thus allowing the mice to survive infection.

Marnell *et al* (2005) confirmed that CRP can also protect against *Haemophilus influenza* infection in both transgenic mice and wild-type mice treated by passive inoculation. This was stated to be because CRP binds the pneumococcal C-polysaccharide of bacteria and opsonises them for phagocytosis. This process does not require the use of the Fcγ receptors meaning that it is not primarily protective by direct opsonisation but more likely through the activation of complement and subsequent opsonophagocytosis. Marnell *et al* (2005) also stated that CRP was needed for serum bactericidal activity against the *Haemophilus influenza* pathogen but that complement activation was more effective.

Kinglsey and Jones (2008) tested the ability of CRP as a marker of inflammation and if it could be used to distinguish different infection types. They discovered that mean CRP levels in a spreading infection were higher than those in the other colonised, critically colonised and locally infected groups. All cases of infection showed an increase in CRP levels compared to non-infected controls but CRP levels could not distinguish between the infection types, showing that it is the infection in general that causes CRP levels to rise, rather than the type of infection. This was also noted by Healy & Freedman (2006) who showed that CRP levels can only be used as a method of detecting infection, rather than distinguishing it.

This evidence shows that CRP is not only a marker of infection and inflammation but that the CRP also has a protective role against bacterial infections, principally through the activation of complement and subsequent opsonisation of pathogens.

### 8.1.2 Methicillin-resistant Staphylococcus aureus (MRSA)

*Staphylococcus aureus* is a gram-positive coccal bacterium that is frequently found in the respiratory tract and on the skin. It is considered part of the normal host skin flora and so is not normally pathogenic but is a common cause of wound infections and abscesses, with most strains promoting infection through the release of toxins that bind and inactivate host antibodies (Chambers, 2001; Stryjewski & Corey, 2014).

Antibiotics, such as penicillin, are the main treatment for infection with *Staphylococcus aureus* but this has conferred antibiotic resistance in the pathogen. In 1959, methicillin was introduced as a treatment for penicillin-resistant *Staphylococcus aureus* but the first case of methicillin-resistant *Staphylococcus aureus* (MRSA) was documented just two years later in the United Kingdom. Cases of MRSA remained rare until the 1990s when there was an increase of cases leading to a worldwide problem (Enright *et al,* 2002). There are two types of MRSA infection; hospital acquired and community acquired. Hospital acquired infections tend to be contracted in the healthcare system from one patient to another whereas community acquired infections tend be found in places where there are a lot of people in an enclosed area, with lots of contact and humidity as well as in conditions of poor hygiene. Community acquired MRSA infections are common in schoolchildren, prisoners, soldiers and athletes (Köck *et al,* 2010).

MRSA can live in the host undetected and without causing symptoms until the host becomes immunocompromised or if the pathogen is introduced into the bloodstream which can lead to endocarditis, meningitis and sepsis. The only antibiotic used to treat MRSA is vancomycin however resistance to this is now being observed (Stryjewski & Corey, 2014).

*Staphylococcus aureus* develops resistance to methicillin via a part of the Staphylococcal cassette chromosome mec known as the mec operon (SCCmec). The resistance is conferred upon the penicillin binding protein 2A (PBP2a) leading to lower affinity of binding (Enright, 2003). This prevents the ring structure of

antibiotics to bind to the enzymes that help to form the cell wall of the bacterium (Enright, 2003). The SCCmec genomic island is of unknown origin and contains the cytolysin gene PSM-mec which suppresses virulence as well as ccrA and ccrB that mediate site specific integration and excision of the SCCmec element (Enright, 2003).

### 8.1.3 C-reactive Protein and MRSA

There is little research into the direct effect of CRP on MRSA. It is known that CRP levels rise when the host is infected with MRSA. Povoa *et al* (2005) stated that the normal CRP level for the healthy population is about 0.08mg/dL and this rises to more than 8.7mg/dL during chronic infection. Thus CRP can be used as an indicator of infection, alongside a body temperature of more than 38.2$^{o}$C.

CRP mediates host responses to *Staphylococcus aureus* including some protective function against infection and an increase in phagocytosis of this pathogen.

Patterson and Mora (1965) observed that enhanced resistance to intra-articular infection with *Staphylococcus aureus* in chickens was associated with an increase in serum CRP and that isolated preparations of the protein produced antibacterial activity. Mulholland and Cluff (1964) discovered that endotoxin-induced changes in resistance to local infection with *Staphylococcus aureus* in rabbits was correlated with the circulating levels of leukocytes in the blood. The results of this study showed that the induced resistance was paralleled by the increase in CRP and leukocytes. This finding was supported by Patterson, Harper and Higginbotham (1968) who found an association between CRP and non-specific resistance to infection, including *Staphylococcus aureus*, and showed CRP was acting upon the polysaccharide bacterial cell wall.

Black, Kusher and Samols (2004) stated that CRP enhances the *in vitro* phagocytosis of many microorganisms (including *Staphylococcus aureus*) by leukocytes. Their work confirmed this finding even in the absence of complement,

suggesting that the enhancement of phagocytosis by CRP is due to the interactions with Fcγ receptors.

## 8.2 Aims

The aim of this study was to determine the effects of different isoforms of CRP upon an *in vitro* model of phagocytosis using MRSA as the pathogen of choice since it is associated with chronic wound infections and immunocompromised hosts.

## 8.3 Objectives

The fundamental objectives of this study were:

- To determine whether CRP isoforms mediate the recovery of MRSA colonies *in vitro.*
- To determine whether CRP isoforms have differential effects upon the recovery of MRSA colonies *in vitro.*
- To use of inhibitors where appropriate to block the effects of CRP isoforms on the recovery of MRSA colonies *in vitro*.

## 8.4 Summary of Procedure

An *in vitro* model of macrophage phagocytosis was utilised whereby the recovery of viable bacteria after a defined period of host-pathogen interaction was measured using a fixed number of starting bacteria and CRP-treated macrophages. The methodology was similar to that utilised by Drevets *et al* (2015).

In summary, the U937 monocyte cell line was differentiated into macrophages and activated using LPS and IFN-γ to stimulate phagocytosis. A fixed number of macrophages (host) were then treated with CRP isoforms (with or without inhibitors) for 24 hours before incubation with a fixed number of MRSA (pathogen) for a period of 3 hours to enable host-pathogen interaction to take place. MRSA were subsequently recovered and cultured on agar plates to determine the number of surviving (non-internalised) bacteria following the defined period of host-pathogen interaction. Where CRP treatments caused significant changes in the number of recovered MRSA colonies, the assay was repeated to determine whether the findings could be reversed with the use of pharmacological inhibitors.

## 8.5 Materials

Materials for cell culture was as previously mentioned in Section 2.3.1. However, penicillin-streptomycin was removed from the phagocytosis assay to prevent any anti-bacterial effect on MRSA colonies.

Human monocyte cell line U937 *(Health Protection Agency Culture Collections, Salisbury)*

RPMI-1640 Media *(Lonza, Slough)*

Foetal Bovine Serum *(Sigma-Aldrich, Dorset)*

Lipopolysaccharide *(Sigma-Aldrich, Dorset)*

Phorbol 12-myristate 13-acetate *(Applichem, Darmstadt)*

Inhibitors – Prepared as previously mentioned in Section 2.4.9.

IFN-γ *(Sigma-Aldrich, Dorset)*

Bovine Serum Albumin (BSA) *(Sigma-Aldrich, Dorset)*

C-reactive Protein (CRP), human >95% native, 5mg/ml (*MyBioSource, San Diego)* – Purified as previously stated in Section 2.4.4.

Methicillin Resistant *Staphylococcus aureus* (MRSA) – Strain 11 is a patient isolate collected at Withington Hospital, Manchester. Many thanks to Dr K Whitehead (Manchester Metropolitan University) for this gift.

Nutrient Broth *(Oxoid, Basingstoke)*

Nutrient Agar *(Oxoid, Basingstoke)*

Saline *(Sigma-Aldrich, Dorset)*

Trypsin *(Thermo Fisher Scientific, Loughborough)*

## 8.6 Methods

### 8.6.1 Cell Culture

U937 cells were cultured and prepared as described in Section 2.4.1. U937 cells at a concentration of $1x10^6$ cells, were differentiated with PMA for 72 hours as previously described (Section 2.4.3) before being activated for 2 hours with 1µg/ml LPS and 100ng/ml IFN-γ.

### 8.6.2 C-reactive Protein Isoforms

CRP isoforms were purified and quantified as previously described in Section 2.4.4.

### 8.6.3 MRSA Assay

Activated macrophages prepared in Section 8.6.1 were treated for 24 hours with physiological concentrations of mCRP (100µg/ml and 250µg/ml) or nCRP (500µg/ml and 1000µg/ml) to mirror levels found in humans during acute inflammatory responses. All sample wells and controls were then incubated with MRSA bacteria at 5000 colony forming units/ml (CFU/ml), to a total of 5000 colonies, for 3 hours to simulate a fixed period of host-pathogen interaction. Controls included a phagocytosis-negative control lacking macrophages to provide a measure of maximum bacterial recovery in the absence of any host-pathogen interaction. Background phagocytosis was determined from measuring bacterial recovery following a period of host-pathogen interaction using untreated macrophages (i.e. macrophages lacking treatment with CRP isoforms). Additional negative controls were prepared using BSA as an alternative (control) protein at the same concentrations as the CRP isoforms (100µg/ml, 250µg/ml, 500µg/ml and 1000µg/ml) to confirm effects on bacterial recovery were CRP isoform specific and not simply due to the presence of an arbitrary polypeptide.

After a 3 hour host-pathogen incubation period all wells were treated with trypsin to promote detachment and recovery of non-internalised bacterial cells. Ten-fold dilutions were plated onto nutrient agar plates and incubated at 37°C overnight to recover viable bacterial colonies. The number of colony forming units were counted to calculate the bacterial recovery as a measure of bacteria that have not undergone phagocyte internalisation.

### 8.6.4 Inhibitors

Where appropriate (i.e. for those CRP treatments that significantly mediated bacterial recovery), the assay was repeated with the addition of the pharmacological inhibitors wortmannin, LML, MEK and nystatin to assess their ability to reverse the effects of CRP isoforms.

### 8.6.5 Statistical Analysis

Graphs were drawn using Microsoft Excel and all statistics performed using IBM SPSS Statistics (Version 22). The Shapiro-Wilkes normality test was conducted on the data before performing a one way ANOVA with Tukey pairwise comparisons.

## 8.7 Results

### 8.7.1 Microbiology Assay – General Effects of CRP on the Recovery of MRSA Colonies

Figure 8.1 shows the recovery of MRSA colonies after treatment of activated macrophages with CRP isoforms. A significantly decreased number of MRSA colonies were recovered when macrophages were present (background phagocytosis) compared to the negative control (bacteria alone) that lacked phagocytic macrophages. This is expected since macrophages activated with LPS and IFN$\gamma$ are known to carry out effective phagocytosis (Patel *et al,* 2012).

The negative BSA controls, regardless of BSA concentration, showed no significant change ($P>0.05$) in the number of MRSA CFUs recovered compared to the background level of bacterial recovery obtained with untreated macrophages, thus indicating that observed changes in recovery were due to specific CRP isoforms rather than just the presence of an arbitrary polypeptide.

The treatment with mCRP showed no significant difference (P>0.05) in the recovery of MRSA colonies compared to the background level of MRSA recovery, suggesting mCRP had no detectable effect on phagocytosis.

However, both concentrations (500µg/ml and 1000µg/ml) of nCRP showed a significant (P=0.01) reduction in the recovery of the MRSA CFUs. The nCRP concentration of 500µg/ml was subsequently taken forward to see if the addition of pharmacological inhibitors could reverse the nCRP-induced reduction in MRSA recovery.

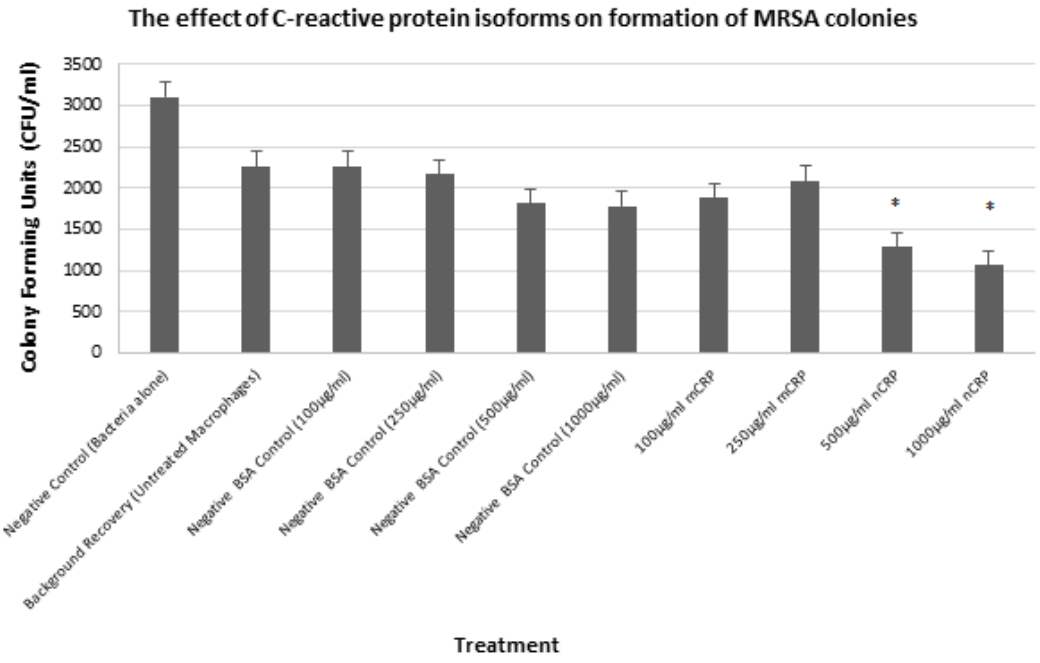



**Figure 8.1 – The recovery of MRSA colonies after treatment with C-reactive protein isoforms.** * denotes significant decrease in bacterial recovery (P<0.05) compared to the background level of bacterial recovery with untreated macrophages.

### 8.7.2 The Effect of Pharmacological Inhibition on the Reduction of MRSA Recovery Produced by 500µg/ml nCRP.

Figure 8.2 indicates the effect on four inhibitors on the recovery of MRSA colonies after treatment with nCRP at 500µg/ml. Treatment with the inhibitors showed a significant increase ($P<0.01$ in all cases) in the number of MRSA colonies recovered following treatment with nCRP at 500µg/ml. There were no significant ($P>0.05$) differences between the background level of bacterial recovery and results obtained from negative controls that contained activated macrophages incubated with just inhibitors alone (i.e. no treatment with CRP isoforms), thus confirming that the pharmacological inhibitors were blocking the biological action of nCRP rather than directly influencing bacterial recovery themselves.

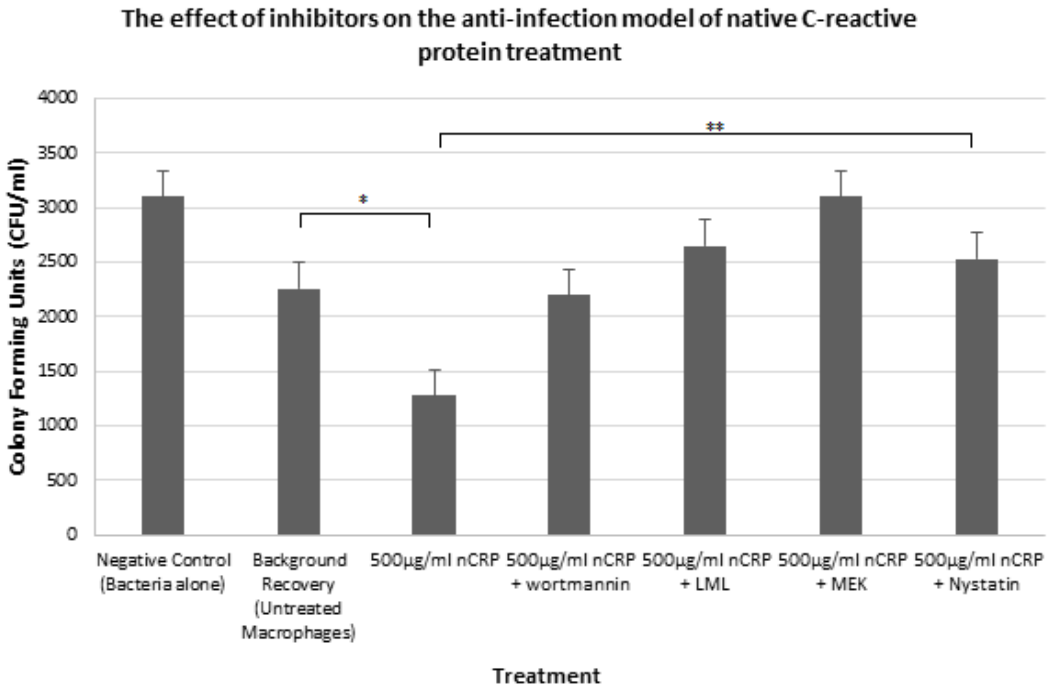



**Figure 8.2 – Recovery of MRSA colony forming units (CFUs) after treatment with nCRP at 500µg/ml and pharmacological inhibitors**. * indicates significant decrease ($P<0.05$) when compared to the background level of MRSA recovery with untreated macrophages. ** indicates significant increase in bacterial recovery ($P<0.05$) when compared to treatment with nCRP 500µg/ml alone.

## 8.8 Discussion

### 8.8.1 General Effects of CRP Isoforms on the Recovery of MRSA colonies

The phagocytosis assay showed a decrease in the number of MRSA colonies recovered in the presence of activated macrophages. This confirmed that host-pathogen interaction was indeed taking place within the assay.

Furthermore, the addition of the various concentrations of negative control (BSA) protein did not significantly alter the recovery of the MRSA colonies compared to the background level indicating that any changes observed with the CRP isoforms were protein-specific.

When the macrophages were treated with the nCRP isoform there was a significant ($P<0.05$) decrease in the number of MRSA colonies recovered compared to the background level of bacterial recovery, suggesting nCRP stimulates phagocytosis. This increase in host-pathogen interaction could be to the activation of the complement pathway. The nCRP can activate the classical complement pathway by binding to the C1q with the opposite face of the binding site (Du Clos, 2004). When C1q becomes enzymatically activated it triggers the binding and cleavage of C4 and C2, resulting in the C3b2b complex being formed. The C3b2b complex becomes covalently attached to the microbial surface and triggers the conversion of C3 to C3b, which also binds to the microbial surface. C3b can also bind to the complex to form the C5 convertase, which helps to create the membrane attack complex (C5b-C9). This process is known as opsonisation and will promote phagocytosis by macrophages (Mold *et al*, 1999). This process is shown in Figure 8.3.

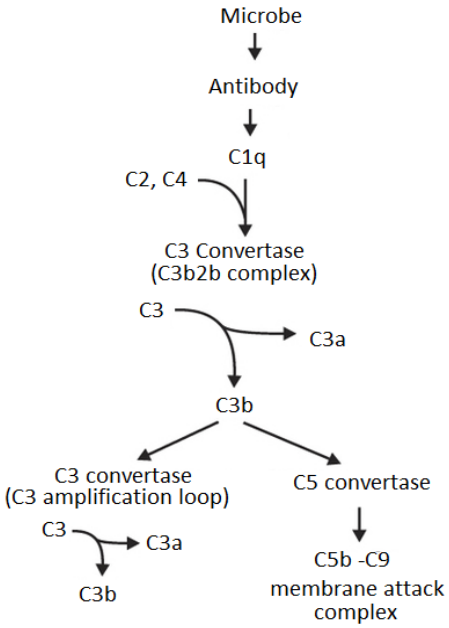



**Figure 8.3 – The classical complement pathway leading to opsonisation and phagocytosis.** Opsonisation occurs with the generation of C3b. Phagocytosis occurs when the membrane attack complex is formed. Modified from Xu *et al,* 2013.

The nCRP isoform can also act in a different manner as it is directly opsonic through interaction with Fcγ receptors. CRP was named for its ability to bind to the C-polysaccharide of *Streptococcus pneumoniae*. This reaction is mediated through the phosphocholine (PCh) moiety of the polysaccharide. This means that the nCRP can bind to the PC-containing phospholipids of the microbial polysaccharides and further opsonisation utilises FcγRI and FcγII (Mold *et al*, 2002).

This would suggest that the nCRP increases the opsonisation of the microbial surface and promotes the phagocytosis of bacterial pathogens. This is supported by other research findings including those of Black *et al* (2004) that showed CRP increases phagocytosis of *Staphylococcus aureus* even in the absence of complement. This study suggested the use of Fcγ receptors in the phagocytosis of pathogen. This study however did not state which isoform of CRP was responsible for the result.

When the results of the mCRP isoform are compared to those of nCRP there is a very different outcome. There were no significant differences observed in the recovery of MRSA colonies compared to the background level of bacterial recovery following mCRP treatment. This could be explained via the fact that when the native protein was dissociated into its monomeric form the opposite face, which is the C1q binding site, is lost and therefore there is no subsequent binding to the microbial surface to trigger opsonisation. The fact that the recovery of MRSA colonies was very similar to that of the background level would indicate that the mCRP isoform is not having any effect upon phagocytosis.

Overall, our findings indicate that the protective effect of CRP against pathogens seen in previous studies such as Mold (1981) and Szalai (2000) is likely to be due to the native protein rather than the monomeric isoform. The next stage was to observe if the addition of pharmacological inhibitors could reverse the effects of nCRP on MRSA recovery.

### 8.8.2 The Effect of Inhibitors on the Recovery of MRSA Colonies Following Treatment with 500µg/ml nCRP

All four of the chemical inhibitors significantly ($P<0.05$) increased in the recovery of MRSA colonies, suggesting they all blocked the phagocytic effect of nCRP to some degree.

#### 8.8.2.1 The Effect of Wortmannin on MRSA Recovery

Wortmannin is an inhibitor of the PI3 kinase pathway that plays a major role in the regulation of phagocytosis (Figure 1.3). Class I and III are regulators of the process but Class II PI3Ks have no function in phagocytosis (Cox *et al,* 1999). Class I and III PI2 kinases act consecutively in the formation and maturation of the phagosome, the vesicle which is formed around the particle that is to be phagocytosed. These PI3 kinases produce two products; $PI(3,4,5)P_3$ and PI(3)P,

which accumulate throughout phagocytosis at the different stages (Gillooly *et al*, 2001).

The formation of the phagosome requires actin reorganisation to allow the pseudopodia to extend around the particle to be ingested. Pseudopodia are extension of the cell which extend out from the cell and engulf the particle. This engulfment forms the phagosome of which when matured uses a combination of low pH, reactive oxygen metabolites and hydrolytic enzymes to break down the engulfed particle (Stephens *et al*, 2002).

Class I PI3 kinases are involved in the formation of the phagosome by allowing PI(3,4,5)$P_3$ to accumulate in the phagocytic cup in early phagocytosis, allowing for actin reorganisation to engulf large particles over 3µm in size (Tamura *et al,* 2009). This is followed by the accumulation of PI(3)P which seals the vacuole, creating the phagosome.

Class III PI3 kinases are mainly required to help the targeting of the phagosome to the membrane compartments during phagocytosis. This leads to the maturation on the phagosome and digests the large particle (Stephens *et al*, 2002). The engulfment of large particles is dependent upon this mechanism. Small particles however are phagocytosed in a PI3K-independent manner. Evidence has shown that the phagocytosis of large particles has been inhibited through the use of PI3 kinase inhibitors. This has also been correlated though the inhibition of actin reorganisation (Gillooly *et al*, 2001).

Thus the literature would suggest that wortmannin, which is a PI3K inhibitor, may be inhibiting actin reorganisation and therefore inhibiting the phagocytosis of MRSA colonies in this study, thereby accounting for the significant increase in MRSA colonies recovered.

### 8.8.2.2 The effect of LML on MRSA Recovery

The inhibitor LML is a p38 MAP kinase inhibitor. This pathway is also involved in the phagocytosis process (Figure 1.4). There are many genes that regulate the phagocytosis process and upregulation of these genes depends upon p38 activation downstream of TLR signalling (Tricker & Cheng, 2008). The main target for this activation is the actin cytoskeleton (May & Machesky, 2001).

As with the PI3 kinase pathway, the p38 MAP kinase pathway is involved in the remodelling of the actin cytoskeleton that is required for phagocytosis to occur. This is because p38 is a modulator of actin polymerisation which is downstream from Rac1. By inhibiting p38 MAPK phosphorylation, actin polymerisation cannot occur, which leads to the macrophage being unable to internalise the pathogen (Ninković & Roy, 2012). Also by inhibiting the p38 MAP kinase pathway in this manner it does not have any effect upon the ERK1/2 pathway nor does inhibiting ERK1/1 have any effect upon inhibition of the actin cytoskeleton (Ninković & Roy, 2012).

If p38 is being inhibited in this assay, it could block the polymerisation of the actin cytoskeleton and as a result inhibit the engulfment of the pathogen during phagocytosis.

### 8.8.2.3 The Effect of MEK on MRSA Recovery

In the MEK pathway, MEK 1 and MEK 2 are phosphorylated and trigger the activation of the ERK proteins (Figure 1.4). The MEK inhibitor inhibits these two enzymes and therefore inhibits the activation of ERK. When MEK 1 and 2 have are inhibited some anti-microbial functions are inhibited, including the oxidative burst and phagocytosis (Downey *et al,* 1998).

During phagocytosis, especially IgG-mediated phagocytosis, ERK 1 and ERK 2 are tyrosine phosphorylated in the preceding steps to ingestion. This activation can also be Fcγ receptor mediated through MAP kinase phosphorylation and activation

when the receptor is engaged and signal transduction occurs. It is the activation of this pathway that leads to phagocytosis (Suchard *et al,* 1997).

IgG is known to activate the classical complement pathway through C1q which can also be achieved through CRP binding. This would suggest that nCRP is activating phagocytosis through this pathway (Du Clos, 2004). The MEK inhibitor blocks this pathway by preventing the phosphorylation of MEK 1 and MEK 2, which in turn inhibits ERK. This type of inhibition does not affect the PI3 kinase pathway or the p38 MAP kinase pathway. This would suggest that in this assay, the MEK inhibitor was preventing the signalling pathway and preventing phagocytosis.

#### 8.8.2.4 The Effect of Nystatin on MRSA Recovery

Nystatin is an inhibitor of the lipid raft-caveolae endocytosis (Razonable *et al,* 2005). Endocytosis is the process by which cells can engulf molecules. Phagocytosis is one type of endocytosis; however, lipid raft-caveolae endocytosis is via a different mechanism. Lipid raft-caveolae are membrane domains that are rich in cholesterol and glycosphingolipids which are involved in the internalisation of many molecules such as certain toxins, GPI-anchored proteins and other cargos. These membrane domains are also involved in cholesterol trafficking. Caveolae are 'flask-shaped' pits in the membrane and are non-clatharin coated.

Nystatin is known to bind to ergosterol in fungi, which is the equivalent of cholesterol in humans. Nystatin binds to this and forms pores in the membrane to allow the crossing of potassium and other cell constituents to cross the membrane (Coutinho & Prieto, 2003).

Evidence would suggest that instead of targeting the pathways that control phagocytosis, nystatin is targeting the lipid-rafts in the cell and preventing the lipid rafts from assembling signalling molecules to influence protein trafficking (Razonable *et al,* 2005).

### 8.8.3 The Role of Inhibitors on MRSA Colony Recovery

This assay does not indicate that just one single pathway can be targeted to inhibit the effects of the native protein on the recovery of MRSA colonies. The results would suggest that the native protein is having a significant effect on phagocytosis by upregulating various potential pathways, and that pharmacological inhibition of one or more of these mechanisms reverses the nCRP-induced phagocytosis.

This could have future implications for healthcare interventions. Levels of CRP increase dramatically during bacterial infections and this study would suggest that the native protein is promoting pathogen uptake and destruction by phagocytosis. Thus, pharmacological inhibition of nCRP-mediated pathways or the conversion of nCRP to mCRP during the inflammatory response should be avoided in the presence of infection since this may impede pathogen destruction by impairing phagocytosis. Diseases and inflammatory conditions that induce the mCRP isoform and/or reduce the presence of nCRP are likely to be more prone to infection as a comorbidity in a non-sterile environment due to impaired phagocytosis. Furthermore, therapeutic strategies that would prevent the disassociation of nCRP to mCRP could potentially promote phagocytosis and pathogen elimination.

## 8.9 Future Work

The findings of this assay are indicative of phagocytosis taking place but confirmation of phagocytosis has not been demonstrated. The assay could be enhanced by adding a fluorescent label/tag the bacterial colonies using a fluorescent dye, such as fluorescein isothiocyanate, so that the process of phagocytosis can be observed by microscopy during the three hour incubation period. This would provide real time evidence of host-pathogen interaction and bacterial internalisation.

Future work could identify how the nCRP is triggering phagocytosis. This could consist of observing the effects of nCRP on phagocytosis in the presence of inhibitors of complement or the FCγ receptors to analyse which particular pathways are being utilised.

The assay could also be repeated with a number of pathogens. MRSA is only one of many pathogens that can infect a wound or abscess. Other potential pathogens include *Pseudomonas aeruginosa, Enterococcus faecalis and Streptococcus pyogenes* that are commonly associated with leg ulcers and chronic wounds (Siddiqui & Bernstein, 2010).

Further work could be conducted to confirm precisely how the mechanisms of phagocytosis are being inhibited by the four pharmacological inhibitors used in the assay. This could confirm how nCRP is acting upon those pathways and potentially lead to therapies that can promote pathogen destruction.

## 8.10 Summary

In summary, it is the native form of CRP that exhibits an effect upon the recovery of MRSA colonies, suggesting that nCRP enhances host-pathogen interaction via increased phagocytosis. This may be due to nCRP remaining intact and therefore enabling C1q binding to occur, triggering the complement pathway. This pathway would lead to the opsonisation of the microbial surface and the promotion of phagocytosis, supporting evidence that nCRP exhibits anti-microbial traits. The monomeric CRP has no effect upon the recovery of MRSA colonies suggesting that it has no affect upon phagocytosis.

The four pharmacological inhibitors all showed a significant increase in the recovery of MRSA colonies suggesting that these inhibitors are affecting the pathways controlling nCRP-induced phagocytosis. Further work would be needed to establish the exact mechanisms involved in enhancement of bacterial destruction by nCRP.

# Chapter 9: The Effect of Estrogen on *in vivo* C-reactive Protein Levels

## 9.1 Introduction

### 9.1.1 Age-related Impaired Healing

Age is a major factor that can impair the normal healing process (Guo & DiPietro, 2010). The main effect of age is that wound healing is delayed but does not cause an impairment to the quality of the healing (Guo & DiPietro, 2010). The main association with age-impaired healing is that there is an altered inflammatory response as well as an altered skin morphology. As skin ages there is a decline in dermal thickness as well as a decrease in the absolute number of cells (Ashcroft *et al*, 2002). Specific age-related changes to inflammation also occur including altered cell adhesion, migration and function responses (Ashcroft *et al*, 2002) leading to the impairment of the healing process; delayed T-cell infiltration to the wound area as well as changes in the production of chemokines and a reduced ability of macrophages to phagocytose (Swift *et al,* 2001). Cohen *et al* (1987) provided *in vivo* evidence that with age there was an increase in wound inflammatory cell numbers. Studies with aged mice have also shown delayed re-epithelialisation, collagen synthesis and angiogenesis when compared to young mice (Swift *et al,* 1999) and that there was increased production of pro-inflammatory cytokines such as IL-6 and TNF-α from human donors (Fagiolo *et al*, 1992).

### 9.1.2 Estrogen and Inflammation

There has been increasing evidence of the role of sex-steroid hormones in the wound healing and inflammation (Gulshan *et al*, 1990; Ashcroft *et al,* 1997; Guo & DiPietro, 2010). This is especially seen in age-related research into healing where

there is a substantial influence of these hormones on the inflammatory response *in vivo.*

Estrogen is mainly produced by the ovaries (Nelson & Bulun, 2001) and is released into the bloodstream however there is evidence that other peripheral tissues such as adipose tissue (Hemsell *et al*, 2001), skin fibroblasts (Sawaya & Penneys, 1992) and osteoblasts (Yaffe *et al,* 1998) are also sources of estrogen. Postmenopausal women see a depletion in their levels of estrogen and a result start to see impaired healing as well as other effects such as dry skin that bruises easily. These effects can be reversed with hormone replacement therapy (HRT) which also deposits collagen in the skin and thickens the skin (Ashcroft *et al,* 1999).

Estrogen has been shown to increase the rate of healing of acute wounds in both male and female patients by reducing the inflammatory response (Ashcroft *et al,* 1997; Ashcroft *et al,* 1999). In contrast testosterone delays acute wound healing and increases the inflammatory response (Ashcroft & Mills, 2002). As circulating testosterone levels do not tend to alter much with age, this could provide a reason as to why males tend to heal slower than females even after treatment with estrogen (Ashcroft *et al,* 1999).

Evidence also indicates that women have an enhanced immune system in comparison to a male immune system which can be depressed in cases such as trauma haemorrhage (Wichmann *et al,* 1996). This difference is attributed to the regulation of the immune system by hormones, with the male hormones causing a depression (Grossman, 1989). Other studies have shown that estrogen has an immune-enhancing effect during stress and by providing resistance to some bacterial infections (Yamamoto *et al,* 1991).

Human skin and inflammatory cells express estrogen receptors (ER) which provides evidence that local levels of estrogen may influence the wound healing and inflammation processes (Gulshan *et al*, 1990). This is achieved through the modulation of the inflammatory response, cytokine expression and matrix deposition leading to accelerated re-epithelialisation, stimulated angiogenesis and

wound contraction (Ashcroft & Ashworth, 2003; Hardman & Ashcroft, 2008). It has also been shown that topical and systemic estrogens improve the rate of wound healing in the elderly.

Estrogen modulates the inflammatory process by causing the inhibition of neutrophil migration through an alteration of the expression of neutrophil adhesion molecules (Ashcroft *et al,* 1999). This leads to increased wound collagen and fibronectin levels as a result of reduced neutrophil numbers in the wound area and consequently reduced neutrophil-derived elastase. In chronic wounds such as venous ulcers there is an elevated production of neutrophil-derived elastase and associated tissue breakdown (Herrick *et al,* 1997). Ashcroft *et al* (2003) also indicated that estrogen may have a direct effect upon monocytes and macrophages due to the estrogen receptors on their cell surface. However, the effect of estrogen upon these cells varies dependent upon their state of differentiation and local concentrations of estrogen. Estrogen causes the infiltration of immature monocytes to wound sites by increasing the secretion of MCP-1 but this effect is reversed in mature tissue macrophages, causing a downregulation of inflammation (Ashcroft *et al,* 2003). It is also suggested the estrogen can have an effect on the differentiation of monocytes into mature macrophages during the inflammation process (Calvin *et al,* 1998).

### 9.1.3 C-Reactive Protein and Estrogen

There has been tentative evidence of correlations and interactions between estrogen and CRP within inflammatory processes. As with most other previous research into CRP and its effects, the research conducted has referred to CRP as just a single entity and not differentiated between the two isoforms. Numerous studies have confirmed that CRP is a predictive marker for cardiovascular disease and that HRT use in post-menopausal women increases the risk of stroke and blood clots (Ridker *et al,* 1999; Ridker *et al;* Decensi *et al,* 2002; Vongpatanasin *et al,* 2003; Silvestri *et al,* 2003). Ridker *et al* (1999) found that healthy postmenopausal women

had nearly two-fold increased levels of circulating CRP when they were taking HRT and that CRP was the most affected inflammatory marker. As CRP is a marker of cardiovascular risk, this suggested that those on HRT were at a higher risk of cardiovascular implications such as stroke and blood clots (Ridker *et al,* 2000). However, there is some debate on the effect on CRP levels with HRTs where estrogens are combined with progestogens, with those taking oral HRT showing an increase in CRP levels in the first 12 months of therapy compared to those with transdermal therapy demonstrating no change in circulating CRP levels (Decensi *et al,* 2002). However, Vongpatanasin *et al* (2003) also showed that estrogen administered orally increases CRP levels twofold whereas estrogen administered transdermally had no effect upon circulating CRP levels. Furthermore, Silvestri *et al* (2003) showed that the effect of HRT on CRP decreased over time.

Corcoran *et al* (2010) found a variable response in CRP levels relating to the estrogen levels in both older men and postmenopausal women. No effect on CRP levels was seen when estrogen was replaced with testosterone, another steroid sex hormone found in circulation and peripheral tissues of both genders but produced at higher circulatory levels in males (Corcoran *et al,* 2010). This study highlighted a relationship between CRP and estrogen (Corcoran *et al,* 2010) which is concordance with work by Ashcroft *et al* (1999) showing that estrogen is the predominant sex steroid hormone mediating inflammation and wound healing processes.

Collectively, these studies suggest that estrogen increases circulating levels of CRP, leading to a more pro-inflammatory environment in circulation and thus an increased risk of certain cardiovascular events such as stroke. The effect of estrogen on local CRP levels in many peripheral tissues including skin has not been elucidated and studies using transdermal administration of estrogen have presented conflicting findings in terms of circulating levels of CRP. Multiple studies have shown that estrogen administered transdermally has no effect on circulating CRP levels (Decensi *et al,* 2002; Vongpatanasin *et al,* 2003). In contrast, other research has shown that circulating CRP levels are reduced in humans treated with transdermal estrogen (Giltay *et al*, 2000; Dencensi, Omodei & Robertson, 2002;

Nunomura *et al*, 1994). A reduction in CRP levels following an increase in peripheral estrogen concentration supports the findings of Ashcroft *et al* (1999) demonstrating that estrogen reduces the inflammatory response in wounds. Most CRP research in the literature has focused on cardiovascular risk and measured circulating CRP levels, principally in plasma or serum rather than in whole blood that contains leukocytes and platelets involved in the inflammatory response. Moreover, local production of CRP in many peripheral tissues like skin has not been investigated to date. Therefore, this study has investigated the effect of estrogen on peripheral levels of both CRP isoforms in murine wound and whole blood samples using an ovariectomised (OXV) model of age-related (estrogen-deprived) impaired healing with and without estrogen supplementation.

## 9.2 Aims

The aim of this study was to assess the levels of CRP isoforms in murine wound samples and determine the effect of ageing on CRP levels in an *in vivo* model of age-related (estrogen-deprived) impaired healing

## 9.3 Objectives

The fundamental objectives of this section were:

- To measure circulating amounts (both cellular and extracellular) of CRP isoforms in whole blood an *in-vivo* murine model of age-related impaired healing.
- To measure peripheral concentrations of CRP isoforms in the wounds of *in-vivo* murine models of age-related impaired healing.

## 9.4 Summary of Procedure

The *in-vivo* murine model of age-related impaired healing uses ovariectomised (OVX) female mice that have had both their ovaries surgically removed. This surgical procedure blocks the production of estrogen (since peripheral estrogen production is absent in mice) and represents a model of impaired healing typically found in elderly humans such as post-menopausal women. The supplementation of estrogen in OVX mice (OVX+E) reverses the impaired healing due to estrogen-deprivation found in ageing and is a model of hormone replacement therapy (HRT) in post-menopausal women. Blood samples from the OVX and OVX+E mice were used to measure total (both cellular and extracellular) circulating levels of CRP isoforms at day 7 post wounding. Normalised wound protein obtained from wound samples were used to determine peripheral levels of CRP isoforms in wounds of OVX and OVX+E mice at day 7 post wounding.

## 9.5 Materials

Mouse blood samples – OVX and OVX+E2 (provided by Dr M Hardman, University of Manchester)

Mouse wound protein samples – OVX and OVX+E2 (provided by Dr M Hardman, University of Manchester)

C - reactive protein, human >95% native, 5mg/ml (*MyBioSource, San Diego)* Purified as previously stated in Section 2.4.4.

Nitrocellulose membrane *(Scientific Laboratories Supplies, Nottingham)*

C-reactive protein buffer, adjusted to pH 7.4 (pH is varied for dissociation assay)

140mM Sodium Chloride *(Sigma-Aldrich, Dorset)*

20mM Tris-hydrochloride (*Fisher Scientific, Cheshire)*

2mM Calcium Chloride *(Sigma-Aldrich, Dorset)* – Calcium concentration is varied for dissociation assay

Primary anti-monomeric-CRP made up 1:10 ratio with blocking buffer

Anti-monomeric-CRP 8C10 culture supernatant *(this was a kind gift from Dr LA Potempa)*

Primary anti-native-CRP made up 1:10 ratio with blocking buffer

Anti-native-CRP 1D6 culture supernatant *(this was a kind gift from Dr LA Potempa)*

TBS/Tween buffer, adjusted to pH 7.4

10mM Tris-base *(Fisher Scientific, Cheshire)*

140mM Sodium Chloride *(Sigma-Aldrich, Dorset)*

0.1% Tween-20 *(Sigma-Aldrich, Dorset)*

Blocking buffer, adjusted to pH 7.4

1% Albumin from Bovine serum *(Sigma-Aldrich, Dorset)* in TBS-Tween

Milk Solution, adjusted to pH 7.4

5% semi-skimmed milk powder in TBS-Tween

Polyclonal rabbit, anti-mouse immunoglobulins/HRP *(Dako, Cambridge)* 1:1000 dilution in milk solution

EZ-ECL chemiluminescence detection kit *(Geneflow, Lichfield)*

## 9.6 Methods

### 9.6.1 Mouse Model

Murine blood and wound protein samples were kindly provided by our collaborators (Dr M Hardman *et al*) at the University of Manchester. Samples (n=4) came from a murine model of age-related impaired healing that had undergone ovarectomomy (OVX) and an OVX model supplemented with a slow release (0.05 mg/21 day) 17β-estradiol pellet (E2) (Innovative Research, USA) inserted subcutaneously in the scapula region, using a trocar, on the day of wounding. Wounding (day zero) was conducted in the form of a 1cm scalpel incision on the dorsum of each mouse as described previously in Section 4.6.1 and blood samples and wounds were collected at day 7 post-wounding and the whole blood samples were lysed by storage at -80$^{o}$C prior to subsequent analysis. Total protein was extracted from wound samples via Trizol extraction and total protein concentrations normalised (Section 9.6.2) prior to storage at -80$^{o}$C.

### 9.6.2 Total Wound Protein Normalisation

Total wound protein was normalised to 20mg/ml using CRP buffer (150mM NaCl, 20mM Tris-Hcl and 2mM $CaCl_2$, pH 7.4) in preparation for dot blotting.

### 9.6.3 C-reactive Protein Isoforms

CRP isoforms were purified and quantified as previously described in Section 2.4.4.

### 9.6.4 Dot Blots

Dot blots were conducted as previously described in Section 2.4.7. An example of a dot blot for this section is located in Appendix 1.

### 9.6.5 Statistical Analysis

Graphs were drawn using Microsoft Excel 2013 and all statistics performed using IBM SPSS Statistics (Version 22). Data were analysed with the Shapiro-Wilkes normality test before t-tests or Mann-Whitney tests were conducted upon all the data. Images from the Bio-Rad ChemiDoc™ Touch Imaging System were analysed using the Bio-Rad Image Lab Software (Version 5.2.1).

## 9.7 Results

### 9.7.1. The Effect of Estrogen on Levels of C-Reactive Protein in the Circulating Blood

Figures 9.1A and 9.1B show the differing levels of the CRP isoform in the lysed whole blood of OVX and OVX+E mice (n=4). There was a higher amount of circulating mCRP than nCRP, with only small levels of nCRP detected in lysed whole blood. With the addition of estrogen (OVX+E), the concentration of both isoforms in lysed whole blood increased significantly ($P<0.001$ in both cases) compared to the model of age-related impaired healing (OVX). The mean level of mCRP increased from 449µg/ml to 576µg/ml while the level of nCRP increased from 2µg/ml to 5µg/ml.

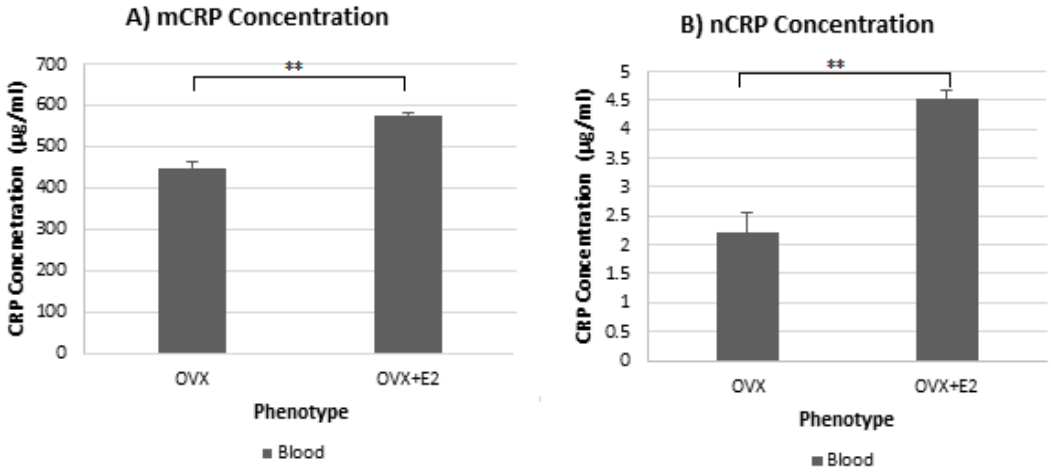



**Figure 9.1 – The concentration of CRP isoforms in lysed whole blood from a murine model of age-related impaired healing (OVX) and the reversal of age-related impaired-healing by estrogen supplementation (OVX + E2).** A) The concentration of total circulating mCRP B) The concentration of total circulating nCRP. Error bars represent standard error of the mean. ** denotes significant difference (P<0.001).

### 9.7.2. The Effect of Estrogen on Levels of C-Reactive Protein in Murine Wounds

Figures 9.2A and 9.2B show the differing levels of the CRP isoform in the murine wounds from OVX and OVX+E mice (n=4). There was a significantly ($p<0.001$ and $p<0.001$) in OVX and OVX+E mice respectively) lower concentration of each CRP isoform in wounds compared to total circulating levels measured from lysed blood. Again there was a higher concentration of mCRP than nCRP, with only small levels of nCRP being detected in wound samples. The concentration of both CRP isoforms decreased significantly ($P=0.003$ in the case of mCRP and $P<0.001$ for nCRP) following estrogen supplementation (OVX+E) to reverse the effects of age-related impaired healing in OVX mice. The level of mCRP decreased from 89µg/ml to 53µg/ml while the level of nCRP decreased from 0.86µg/ml to 0.45µg/ml following estrogen supplementation.

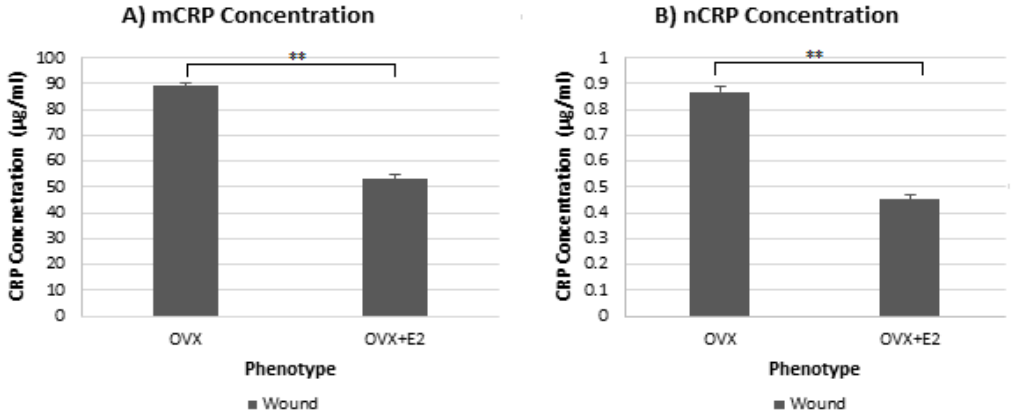



**Figure 9.2 – The concentration of CRP isoforms in wounds of a murine model of age-related impaired healing (OVX) and the reversal of age-related impaired-healing by estrogen supplementation (OVX + E2).** A) The concentration of mCRP levels found in murine wounds. B) The concentration of nCRP levels found in murine wounds. Error bars represent standard error of the mean. ** denotes significant difference ($P<0.001$).

## 9.8 Discussion

Research has suggested that estrogen plays a vital role in wound healing as well as estrogen and CRP levels being linked. The data in this study provides evidence to this theory.

### 9.8.1 Circulating Blood C-Reactive Protein Levels

Figures 9.1A and 9.1B show total circulating (cellular and extracellular) levels of CRP isoforms in lysed whole blood of mice. In estrogen-deficient mice (OVX), the level mCRP and nCRP was significantly ($P<0.001$) lower than when estrogen was supplemented indicating that the presence of estrogen induces circulating levels of CRP. Moreover, the levels of mCRP were 1.3 times higher in the estrogen-supplemented (OVX+E) mice compared to the OVX mice, suggesting a more pro-inflammatory environment in circulation due to the fact that mCRP tends to exhibit

more pro-inflammatory functions than nCRP (Zhao & Shi, 2010). These data support evidence that pre-menopausal women are at greater risk of certain cardiovascular events such as stroke due to elevated CRP levels (Kurtz *et al*, 2011). Thus in circulation, estrogen is working in a pro-inflammatory manner, elevating total CRP levels. These data supported by previous research indicating that post-menopausal women taking hormone replacement therapy (HRT) had much higher levels of CRP than women who were pre-menopausal or had no HRT treatment (Kurtz *et al*, 2011; Liukkonen *et al*, 2010).

### 9.8.2 Wound C-reactive Protein Levels

Figures 9.2A and 9.2B show the effect of estrogen supplementation on levels of CRP isoforms in a model of age-related impaired healing. When estrogen is supplemented back there is a significant ($P<0.001$) decrease in the concentration of both CRP isoforms.

The reduction in CRP levels, particularly the substantial decrease (40.5%; 36µg/ml) in mCRP, suggests that in peripheral tissues such as skin estrogen is behaving in an anti-inflammatory manner during wound healing. This supports extensive findings within the literature indicating that estrogen reduces the inflammatory response during animal and human wound healing (Ashcroft & Ashworth, 2003).

### 9.8.3 The Effect of Estrogen on CRP Levels

These results confirm that estrogen has a dual nature, exhibiting both pro- and anti-inflammatory properties at the same time in different tissues. In circulation, estrogen behaves in a pro-inflammatory manner by increasing the levels of CRP (mCRP in particular). In murine wounds that have a propensity for impaired healing and excessive inflammation, estrogen acts in an anti-inflammatory manner by reducing CRP levels (mCRP in particular) at the wound site.

Previous research has shown that gender has a substantial influence on the risk of cardiovascular events with higher estrogen levels during youth elevating the risk of blood clots and HRT use in post-menopausal increasing the risk of stroke. The findings of this study showing greater levels of CRP isoforms (particularly mCRP) associated with circulating whole blood following estrogen supplementation are in concordance with the literature. For example, it is known that mCRP plays a role in the pathology of cardiovascular disease due to its pro-angiogenic nature and its ability to promote thrombus formation (Slevin & Krupinski, 2009). However, in direct contrast to the situation found in circulation, this study has shown that in skin tissues estrogen reduces local (peripheral) wound levels of CRP isoforms (mCRP in particular). Estrogen promotes healing by dampening inflammation, regulating cytokine expression, stimulating angiogenesis and promoting wound contraction and re-epithelialisation (Ashcroft & Ashworth, 2003; Hardman & Ashcroft, 2008). The postmenopausal decline in estrogen has been shown to lead to impaired healing due to an excessive inflammatory response. The decline in estrogen is accompanied by the overproduction of pro-inflammatory cytokines including IL-6 which is known to stimulate CRP production (Chakrabarti *et al*, 2008). Thus, the findings of this study suggest that estrogen may inhibit the inflammatory response in wounds at least in part by reducing local CRP levels, particularly mCRP, through the down-regulation of cytokines such as IL-6. The findings of this study help to clarify why HRT can be beneficial to wound healing but at the same time can be linked to a higher risk of particular cardiovascular events such as stroke. The results have confirmed the dual nature of estrogen in promoting or inhibiting inflammation at the same time in different tissues (Chakrabarti *et al*, 2008) and highlights a potential role for CRP isoforms during wound healing processes.

## 9.9 Future work

Future work could investigate the effect of ageing (estrogen deprivation) on CRP isoforms in CRPtg mice that can produce human CRP via a transgenic gene (Szalai *et al*, 2002). This transgenic model could be used to assess whether estrogen mediates the expression of human CRP in a similar manner to that shown in this study. *In vitro* studies using human cells typical of the wound environment, particularly macrophages and neutrophils, should be performed to determine whether exogenous estrogen treatments are able mediate CRP isoform levels.

Other steroid sex hormones, such as testosterone, that are known to mediate the inflammatory response during wound repair (Gilliver *et al*, 2007) should be investigated to determine their effects on CRP levels in wounds.

## 9.10 Summary

In summary, this study has shown that estrogen can affect levels of both isoforms of CRP in opposing directions in circulation and wounds. The findings suggest that estrogen may play a pro-inflammatory role in circulation by stimulating CRP levels but appears to be anti-inflammatory in wounds by inhibiting CRP levels. The mechanism by which estrogen mediates CRP levels in not fully elucidated but may involve the regulation of cytokines such as IL-6. The findings suggest CRP isoforms may have a role in wound healing processes, particularly the inflammatory response.

# Chapter 10 - Discussion, Future Work and Conclusions

## 10.1 Discussion

### 10.1.1 Results Summary

This study has provided novel data on the effects of CRP isoforms on inflammation in the context of wound healing processes, particularly age-related impaired healing. Understanding the effect of CRP isoforms on monocytes and macrophages *in vitro* will form the basis for future work confirming these findings in animal models and investigating the precise mechanisms and pathways involved. Ultimately, a body of evidence confirming a role for CRP in wound repair may lead to therapeutic strategies that may mediate age-related impaired healing and chronic wounds in elderly humans.

#### 10.1.1.1 Stability of C-Reactive Protein

The preliminary results of Chapter 3 indicated that calcium concentration of local tissues is a major factor in the stability of the pentameric protein with pH playing a lesser but detectable role. The stability of nCRP is a key factor maintaining the balance of the two CRP isoforms and the resulting inflammatory response in tissues.

#### 10.1.1.2 Immunohistochemistry

The data in Chapter 4 of this study confirmed that both isoforms of CRP were differentially localised to inflammatory cells (neutrophils and monocytes/macrophages) in both the early (D3) and late (D7) inflammatory stage of murine wound healing. By observing changing levels of the CRP isoforms, notable differences in CRP localisation were identified between models of acute healing in young (INTACT) mice and age-related impaired healing in OVX mice.

#### 10.1.1.3 Growth and Apoptosis

The assays in Chapter 5 provided evidence that both CRP isoforms can affect the cell growth, having a detrimental effect upon the growth and/or viability of inflammatory cells at high concentrations. Both isoforms caused a decline in the number of monocytes and macrophages but only nCRP caused apoptosis. Evidence would suggest that the nCRP is causing apoptosis through a variety of pathways potentially through increased capase-3 activity (Blaschke *et al,* 2004).

#### 10.1.1.4 Nitric Oxide Production

The findings in Chapter 6 suggested that both mCRP and nCRP had the ability to differentially mediate NO levels in monocytes and macrophages in a cell type/state- and concentration-dependent manner. The response of cells to CRP isoforms were dependent on the differentiation state of the cells and whether there was pre-stimulation with bacterial endotoxin. Treatment with nCRP had an inhibitory effect on NO production in macrophage-like cells, displaying anti-inflammatory properties, whereas mCRP had a stimulatory effect on NO synthesis in monocyte-like cells promoting the inflammatory response.

#### 10.1.1.5 Cytokine Production

The results in Chapter 7 provided evidence that the two CRP isoforms had differential effects upon the production of pro-inflammatory cytokines in a cell type/state-dependent manner. The findings indicate that estrogen plays a substantial role in how CRP isoforms function during the inflammatory response.

#### 10.1.1.6 Phagocytosis

In Chapter 8, nCRP reduced the recovery of MRSA colonies, suggesting nCRP enhances host-pathogen interactions via increased phagocytosis. The literature suggests this occurs due to nCRP triggering the complement pathway and leading to the opsonisation of the microbial surface. Treatment mCRP had no effect upon the recovery of MRSA colonies suggesting that it has no effect upon phagocytosis.

#### 10.1.1.7 Estrogen Deficiency

Chapter 9 provided evidence that estrogen mediates levels of both isoforms of CRP in both murine circulating blood and wounds. The findings indicated estrogen induces a pro-inflammatory response in circulating blood but acts in an anti-inflammatory manner in wounds by differentially altering the profile of CRP isoforms.

### 10.1.2 General Discussion

#### 10.1.2.1 Presence of CRP isoforms on Inflammation

The results of the immunohistochemistry have shown that both isoforms of CRP are localised in neutrophils, monocytes and macrophages at the site of inflammation during murine wound repair. This corroborates the use of U937 cells in subsequent *in vitro* investigations since it has been confirmed that the U937 cell-line can produce CRP (Ciubotaru *et al*, 2005). The fact that there are changes in the temporal and spatial localisation of CRP isoforms during the inflammatory phase of wound repair suggests that CRP isoforms may play an active role in inflammatory processes during wound repair. There is existing evidence that CRP can activate the classical complement pathway (Du Clos, 2004) leading to the opsonisation and phagocytosis of cell debris and invading organisms (Mold *et al*, 1999).

This study has shown that both CRP isoforms are present in inflammatory cells throughout the inflammatory phase of acute wound healing at both D3 and D7. There was a significantly reduced amount of the CRP isoforms at the D7 time point but this could be accounted for due to the apoptosis of neutrophils (Li *et al,* 2007) following their peak at D3 and the subsequent recruitment of tissue macrophages (Kaplanski *et al,* 2003). However the results indicted that the D7 macrophages still had the ability to produce both isoforms and thus providing evidence for an enhanced role in the inflammation process.

#### 10.1.2.2 Biological Functions of CRP Isoforms

The findings from *in vitro* studies have confirmed that the two CRP isoforms have differing biological functions in monocytes and macrophages.

The nCRP isoform exhibited more anti-inflammatory effects such as promoting apoptosis, decreasing nitric oxide secretion, reducing pro-inflammatory cytokines levels and increasing phagocytosis. These findings are in concordance with published studies indicating that nCRP is more anti-inflammatory in nature than mCRP (Thiele *et al,* 2014). Further corroboration of the differing roles of CRP isoforms comes from the distinct receptors they bind in order to exert biological function. The proposed receptor for mCRP is FcγRIII (CD16), whereas the proposed receptors for nCRP are FcγRIIa (CD32) and FcγRI (CD64) (Khreiss *et al* 2002). *In vitro* studies have proposed mCRP interacts with lipid raft microdomains within the plasma membrane since interruption of these with nystatin abrogates mCRP responses including cytokine secretion, ROS formation and upregulation of adhesion molecules (Ji, *et al.,* 2009).

The results of this study have shown that inflammatory cells exhibit different functions in response to CRP isoforms, in a cell type/status manner. This may be explained by the expression of varying cell receptors on each of the cell types as well as varying activation processes (Geissmann *et al,* 2010).

The results in Chapter 5 show that high concentrations of nCRP (1000µg/ml) can cause apoptosis of cells while high concentrations of mCRP (250µg/ml) inhibits apoptosis. It is suggested that both of these isoforms are utilising the caspase cascade in which to trigger their effects. The nCRP isoform is most likely increasing caspase-3 activity to induce apoptosis (Blaschke *et al,* 2004) while the mCRP isoform is inhibiting apoptosis through the p38 MAPK pathway to prevent the triggering of the caspase cascade (McIlwain *et al*, 2015).

Chapter 6 indicated the differing functions of the isoforms on nitric oxide production. The mCRP isoform stimulated NO production in monocyte-like cells but had no effect in macrophage-like cells, regardless of activation by LPS (bacterial endotoxin). The nCRP isoform reduced NO production in macrophage-like cells both in the presence and absence of endotoxin activation and furthermore, in monocytes activated by bacterial endotoxin nCRP reduced NO levels in a concentration-dependent manner. These results suggest that mCRP may stimulate NO production in both the presence and absence of infection leading to a pro-inflammatory response whilst nCRP often opposed the actions of mCRP reducing the overall inflammatory response (Ciubotaru *et al*, 2005).

Chapter 8 provided evidence that high concentrations of nCRP (500µg/ml and 1000µg/ml) increased phagocytosis of MRSA while treatment with mCRP did not have any effect upon phagocytosis. When the macrophages were treated with the nCRP isoform there was a significant ($P<0.05$) decrease in the number of MRSA colonies recovered compared to the background level of bacterial recovery. This increase in host-pathogen interaction could be to the activation of the classical complement pathway by nCRP. When nCRP dissociates into the monomeric form there is a loss of the C1q binding site on the opposite face of the CRP molecule, meaning that opsonisation and phagocytosis cannot occur (Du Clos, 2004; Mold *et al*, 1999).

The findings from this study indicate that the balance between the two CRP isoforms (mCRP:nCRP ratio) may mediate the resulting inflammatory response and that this balance may be disrupted in impaired healing, including age-related

impaired healing and chronic wounds that affect the elderly. The balance of CRP isoforms could be disrupted by an increase in the dissociation of nCRP into mCRP, leading to a more pro-inflammatory effect. This imbalance in CRP isoforms could be brought about by detrimental changes in hormone levels (particularly estrogen deprivation) and calcium levels with increasing age. .

#### 10.1.2.3 Mechanisms of Action of C-Reactive Protein

The evidence provided by this study shows that CRP plays a more extensive role in inflammation than just acting as a marker of inflammation or activating complement. There is a potential link between the processes that have been investigated in this study. The results in Chapter 5 indicated that the CRP isoforms mediate the nitric oxide (NO) pathway. The nCRP isoform generally decreased NO levels whereas mCRP increased NO levels. The other results seen in Chapter 5 (apoptosis), Chapter 7 (pro-inflammatory cytokine secretion) and Chapter 8 (phagocytosis) can all be linked to the NO results obtained in Chapter 6. The NO pathway is utilised in all these processes (as well as many others). The pro-inflammatory mCRP isoform increased the amount of NO produced, especially in monocytes. In contrast, the amount of NO produced decreased following treatment with nCRP. When NO levels decrease this leads to increased apoptosis (Verma et al, 2004), altered phagocytosis regulation (MacMicking *et al*, 1997) and reductions in pro-inflammatory cytokine production (Andrew *et al*, 1995; Khreiss *et al,* 2004; Wetzler *et al,* 2000; Schwentker *et al,* 2002), as shown within this study.

Previous research has shown that CRP downregulates eNOS transcription in endothelial cells by destabilising eNOS mRNA, thereby decreasing NO release (Verma *et al,* 2002). However, this research only referred to CRP with no reference to the isoform involved but it can be deduced it was the nCRP isoform. This research is supported by the findings of this study showing the nCRP isoform reduces levels of NO in monocytes and macrophages. However, the NO synthases involved in inflammatory cells have not been confirmed.

Decreased NO production is known to facilitate endothelial cell apoptosis (Verma *et al*, 2004). In line with this, the nCRP isoform reduced levels of NO and led to apoptosis (Chapter 5). In contrast, the mCRP isoform did not induce apoptosis, possibly because it increased NO production. Since high concentrations of mCRP (250µg/ml) led to some cell death that could not be identified as apoptosis (Chapter 4), necrosis was highlighted as a potential cause of cell death.

From Chapter 5 it was shown that mCRP did not induce apoptosis and inhibited the process of apoptosis by preventing the activation of the caspase cascade (Figure 5.18) (Khreiss *et al,* 2002). However apoptosis can be NO-dependent or NO-independent (Li & Wogan, 2005) showing that NO is a regulatory mechanism in these processes. Thus, inhibiting this pathway via increased NO production leads to the inhibition of apoptosis.

NO is also known to directly inhibit caspase proteases which are the main mediators in cell death (Kim *et al, 2001)*. This is achieved by NO directly nitrosylating the active site of the caspases and preventing protein function (Kim *et al*, 1997). As mCRP has been shown to increase NO levels, this would have a direct inhibitory effect upon the apoptotic pathway.

NO inhibits apoptosis by preventing activation of caspase-3 activation by regulating the expression of death receptors in a cyclic guanosine monophosphate (cGMP)-dependent manner (Brüne *et al*, 1998). This leads to altered expression of proteins that trigger the early signalling events in death receptor signalling and thus reduced death-inducing signalling complexes. NO can also alter the expression of Bcl-2 proteins, which can be both pro- and anti-apoptotic (Brüne *et al*, 1998). This process alters the release of further downstream mediators and is regulated through the production of cGMP (Brüne, von Knethen & Sandau, 1998).

Nitric oxide is a key regulator of phagocytosis in macrophages (MacMicking *et al*, 1997). Usually high levels of NO are required within the phagosome to aid the breakdown of material without leading to a toxic effect within the cell (MacMicking *et al*, 1997). The results from Chapter 8 and Chapter 6 show that nCRP caused a

reduction in the recovery of MRSA and reduction in NO levels. This suggests the ability of nCRP to improve phagocytosis most likely occurs though increasing activation of the complement pathway. Thus, nCRP is probably causing the activation of the C1q molecule, thus triggering the complement cascade and leading to opsonisation of bacteria and increased phagocytosis by macrophages (Mold *et al*, 1999).

The release of cytokines by inflammatory cells initiates inflammation and NO is a key modulator of cytokines. NO can activate the promotor of the IL-8 gene but also in turn IL-8 can suppress the activation of NO (Andrew *et al*, 1995). The results of Chapter 6 indicated that CRP has an effect upon the release of IL-8, highlighting a potential pathway for further investigation. Significantly higher levels of IL-8 were detected when cells were treated with mCRP, indicating a more pro-inflammatory effect, which corresponds to the effect of the mCRP (Khreiss *et al*, 2004).

NO has also been implicated in the downregulation of the inflammatory phase of wound healing through the suppression of MCP-1 (Frank *et al,* 2000). In Chapter 5, NO levels were elevated in monocytes following treatment with the pro-inflammatory mCRP isoform. This highlights a possible mechanism by which elevated NO levels in monocytes may move the process of wound repair forward by subsequently inhibiting MCP-1 s (Wetzler *et al,* 2000).

Other studies have indicated correlations between the production of NO and levels of IL-6 and TNF-α within wound healing and inflammation processes. NO has previously been found to directly affect the expression of TNF-α through a cGMP-independent mechanism (Lander *et al,* 1993). There has been debate regarding the effect of NO on IL-6 production, with some studies showing stimulation and others showing suppression of IL-6 (Schwentker *et al,* 2002).

When all of the studies are considered collectively it appears the nitric oxide pathway may possibly be a link between the various effects of CRP isoforms. To determine if the NO pathway mediates other functions associated with CRP

isoforms future work could utilise specific inhibitors of the NO pathway to see if other functions such as phagocytosis, apoptosis and cytokine production are significantly perturbed.

#### 10.1.2.4 CRP and Estrogen

The results of this study have shown that estrogen supplementation causes an increase in CRP levels in murine circulating blood and a decrease in wounds (Chapter 9). The addition of estrogen mediates the CRP-induced changes in cytokine levels (Chapter 7)

Estrogen is a key regulator of healing, with impaired healing occurring in its absence (Gulshan *et al*, 1990). The OVX *in vitro* model used in this study represents this age-related impaired healing and the effects seen when estrogen is not present in these inflammation processes. The use of this model also allowed for the use of an addition of an exogenous source of estrogen (E2) in order to observe the representative effect of HRT in this model (Chapter 9).

Previous research has shown that by using HRT in post-menopausal women there is a sustained increase in CRP levels in circulating blood (Ridker *et al,* 1999). However, at wound sites estrogen reduces CRP (particularly mCRP) levels. This highlights the the dual nature of estrogen; functioning in a pro-inflammatory manner in circulating blood but functioning in an anti-inflammatory manner in peripheral tissues such as wounds. In terms of the anti-inflammatory nature of estrogen, research has shown estrogen can reduce NO production in monocytes (Vegeto *et al,* 2000; Vegeto *et al;* 2001). The results in Chapter 6 have shown that estrogen supplementation generally reduced NO production in monocytes but increased levels in the macrophages following treatment with mCRP or nCRP.

The ratio of mCRP:nCRP should be considered when analysing the effects of the CRP isoforms on the wound healing process. From the results, the mCRP isoform is considered to be more pro-inflammatory (Khreiss *et al,* 2004) than the nCRP isoform. The results in Chapter 4 indicated that in the age-related impaired

(OVX) model of healing, both time points indicated a higher mCRP:nCRP ratio and therefore a higher number of mCRP-positive cells. This high ratio indicates that the inflammation in impaired healing is excessive and prolonged, while corresponding wounds from INTACT mice have a lower ratio of mCRP:nCRP indicating fewer cells with the pro-inflammatory CRP isoform. It can be suggested that in the INTACT wounds this lower ratio of the two CRP isoforms leads to a resolution of inflammation, thus allowing the wound to heal more rapidly and successfully (Eming *et al*, 2007).

#### 10.1.2.5 Inhibition of Function

Nystatin is known to disrupt the plasma membrane of cells allowing for membrane leakage. These barrel-like pores allow for potassium and other cell constituents to pass across the membrane and cause cell death (Razonable *et al, 2005;* Coutinho & Prieto 2003). C-reactive protein, especially mCRP, is known to associate with the cell membrane and therefore any disruption of the cell membrane can lead to a decline in the function of the CRP isoforms. The use of nystatin as an inhibitor of the effects of CRP could be a potential therapeutic target in terms of disrupting the negative effects of the mCRP, thus reducing the pro-inflammatory actions of the CRP and helping to reduce inflammation.

The other three pathways; the PI3K, MEK and p38 pathway showed some inhibition over the effects of the CRP isoforms. One potential downstream target of these three pathways is nuclear factor-κβ (NF-κβ). NF-κβ is a protein complex that is involved in the transcription of DNA, the production of cytokines and is involved in cell survival (Tak & Firestein, 2001). Studies have shown that it plays a key role in the regulation of inflammation and incorrect regulation leads to inflammatory disorders including atherosclerosis and sepsis (Tak & Firestein, 2001).The results show a possibility that the CRP isoforms could be utilising these three inflammatory pathways to regulate NF-κβ and therefore regulate many inflammatory process. However any potential link between CRP and NF-κβ has not been identified.

There is the possibly a variety of pathways maybe utilised by CRP isoforms and therefore these pathways need to Interrogated in future work investigating CRP isoforms.

#### 10.1.2.6 Clinical Implications

The results of this study have shown that CRP should be a potential therapeutic target for treatment of inflammation. Prevention of the dissociation of the native CRP to the monomeric isoform can be a potential target of clinical significance.

CRP is a marker of inflammation and is implicated in a variety of different disease states such as atherosclerosis (Osman *et al,* 2006), arthritis and meningitis (Clyne & Olshaker, 1999). Research has shown that CRP plays a role within these aetiologies rather than just indicating the presence of disease. These responses include the activation of complement, induction of cytokines and alteration of cell adhesion proteins.

This study shows that CRP is a major component in inflammation and dysregulation of CRP-mediated responses may be involved in impaired healing and the pathogenesis of chronic wounds. The pro-inflammatory effects of mCRP relative to nCRP could acerbate the inflammation associated with chronic wounds. The mCRP:nCRP ratio is indicative of the effects of the CRP isoform on the wound healing process. In the age-impaired model there was a high mCRP:nCRP ratio, indicating a prolonged and excessive inflammation process due to a high number of mCRP positive cells. By targeting the mCRP isoform and potentially increasing the amount of the anti-inflammatory nCRP, could potentially accelerate repair in impaired wounds as this would lower the mCRP:nCRP ratio and bring the two inflammatory effects into balance and allowing the resolution of healing (Eming *et al*, 2007).

Targeting the dissociation of nCRP, potentially with calcium supplementation or pharmacological inhibition, or mediating CRP-mediated

responses using estrogen (or selective estrogen receptor modulators) in the elderly may reverse age-related impaired healing and offer therapeutic strategies to treat chronic wounds.

## 10.2 Future Work

Future investigations could use the compound 1,6 bis-phosphocholine (Habersberger *et al,* 2012) that has the ability to prevent the dissociation of the nCRP *in vitro.* This compound works by combining two nCRP molecules to prevent them from binding to the cell membrane (Habersberger *et al,* 2012). By using this compound, the effects observed following treatment with nCRP could be attributed entirely to nCRP, excluding the possibility of dissociation into mCRP. To determine if the NO pathway is a key mediator of other functions associated with CRP isoforms future work could utilise specific inhibitors of the NO pathway to see if other functions such as phagocytosis, apoptosis and cytokine production are significantly perturbed. Additional work should also be done with selective estrogen receptor modulators (SERMs) to determine which estrogen receptors are mediating the estrogen-induced changes in CRP isoform profile and biological activity.

This study has only mainly considered the effects of the CRP isoforms upon the monocytes and macrophages in the inflammation process. This means that there is the potential for this work to be repeated utilising other cell types including the use of neutrophil cell models, such as HL60, or an endothelial cell models, such as HUVEC. This would help to build a profile of cell-specific responses and functions mediated by CRP isoforms. This study can be progressed further by considering many other processes and functions of inflammation and wound healing including assays to investigate neutrophil/monocyte cell adhesion and chemotaxis, keratinocyte proliferation and migration, angiogenesis, fibroblast proliferation, extracellular matrix production and wound contraction). Further work with is also

needed to identify the precise mechanisms and pathways by which CRP isoforms may exert biological effects.

The findings from cell culture experiments conducted *in vitro* could be verified *in vivo* using animal models of wound repair and providing exogenous CRP isoforms locally to wounds. A mouse model that can express the human CRP gene (CRPtg mice) could also be adopted to investigate the effect of endogenous CRP production on wound healing processes. Additional studies could take *ex vivo* samples (e.g. inflammatory cells and skin biopsies) from human subjects to confirm findings in human tissues. Replication of findings from this study using *in vivo* and *ex vivo* models would provide a growing body of evidence confirming the extent of involvement of CRP isoforms in wound repair and may ultimately indicate potential therapeutic strategies for the treatment of age-related impaired acute wound healing and chronic wounds in the elderly.

## 10.3 Conclusion

C-reactive protein appears to be not just a marker of inflammation; the findings of this study indicate CRP isoforms are present in inflammatory phases of murine wounds and may differentially mediate key aspects of the inflammatory process in human cells; including phagocytosis, apoptosis, cytokine profile and NO production. The findings show that CRP isoforms regulate biological processes in a cell type/status-specific manner.

This novel research has shown that the ratio of CRP isoforms (mCRP:nCRP) reflects the degree of inflammation present in wounds with higher values indicating a more pro-inflammatory response that is associated with age-related impaired healing. These findings suggested the nCRP isoform is a potential therapeutic target and that preventing dissociation of nCRP or increasing the stability of nCRP may shift the balance toward reduced inflammation and accelerated wound repair.

This study has identified the NO pathway as a potential mechanism that could be mediating many of the functions associated with CRP isoforms during the inflammatory response. Finally this research has highlighted a relationship between estrogen and the activity of CRP isoforms during inflammation and the effect of estrogen on CRP isoform localisation in murine circulating blood and wounds. Estrogen mediated CRP-induced responses in a cell type/status-specific manner, leading to a more pro-inflammatory phenotype in circulation and a more anti-inflammatory phenotype in wounds. Estrogen deprivation in age-related impaired healing was associated with a more pro-inflammatory CRP isoform profile, with higher levels of mCRP than nCRP.

In conclusion, this study has provided novel data implicating CRP isoforms as important regulators of the inflammatory response in relation to wound healing. The work highlights the need to investigate CRP isoforms further to determine their precise roles during wound repair, particularly in age-related impaired healing and chronic wounds in the elderly.

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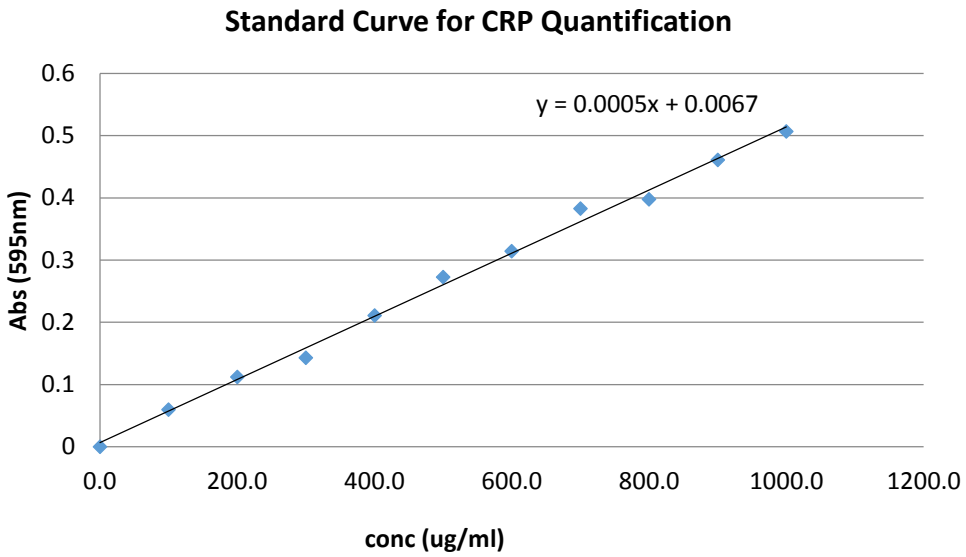
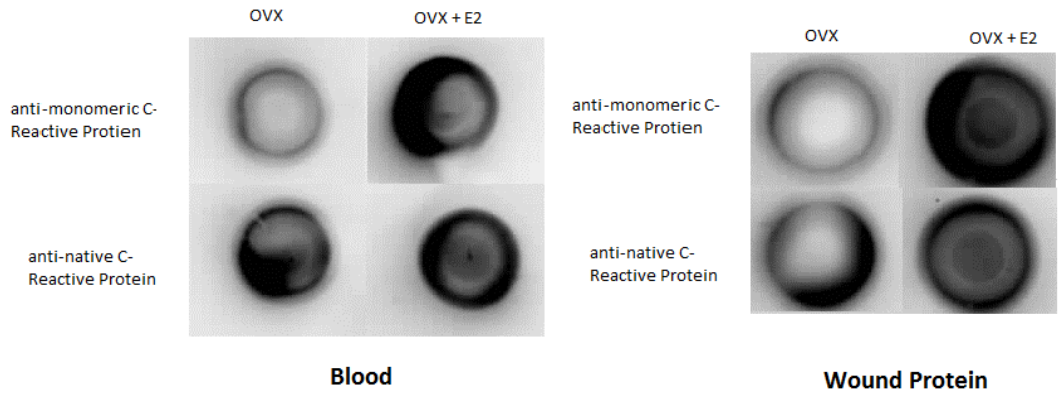
# Appendix

Appendix 1



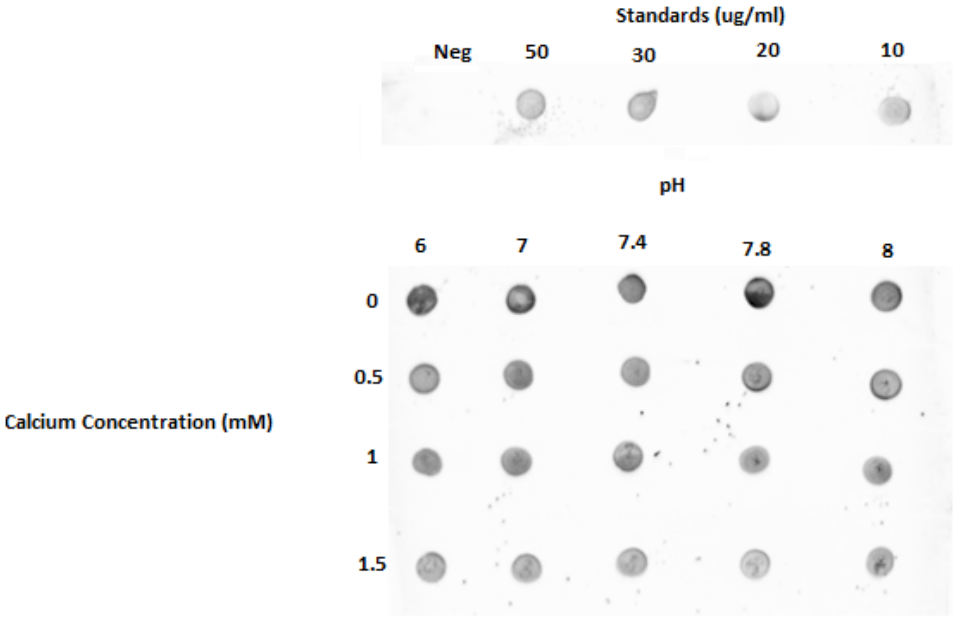

Appendix 1 – An example standard curve used to quantify the concentration of purified mCRP and purified nCRP. Line of best fit is **CRP Concentration = (0.0005 x absorbance at 595nm) + 0.0067**

Appendix 2



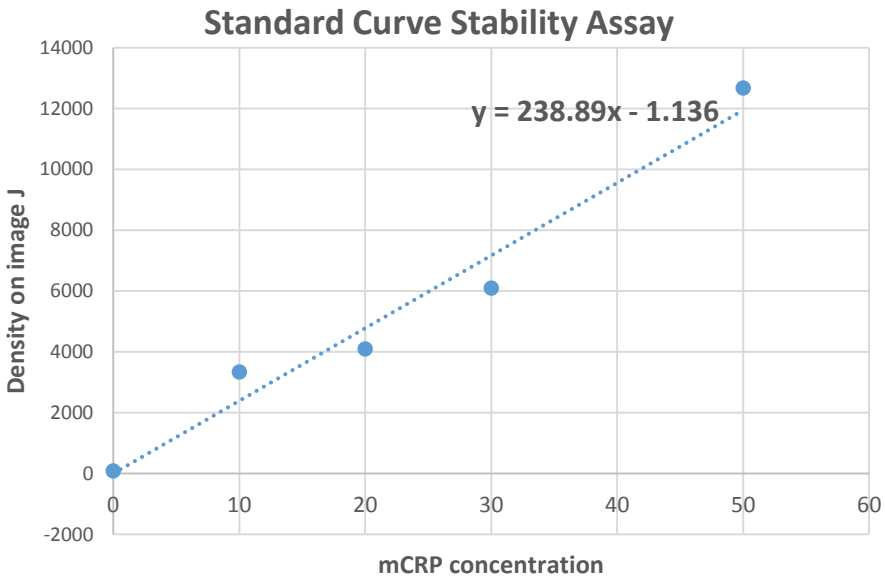

Appendix 2 – Example of annotated dot blot showing mCRP and nCRP presence in blood and wound protein (data collected for Chapter 9)

Appendix 3



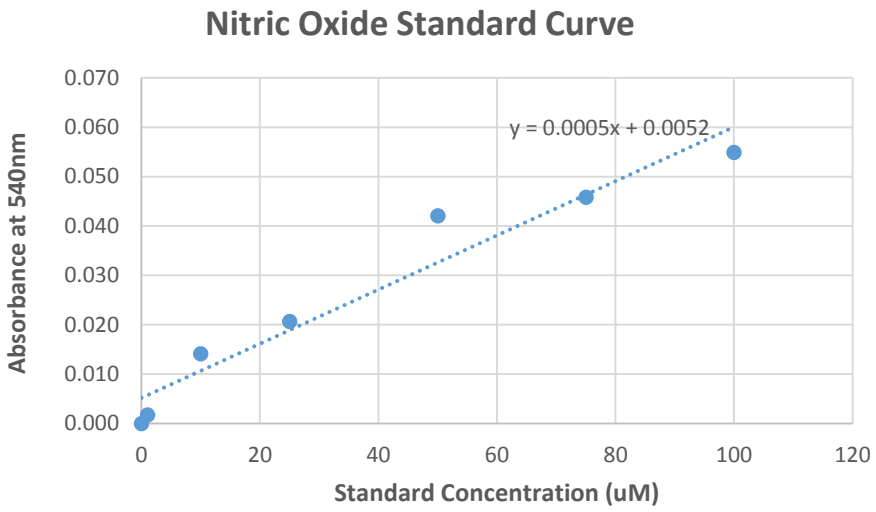

Appendix 3 - an annotated example dot blot showing the layout of for the cacium test in Chapter 3

Appendix 4




Appendix 4 – An example of a standard curve used to cacluate mCRP concentration in Chapter 3. Line of best fit is **density = (238.89 x mCRP concentration) - 1.136**

Appendix 5




Appendix 5 – An example of a standard curve used to calculate nitric oxide concentration in Chapter 6. Line of best fit is **Nitric oxide concentration = (0.005 x absorbance at 540nm) + 0.0052**