Investigation of serum monomeric C-reactive protein and associated auto-antibodies in rheumatoid arthritis

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Abstract

The research described here considers molecular variations of the acute phase response protein, C-reactive protein (CRP), and the presence of CRP and anti-CRP auto-antibodies in rheumatoid arthritis (RA) patient serum.

Monomeric CRP (mCRP) was generated *in vitro* by 2M urea induced dissociation of native pentameric CRP (pCRP), over a 10 week period in the absence of calcium. The subunit size (23kDa) and identity were confirmed by size exclusion chromatography, and western blotting with mCRP specific antibodies. Once dissociated, it was not possible to induce re-association.

Human RA (n=30) and healthy control samples (n=30) were tested for the presence of serum mCRP. RA patients had higher mean mCRP levels than non RA (0.092mg/l, 0.069mg/l), however no samples were elevated above the calculated normal threshold for mCRP (≥ mean + 2SD, 30 control samples). There was no correlation between serum levels of mCRP and pCRP, suggesting physiological dissociation of pCRP may not be solely responsible for the presence of mCRP.

Auto-antibody detection by competitive ELISA confirmed the presence of anti-mCRP and pCRP auto-antibodies of the classes IgG, IgA and IgM in both RA and control groups. Anti-mCRP and pCRP auto-antibodies in RA samples were significantly higher than controls in the female cohort in all but anti-mCRP IgM, with only anti-m/pCRP IgA significantly higher for the males ($P<0.01$). Both mCRP and pCRP were found to interact directly with anti-IgG at high concentrations of both. A greater proportion of RA samples contained all three auto-antibody classes, anti-pCRP - RA 66.6%, control 50%; anti-mCRP- RA 50%, control 13%. Auto-antibody profile varied between RA and control groups, elevated anti-mCRP IgA antibodies being a key predictor of RA risk $P<0.0001$,

more so when combined with advancing age although no correlation between CRP and auto-antibody concentrations was found. This may prove useful diagnostically.

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Abbreviations

ACPAs	Antibodies to citrullinated protein antigens
AD	Alzheimer's disease
Anti-CCP	Anti-cyclic citrullinated peptide
Anti-CII	Anti-collagen type II
APR	Acute phase response
APC	Antigen presenting cell
APP	Acute phase protein
BSA	Bovine serum albumin
BAFF	B cell activating factor
CCL2	Monocyte chemotactic protein (MCP-1)
CCL5,	Chemokine (C-C motif) ligand, also known as RANTES
C/EBPβ	(CCAAT/enhancer-binding protein beta)
CRP	C-reactive protein
CVD	Cardio vascular disease
DAS28	Disease Activity Score in 28 joints
DMARDs	Disease-modifying antirheumatic drugs
ESR	Erythrocyte sedimentation rate
HRP	Horseradish Peroxidase
HAS	Human serum albumin
HNF1	Hepatocyte nuclear factor
HUVEC	Human umbilical endothelial cells
HVSMC	Human vascular smooth muscle cells
ICAM	Intercellular adhesion molecule
IDDM	Insulin-dependent diabetes mellitus

IFNγ	Interferon gamma
Ig	Immunoglobulins
iNOS	Inducible nitric oxide synthase
IP-10	IFN-γ-inducible protein 10
ITAMs	Immunoreceptor tyrosine-based activation motifs
ITIMs	Immunoreceptor tyrosine-based inhibitory motifs
LDL	Low density lipoprotein
LPS	lipopolysaccharide
MCP-1	Monocyte chemotactic protein (CCL2)
MCP-2	Monocyte chemotactic protein (CCL8)
M-CSF	Macrophage colony-stimulating factor
mCRP	Monomeric C-reactive protein
MHC (I or II)	Major histocompatibility complex I or II
MMP	Matrix metalloprotein
MTX	Methotrexate
NAb	Naturally occurring antibodies
NADPH	Nicotinamide adenine dinucleotide phosphate
NETs	Neutrophil extracellular traps
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
OPG	Osteoprotegerin (osteoclastogenesis inhibitory factor)
PAD	Peptidyl arginine deaminase
PAF	Platelet activating factor
PAMPS	Pathogen associated molecular patterns
PC	Phosphocholine

pCRP	Pentameric C-reactive protein
pI	Isoelectric point
PMA	Phorbol 12-myristate 13-acetate
PMPs	Platelet derived microparticles
PRRs	Pattern recognition receptor proteins
RANTES	Regulated on activation, normal T cell expressed and secreted, also known as CCL5, chemokine (C-C motif) ligand
RF	Rheumatoid factor
SF	Synovial fibroblasts
SLE	Systemic lupus erythematosus
STAT3	Signal transducer and activator of transcription 3
TCL	T cell receptor
TGFβ	Transforming growth factor beta
Th	T helper cell
Tc	Cytotoxic T cell
TINU	Tubulointerstitial nephritis and uveitis syndrome
TLRs	Toll like receptors
TNF	Tumour necrosis factor
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor

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Almost nine years ago I began my PhD, little did I know then it would be one of the hardest things I have ever undertaken, however, it has also become one of the most satisfying. My PhD has taught me so much about myself and also about the people closest to me, without whom I would never have made it this far, I am truly grateful to you all.

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Chapter 1 - Introduction

1.1 Overview

C-reactive protein (CRP) is an evolutionarily conserved member of the pentraxin family of proteins and has been identified within a variety of organisms including mammals, fish and the horseshoe crab *Limulus polyphemus*. Pentraxin comes from the Greek words *penta* (five) and *ragos* (berries), due to its electron micrograph appearance (Pepys *et al*., 1978). CRP forms part of the acute phase response, with levels rising rapidly in response to inflammation and infection within the body. Upon detection of infection the liver increases transcription of CRP and levels rise in the serum within hours, making this protein a valuable and routinely used, nonspecific marker of infection, inflammation or damage to tissue (Pepys & Hirschfield, 2003).

Patients with chronic inflammatory diseases such as rheumatoid arthritis (RA) have raised levels of CRP, with the increase in serum used to diagnose and monitor levels of inflammation. This pentraxin is involved in several host defensive functions due to its ability to recognise foreign pathogens or damaged cells, leading to activation of the humoral and cellular arms of the immune system. Although serum levels vary due to disease state or genetics variants, there are no records of human deficiency in CRP (Pepys & Hirschfield, 2003), suggesting that, in conjunction with its conserved nature across species, CRP is a protein of great physiological importance.

1.2 The Human Immune System

Immunology may be a relatively new science in terms of the modern day understanding of the body's defence system, however it was first referenced during the plague of Athens in 430 BC, where it was recorded that people who had survived a previous bout of plague did not appear to contract the disease a second time (Retief & Cilliers, 1998). Fast forward nearly 2500 years and perhaps the father of immune system study, Edward Jenner, observed that cowpox appeared to confer protection against smallpox. In 1796 Jenner demonstrated his vaccination technique to initially skeptical peers, who soon recognized the potential of his discovery (Murphy & Weaver, 2017). Louis Pasteur in the late 19th century continued research into the vaccination process (Murphy & Weaver, 2017), and Robert Koch published his findings showing how isolating disease-causing organisms was key to the understanding of infectious disease. We now have a thorough understanding of the pathogens Koch was observing and immune system research follows in the footsteps of these pioneers.

The human and mammalian immune system is broadly split into two interacting systems, the innate immune system and the acquired immune system. The innate system is a fast acting and short lived non-specific line of defence, which recognizes 'non-self' agents and mounts a defence against them. The acquired system provides a more selective response, specific to targeted pathogens. Broadly, the immune system has a series of well-orchestrated tasks which begin with recognition of the pathogen, undertaken by the leucocytes, part of the innate system, and lymphocytes of the adaptive system. The body must then overcome the pathogen and contain it, preventing further infection, before ultimately removing it from the body. The complement system plays an important role at this point which when activated, sets in motion a pathway consisting of more than 30

plasma and membrane bound proteins, essential within the body's defence mechanism in the inflammatory response. One aspect of the complement system response is the activation of antibodies and recruitment of leucocytes. Once the pathogen has been eliminated, the body then instigates immunological memory, allowing a rapid reaction should the host need to respond to a subsequent challenge. Lastly the body must be able to regulate the immune response, ensuring it does not begin to cause harm to itself. Dysfunction of the regulatory and modulatory immune system risks causing harm to the host through autoimmune disease and allergies (Medzhitov & Janeway, 1997; Simon *et al*., 2015).

The human body has two systems of defence in the form of either physical or chemical barriers with which it prevents infectious disease causing agents such as virus, bacteria, fungi, unicellular or multicellular parasites from gaining access to the body. Physical barriers include skin, mucosa and cilia lining the respiratory tract and mucus secreted by epithelial cells of the gut. Chemical barriers include acidic conditions such as the surface of the skin or stomach acid, collectins which attach to sugars on microbial surfaces marking them for destruction and lysozymes, a constituent of tears and sweat. However, should the pathogen gain access, the body must be able to draw on further resources to fight against invasion rapidly (Murphy & Weaver, 2017).

Not all infectious organisms are harmful to the host, with many essential to physiological function. Bacteria living within our gut, known as commensal bacteria, help digest food and play important roles in maintaining intestinal homeostasis. The commensal microbiome aids maturation of the mucosal immune system and as such ensures the protective barrier of the intestinal tract is maintained (Shi *et al*., 2017). There are however many pathogens which constantly threaten our body and without a well-functioning immune system we would be vulnerable to infection by these pathogens on a daily basis.

1.2.1 The Innate Immune System

The innate immune system is an evolutionarily conserved form of defence found in multicellular organisms which allows the body to defend itself rapidly against pathogens such as bacteria and other microbes. This system relies upon the recognition of pathogens in a non-specific pattern recognition manner. The recognition of these patterns allows the host to determine self from non-self quickly and activates the acute phase response which involves the rapid release of platelets, leucocytes and the acute phase proteins including C-reactive protein (Medzhitov & Janeway, 1997; Beutler, 2004; Sjöwall & Wetterö, 2007a; Shishido *et al*., 2012; Simon *et al*., 2015). Organisms are born with innate immunity, with organisms from vertebrates to the fruit fly *Drosophila melaogaster* and the nematode worm *Caenorhabditis elegans*, (Murphy & Weaver, 2017) sharing genes involved within the intercellular signaling pathway of innate defence. This system mounts a defence that although not specific to the particular pathogen detected, is in place within hours of infection.

The innate system can be divided into two distinct arms of defence, the humoral and the cellular. The humoral response contains a diverse assortment of agents capable of instigating an attack on the pathogen by multiple components. Macromolecules including naturally occurring antibodies (NAb), serum proteins, and peptides are secreted into extracellular spaces following recognition of a non-self-entity. The breakdown and breach of external barriers resulting in pathogenic infiltration of tissue, leads to a local increase of blood proteins such as collectins (surfactant protein A and D, mannose binding lectin), C1q, pentraxins (C-reactive protein, PTX3, serum amyloid protein) and ficolins (Janeway *et al*., 2001; Iwasaki & Medzhitov, 2015). Complement activation follows recognition and binding of a pathogen or damaged/apoptotic tissue by C-reactive protein, which in turn activates C1q and the complement cascade (McInnes & Schett, 2007). The accumulation of

proteins resulting from instigation of the inflammatory pathway causes pain, redness and swelling to the area (Janeway *et al*., 2001; Murphy *et al*., 2008; Artis & Spits, 2015). Complement system activation will also be instigated allowing further recruitment of other immune agents such as antibodies and leucocytes, enhancing the response and level of inflammation at the site of infection (Chen *et al*., 2010; Shishido *et al*., 2012; Nonaka, 2014).

Central to the innate system is a network of phagocytic cells and proteins which recognise conserved features of pathogens (Medzhitov & Janeway, 1997; Murphy & Weaver, 2017). The discrimination between self and foreign cells through recognition of these highly conserved motifs called pathogen associated molecular patterns (PAMPS), is crucial to the ability of immune components to function without destruction of self-tissue (Berg *et al*., 2010). PAMPs are present on pathogen molecules and include proteins, nucleic acids, lipids and polysaccharides, often in repeating patterns. Conservation of these molecular structures is key to the success of the pattern recognition system, for instance lipopolysaccharide (LPS) is shared by all gram negative bacteria. PAMPS are detected via pattern recognition receptor proteins (PRRs) which are expressed on the surface of immune cells including dendritic and macrophage cells (Medzhitov & Janeway, 1997; Kawai & Akira, 2011; J. Shi *et al*., 2014), allowing the host to recognise a great variety of molecular structures that are associated with different pathogens. The PRRs are composed of a diverse set of receptors that belong to different protein families. Some like CD14 and collectins recognise PAMPs directly and others such as Toll and complement receptors recognise the products generated by PAMP recognition. Functionally PRRs fall into distinct classes, circulating humoral proteins, endocytic receptors expressed on cell surfaces such as macrophages and dendritic cells, and signaling receptors expressed on the surface or intracellularly (Medzhitov & Janeway, 1997). The exact nature of response will

be dependent upon the cell expressing the PRRs; antigen presenting cells induce activation of lymphocytes specific for pathogen antigens and effector cells of the innate immune system induce expression of cytokines such as IL-12 and IL-4 leading to activation of the adaptive immune system. Transmembrane proteins such as toll-like receptors (TLRs) upon recognition of PAMPs trigger an inflammatory immune response via cytokine signaling (Medzhitov & Janeway, 1997; Kawai & Akira, 2010; Sims & Smith, 2010).

The cellular component of the innate immune system relies on recruitment of immune cells to the point of infection and amplification of the initial inflammation state through local secretion of inflammatory signaling mediators, including cytokines and chemokines. The complex cycle of cytokines and chemokines released by the acute phase proteins and cells at the point of tissue injury can enhance either a pro or anti-inflammatory response (McInnes & Schett, 2007; Artis & Spits, 2015). These cytokine and chemokine releasing leucocytes include dendritic cells, neutrophils, natural killer cells, monocytes, eosinophils and basophils together with mast cells and macrophages (Lacy & Stow, 2011). The coordinated release of soluble mediators including tumour necrosis factor (TNF), interferon gamma (IFNγ), transforming growth factor beta (TGFβ), chemokine (C-C motif) ligand (RANTES) also known as CCL5 and interleukins (IL) such as IL-1β, IL-4,IL-6, IL-10, IL-12 and IL-18 carefully orchestrates further immune responses, which lead to the initial inflammatory response before subsiding (Lacy & Stow, 2011).

Phagocytes recruited during an inflammatory response ingest foreign particles such as bacteria, parasites, foreign debris and dead self-cells and digest them via internal enzymes located within the lysosome. Alternatively phagocytes employ a lysosome independent killing mechanism via expression of NADPH oxidase producing superoxide, hydrogen peroxide and inducible nitric oxide (iNOS), which generates nitric oxide (NO) (Pannen & Robotham, 1995; Robinson, 2008; Baptista *et al*., 2012; Iwasaki & Medzhitov,

2015; Simon *et al*., 2015). The innate immune system whilst different in terms of response time and specificity to the acquired or adaptive immune system, works in synergy with it, with many areas of overlap. The signaling components of the innate system and antigen presenting cells are intrinsic in the implementation of a full adaptive immune response and vertebrates have evolved to initiate both arms of the immune system, working together to protect the host from pathogenic agents.

1.2.2 The Adaptive Immune system

Should the innate immune system be unable to provide a response adequate enough to fully contain an infectious agent. In this instance, vertebrates have evolved an enhanced and sophisticated countermeasure known as the adaptive immune response. This specialised system contains a diverse range of cells capable of responding, albeit in a less rapid manner, to cytokine and chemokine signals originating from the innate system and raises an enhanced defence against the pathogen in question. What this system loses in response time, it gains in specificity, with a capacity to form long lasting protection or memory against previously encountered pathogens. An individual who may have previously encountered an infectious agent, such as measles, and recovered from an associated infection, would be protected against further infection due to the specific recognition ability of the immune system, however they would not be protected against other viruses (Alberts *et al*., 1994; Dörner and Radbruch, 2007).

Where the innate system relies upon PAMPs, the adaptive system is highly specific to a particular pathogen (Alberts, 1994; Kumar *et al*., 2011; Rachner *et al*., 2011). Cytokines and chemokines released by cells of the innate system raise the alarm and signal the adaptive system, which results in migration of immune cells to the site of infection. Of these cells B and T lymphocytes play a crucial role (Murphy *et al*., 2008; Artis & Spits,

2015; Iwasaki & Medzhitov, 2015), with their activation resulting in two classes of response, an immunoglobulin (antibody) and a cell-mediated response. The antibody response is the domain of the B cell which when activated secretes antibodies. These antibodies circulate the bloodstream and perfuse into other body fluids, ultimately binding the foreign antigen initially responsible for their production. B cells contain membrane bound immunoglobulins (B-cell receptors) which allow detection and presentation of an antigen, activation of the B cell follows presentation of an antigen to a helper T cell. Upon binding to the antigen via T cell receptors, the T cell releases cytokines which induce proliferation of B cells into memory cells and antibody secreting plasma cells (Janeway Jr. & Medzhitov, 2002; Berg *et al*., 2010; Sela-Culang *et al*., 2013; Murphy & Weaver, 2017).

Antigen presenting cells (APC), such as dendritic cells and macrophages engulf pathogens, processing and breaking them into peptides which are then presented upon their surface allowing them to interact directly with B cells or T cells. This intereaction occurs via major histocompatibility complexes (MHC) of either type I or II. The presentation of these fragments allows further signals initiating an adaptive immune response and activation of B cells, through specific cytokine signals received from an activated T helper cell. Both B and T cells recognise the same pathogen although not necessarily via the same epitope (Alberts *et al*., 1994; Murphy and Weaver, 2017).

T lymphocytes as a group, work together to provide a direct cell mediated response, attacking foreign pathogens such as a virus or bacteria. T helper cells (Th) work with antigen presenting cells (APC) to activate B cells. $CD4^+$ helper cell/inducer cells (T_H) and $CD8^+$ cytotoxic/suppressor T cells (T_C), express surface T-cell receptors which bind to their respective MHC class II or I ligands, found on antigen presenting cells (Miceli & Parnes, 1993; Artis & Spits, 2015; Hoffman *et al*., 2016; Shi *et al*., 2017). These cells are prevalent in a ratio of between 1.5 to 2.5 $CD4^+/CD8^+$ in healthy hosts, whilst a lower or

inverted ratio presents an immune risk, often associated with immune function alterations and chronic inflammation (McBride & Striker, 2017). T helper cells are responsible for the activation of other immune cells through MHC protein binding. Infected cells present fragments of the foreign pathogen on their surface enabling the T cell to distinguish between healthy and infected cells (Alberts *et al*.,1994; Murphy and Weaver, 2017). When a T_C cell recognises an antigen and becomes activated it initially secretes cytokines, primarily TNFα and IFNγ, both of which have anti tumour and anti-microbial effect, it then eradicates the virus infected, damaged, dysfunctional or cancer cell, inducing its death via a release of perforin, granulysin or granzyme leading to cell lysis or apoptosis. The caspase cascade which results in apoptosis of the cell via Fas/FasL interaction is a further tool with which the Tc cell eliminates infected or problem cells (Alberts *et al*., 1994; Murphy and Weaver, 2017). Additionally, antigen specific T memory cells provide long lasting immunity post infection.

On occasion the immune response against viruses such as hepatitis B and C, Epstein-Barr virus, rabies or even smallpox can cause more damage than the virus itself (Dörner & Radbruch, 2007). The balance between the initiated immune response as a protective or pathogenic measure is key to our survival.

1.2.3 The Acute Phase Response

The acute phase response (APR) is instigated upon tissue injury, trauma and inflammation and encompasses a range of changes to the concentration of many plasma proteins. These proteins are predominantly synthesised within the liver in response to inflammatory signals which bind to receptors on hepatocytes inducing transcription of these acute phase proteins (APP), (Pannen & Robotham, 1995). Whilst non-specific in nature these proteins are used as markers of disease. At the site of injury, pro-inflammatory cytokines are released and

the local vascular system and inflammatory cells are activated, resulting in production of further cytokines which permeate throughout the circulation of the body. The response is characterised by changes to endocrine, metabolic, neurologic and immunologic functions. Cytokines activate key receptors which in turn lead to systemic reactions and onward activation of the hypothalamic pituitary adrenal axis together with a reduction in growth hormone secretion (Gruys *et al*., 2005). In addition to this, levels of adrenocorticotrophic hormone (ACTH) and glucocorticoids increase, complement and the blood coagulation systems are activated and serum levels of calcium, iron, zinc and vitamin A fall (Gruys *et al*., 2005; Dinarello, 2009). The liver synthesis of APP increases and within a few hours levels of C-reactive protein (CRP) and serum amyloid A (SAA) increase. Platelets aggregate, dilation and leakage of blood vessels occurs and granulocytes and monocytes accumulate and are activated. Activated fibroblasts and endothelial cells produce yet more cytokines resulting in leukocytosis and an increased erythrocyte sedimentation rate due to elevated plasma concentrations of fibrinogen and other APP (Castell *et al*., 1990).

1.3 Components of the Immune System

1.3.1 Monocytes and Macrophages

Peripheral-blood mononuclear cells (PBMCs) and macrophages are key effectors and regulators of the innate immune response and inflammation. The monocyte is a class of leucocyte which when mature differentiates into macrophage or dendritic antigen presenting cells and develops from a common myeloid progenitor cell in the bone marrow. In humans, monocytes differentiate from CD34-positive stem cells, leaving the bone marrow to circulate the body for approximately five days until they become macrophages following transmigration from the lumen of blood vessels into tissue (Burmester *et al*., 1997). This translocation follows recruitment to localised sites of inflammation or infection upon detection of monocyte chemotactic protein 1 (MCP-1) (Lundberg & Hansson, 2010).

Macrophages are considerable phagocytic cells which can clear 2×10^{11} erythrocytes each day (Mosser & Edwards, 2008). Macrophage action is independent of immune cell signaling and the clearance of apoptotic cells effects little to no production of immune mediators by unstimulated macrophages suggesting most ongoing phagocytosis occurs independently of other immune cells (Mosser and Edwards 2008). Interestingly the clearance of cellular debris resulting from necrosis leads to changes in the macrophage physiology and production of cytokines and pro-inflammatory mediators. Macrophages can be activated by IFNγ and TNF produced by natural killer and T helper cells (O'Shea *et al*., 2002), which are then responsible for producing IL-1, IL-6 and IL-23 (Langrish *et al*., 2005), thus amplifying the immune response.

1.3.2 Neutrophils

Neutrophils are formed within the bone marrow from the same myeloid progenitor cells as monocytes. An integral part of the immune system the neutrophil contains distinctive cytoplasmic granulocytes and a nucleus divided into three distinct segments. These are the most abundant leukocyte within the human immune system and are rapidly recruited to the site of infection with P selectin observed to play a part in this recruitment (Diacovo *et al*., 1996). These cells can phagocytose foreign microorganisms directly or can produce toxic antimicrobial mediators (Kruger *et al*., 2015). Neutrophils are not only able to phagocytose pathogens but employ neutrophil extracellular traps (NETs) capturing microbes extracellularly and then autophagy to digest them intracellularly (Brinkmann *et al*., 2004; Mantovani *et al*., 2011). Although neutrophils play an intrinsic role within the immune system, impaired clearance of apoptotic neutrophils is linked to autoimmune disease (Vandivier *et al*., 2006). Neutrophils also contribute to the inflammatory response via production of immune stimulating factors including cytokines, proteases and reactive oxygen species (Sampson, 2000).

1.3.3 Lymphocytes

Lymphocytes are a class of leucocyte and form an intrinsic part of the vertebrate immune system (Gasteiger & Rudensky, 2014). They are derived from hematopoietic stem cells within the bone marrow and include T cells, B cells and natural killer cells (NK). The NK cell contains granules with enzymes such as perforin and proteases which when close to a target cell, form a pore in the cell membrane and causing lysis (Berg *et al*., 2010). NK cells target tumour cells or self-cells infected with a virus and are critical to the functioning of the innate immune system. These cells do not require antigen presentation via MHC proteins to elicit an immune response and lack antigen specific cell surface receptors

(Vivier *et al*., 2011). NK cells can exacerbate or limit immune responses; however they are also regulatory cells interacting with dendritic, endothelial, macrophage and T cells through the release of cytokines such as IFNγ, TNFα and IL-10. They also release an array of chemokines, including monocyte chemoattractant protein 1 (MCP-1/CCL2), macrophage inflammatory proteins (MIP) MIP1α/CCL3, MIP1β/CCL4, RANTES/CCL5 and IL-8, helping recruit other immune cells to localised inflammatory sites (Alberts *et al*., 1994; Walzer *et al*., 2005).

T lymphocytes are also derived from precursors generated in the bone marrow which then migrate to the thymus, where they develop fully into T cells (Di Rosa & Pabst, 2005). The T cell can be subcategorised into T helper cells, cytotoxic T cells and T memory cells and have many roles within the immune system. Each T cell has an antigen recognising T cell receptor (TCR) with the potential to recognise a single antigen, but are described as Naïve T cells until they contact this antigen (Hall, 2015). Once activated the T cell replicates during the process of clonal expansion and following cytokine signal differentiate into effector cells. Dependent on the cocktail of cytokines present during activation functionally distinct T cells are induced including Th1, Th2 or Th17. Th17 cells mediate many autoimmune responses including rheumatoid arthritis and can kill target cells directly by their release of cytokines, or indirectly via promoting IgM production (Boniface, Moyet *et al*., 2013). Effector T cells mediate immunity and destroy cells with a recognised antigen and monitor the intracellular contents of the host cell infected with pathogen, or malignant cells expressing tumour associated antigens. In autoimmunity they also kill normal healthy cells (Hall, 2015). T cells are involved in the activation of B lymphocytes when presented with an antigen by the B cell, to their T cell receptors (Alberts *et al*., 1994).

Activation of B cells by T cells results in the release of antigen specific antibodies. B cells originate and mature within the bone marrow, express B cell receptors and are capable of ingesting antigens before presenting fragments on their receptors initiating an immune response. B cells like T cells are also divided into subgroups based on their functional properties within the adaptive immune system, namely the plasma cells, which secrete antibodies and memory cells which provide a more rapid defence against a pathogen which has previously invaded the body (Berg *et al*., 2010). A B1 subgroup secrete natural IgM antibodies which play an important role in homeostasis due to their ability to bind self-antigens, this group also secretes polyreactive IgA antibodies which aid mucosal immunity. Whilst developing within the bone marrow B cells undergo two types of selection leading to correct development, that of positive and negative selection. Positive selection occurs through antigen-independent signaling involving the B cell receptor, where these receptors are unable to bind their ligand the B cell does not receive the correct signal and no longer develops. If a B cell receptor binds a self-antigen with high affinity, negative selection occurs, leading to one of four fates- clonal deletion, receptor editing, anergy or the B cell continues to develop ignoring the signal. The negative selection process is essential within the state of central tolerance whereby mature B cells do not bind with self-antigens (Tobón *et al*., 2013)

1.3.4 Antibodies

Antibodies, also known as immunoglobulins (Ig), are glycoproteins involved within immune system identification and elimination of pathogenic and disease antigens from the host body. These immune proteins are found on the surface of B cells acting as surface receptors, or secreted into the extracellular space circulating within the blood where they

can bind to their target antigen, eliminating it through processes such as agglutination, precipitation or neutralisation (Hoffman *et al*., 2016).

An antibody molecule consists of four protein chains, two heavy and two light, linked together by disulphide bonds in a roughly 'Y' shaped hinged conformation. Each light chain is composed of a single variable and constant domain. The longer heavy chain is made up of one variable but also three constant domains. The antigen binding fragment, Fab, is formed from the variable regions of both a heavy and light chain, together with the light chain constant region and first constant of the heavy chain, it is located at the N-terminus and apex of the 'Y'. This region recognises the specific antigen which stimulated their production. The constant regions are composed of the remaining domains of the heavy chain and form the Fc, or crystallisable fragment region. This Fc region is important in antibody function due to its binding and interactions with Fc receptors on the surface of lymphocytes (Alberts *et al*., 1994; Hoffman, Lakkis and Chalasani, 2016; Murphy and Weaver, 2017). Binding of these Fc receptors activates other parts of the immune system such as macrophages or C1q leading to complement pathway initiation.

Heavy and light Ig chains are encoded by a multigene family, with individual V and C domains encoded by independent elements. Somatic recombination occurs in the early stage of T and B cell maturation resulting in a diverse repertoire of Igs following rearrangements of the variable (V), joining (J) and occasionally diversity (D) segments. V(D)J gene segments encode the variable (V) domains and individual exons the constant (C) domains (Schroeder and Cavacini, 2013).

Antibodies are categorised into five isotypes, or classes (IgM, IgD, IgG, IgA, and IgE), dependent on their overall conformation and heavy chain region C-terminus domain (μ, δ, γ, α and ε respectively) as seen in Table 1.1. There are also four subclasses of IgG antibodies, IgG1, IgG2, IgG3 and IgG4 and two subclasses of IgA antibodies, IgA1 and

IgA2. The heavy chain isotype and Fc binding determines the effector function initiated by each class, for instance IgG3 and IgM are strong complement activators, whilst IgG1 activates macrophages and IgE mast cells proteins (Alberts *et al*., 1994; Hoffman, Lakkis and Chalasani, 2016; Murphy and Weaver, 2017). IgG1 and IgG3 bind FcγR and activate complement, with IgG1 being the most abundant isotype making up 60-75% of serum IgG. The IgG molecule is approximately 150kDa, circulates in a monomer conformation and forms the major Ig in serum (75%). IgA is the second most abundant serum immunoglobulin and is the major class found within tears, saliva and mucus, with IgM the third most common. IgA is found in dimers within secretion and monomers within serum. IgM is the first immunoglobulin to be made by the feotus and also the initial antibody produced by a new B cell when stimulated by an antigen. IgM circulates as a pentamer, although is sometimes found as a monomer. Lastly IgD and IgE both of which are monomers. IgD is found at low levels in serum and IgE, the least common antibody which is involved in defence against parasites but also allergic reactions (Murphy *et al*., 2008; Schroeder *et al*., 2010; Murphy & Weaver, 2017).

Table 1.1 Table detailing differences between antibody structure function and binding, + respresenting weak interaction and +++ strong interaction. Adapted from Schroeder and Cavacini 2010.

Name	Serum (%)	Structure	Complement Fixation	Opsonizing	Cross Placenta	Other Functions	FcR
IgG	75%	Monomer	+	+++	Yes	For all IgG subclasses: Main antibody of secondary response, neutralise toxins and virus	FcγR
IgG1	67% IgG	Monomer	Yes	Yes	Yes		I,II,III
IgG2	22% IgG	Monomer	Yes	Yes	Yes		II
IgG3	7% IgG	Monomer	Yes	Yes	Yes		I,II,III
IgG4	4% IgG	Monomer	No	No	Yes		I,II
IgM	10	Pentamer	+++	+	No	Primary response secreted into blood, monomer attached to B cell surface	
IgA	15	Monomer, Dimer	No	No	No	Mucosal Response, tears, saliva, colostrum	FcαR (CD89)
IgA1		Monomer, Dimer	No	No	No		
IgA2		Monomer, Dimer	No	No	No		
IgD	<0.5	Monomer	No	No	No	Part of B cell receptor, activated by basophils and mast cells	FcδR
IgE	<0.01	Monomer	No	No	No	Allergic response, protects against parasitic worms	FcεR I,II

The Fc region of the antibody can be the site of auto-antibody binding such as rheumatoid factors found within the blood of rheumatoid arthritis sufferers. This class of

auto-antibody recognises the Fc portion of IgG antibodies. Within clinical or research immunological techniques, the Fc section of an antibody bound to an antigen is also the site of secondary antibody binding. Many secondary antibodies have dyes or enzymes covalently bound enabling visualisation of proteins or antigens recognised by the detection antibody (Alberts *et al*., 1994).

1.3.5 Eosinophils and Basophils

Basophils and eosinophils account for less than 1% and 5% of leucocytes respectively and are key players in allergic inflammation (Nadif *et al*., 2013). Eosinophils are responsible for regulation of allergy reactions and for preventing parasite and pathogen infections within the body. They develop in the bone marrow until they mature at which point they circulate the body until reaching a site of inflammation or infection (Murphy *et al*., 2008; Murphy & Weaver, 2017). Activated eosinophils fight viral infections via the release of granular proteins and the production of proinflammatory cytokines. Basophils are the least common white blood cell, they release histamine and following activation produce IL-4 in response to parasites together with IL-13, and are found in increased number within the lungs of patients who die of asthma (Stone *et al*., 2010).

1.3.6 Platelets

Platelets are small (2-3µm diameter), discoid and anucleate cell fragments, which circulate within the blood and were first observed by Giulio Bizzozero in 1882 (Ribatti & Crivellato, 2007; Ghoshal & Bhattacharyya, 2014). Also known as thrombocytes, they are abundant within circulation and originate from megakaryocytes (bone marrow cells), which fragment into 1000's of platelets within the bone marrow in response to thrombopoietin, and are then released into circulation. Each megakaryocyte can produce 5000-10000 platelets, with a healthy adult producing 10^{11} a day (Ribatti & Crivellato,

2007). Their primary role within the body is within wound repair through the initiation of coagulation cascades, preventing uncontrolled bleeding following tissue damage.

Platelets circulate in their inactivated form until they are activated upon damage to a blood vessel, which results in the initiation of coagulation, known as hemostasis. Membrane lipids phosphatidylserine and phosphatidylinositol present in the inner plasma membrane during the inactive state are exposed during activation by ATP-dependent floppases and scramblases, leading to the coagulation cascade.

Pro-aggregation signals known as platelet agonists enhance platelet adhesion to the site of injury and the platelet changes shape, releases granule contents and leads to aggregation with other platelets (Ribatti & Crivellato, 2007; Murphy *et al*., 2008). Surface receptors trigger the release of granule contents which includes α granules (GPIIbIIIa, fibrinogen, P selectin and vWF) and dense granules (catecholamines, serotonin, calcium, ADP and ATP), both of which play a role in normal (coagulation, inflammation, wound healing) and abnormal processes (atherosclerosis and tumourigenesis), (Blair & Flaumenhaft, 2009). The release of platelet granules amplifies the platelet response, leading to recruitment of further platelets and blood cells, formation of a platelet plug and eventually a thrombus preventing bleeding (Berg *et al*., 2010; J. L. Li *et al*., 2017).

1.3.7 Cytokines and Chemokines

Cytokines are a group of small proteins or glycoproteins which serve as chemical messengers involved in cell-to-cell signaling and are involved in a variety of processes such as cell growth and differentiation, tissue repair and immune function. Cytokines play a key role in regulating the magnitude, duration and nature of an immune or inflammatory response and range from 5-50 kDa. During inflammatory or acute phase response they can be easily detectable within serum. Three cytokines of particular importance within

initiation and regulation and communication between the innate and acquired immune systems are IL-1, IL-6 and TNF-α all of which are key to development and propagation of the acquired immune response (Tracey & Cerami, 1994; Brennan & McInnes, 2008; Dinarello, 2009), these cytokines are included in Table 1.2 illustrating cytokines, their key functions, origin and the cells they impact.

Table 1.2 Key cytokines, their originating cell, function and cells impacted.

Type of cytokine	Function / Secreted by	Cell type impacted	References
IL-1 (IL-1α,IL-1β)	Co-mitogen for T lymphocytes. Stimulate liver inducing transcription of acute phase proteins (APP). Leads to increase expression of inflammatory genes. Secreted by monocytes, macrophages, B cells, dendritic cells, endothelial cells.	Endothelial cells, T_H cells, B cells, hepatocytes, macrophages and neutrophils	(Baumann & Gauldie, 1994; Tracey & Cerami, 1994; Gabay & Kushner, 1999; Brennan & McInnes, 2008; Dinarello, 2009)
IL-6	Growth factor for B cells. Stimulate liver inducing transcription of APP. Induces antibody production within B cells. Downregulation of TNF-α, IL-1. Secreted by monocytes, macrophages, T_H2 cells, bone marrow cells.	Hepatocytes, B cells, plasma cells, myeloid stem cells.	(Baumann & Gauldie, 1994; Tracey & Cerami, 1994; Gabay & Kushner, 1999; Brennan & McInnes, 2008; Dinarello, 2009)
TNF-α	Propagates T cell response via T helper cell type 1 (Th1). Stimulate liver inducing transcription of APP. Pro-inflammatory, induces cytokine production, cell proliferation and apoptosis. Secreted by macrophages, NK cells, $CD4^+$ lymphocytes, adipocytes, basophils, eosinophils, dendritic cells.	Endothelial cells, T_H1 cells, hepatocytes.	(Baumann & Gauldie, 1994; Tracey & Cerami, 1994; Gabay & Kushner, 1999; Sun *et al*., 2005; Brennan & McInnes, 2008; Dinarello, 2009; Chen *et al*., 2018)
IFN-γ	Pro-inflammatory, activation of macrophages and NK cells, inhibition of T_H2 differentiation. Secreted by T_H1, CD8 T cells, NK cells.	Macrophages, NK and T_H2 cells	(Chen *et al*., 2018)
IL-7	Pro-inflammatory, role within synovium reducing B cell apoptosis, increasing antibody production. Induces differentiation into progenitor B and T cells. Secreted by bone marrow and	B Cells, hepatocytes, resting T cells, lymphoid stem cells	(Lubberts *et al*., 2005)

	thymic stromal cells.		
IL-10	Anti-inflammatory. Inhibits gene expression of T cell and macrophage pro-inflammatory cytokines, reducing function of antigen presenting cells. Secreted by T_H2 cells, T cells, B cells	Macrophages, antigen presenting cells, T cells.	(de Waal Malefyt, 1991; Petrovic-Rackov & Pejnovic, 2006; Chen *et al*., 2018)
IL-12	Highly inflammatory cytokine and stimulator of TNF-α and IFN-γ. Secreted by macrophages, neutrophils, B cells.	Activated T_c cells, NK and activated T_H1 cells.	(Furst & Emery, 2014; Chen *et al*., 2018)
IL-15	Stimulates intestinal epithelium growth, T cell proliferation, co-mitogen for B cell activation and proliferation. Promotion of CD8 T cell survival, osteoclastogenesis and bone destruction. Secreted by T cells and mononuclear phagocytes.	T cells, intestinal epithelium , NK, activated B cells, osteoclast	(Saeed & Revell, 2001; Cui *et al*., 2014; Lorenzo, 2015)
IL-17	Involved within stimulation of iNOS and chemokine synthesis, inducer of osteoclasts genesis and RANKL expression. Secreted by T cells, including Th17	Osteoclasts, fibroblast, endothelial cells, epithelial cells, macrophages.	(Kotake *et al*., 1999; Ouyang *et al*., 2008; van den Berg & Miossec, 2009; Pickens *et al*., 2010)
IL-18	Inducer of IFN-γ and gene expression in IL-6, iNOS, COX-2 and increase chemokine production in human PBMC. Secreted by macrophages, monocytes, T and B cells, dendritic cells, epithelial cells	Macrophages, monocytic and endothelial cells, T cells and NK cells.	(Kunikata *et al*., 1998; Petrovic-Rackov & Pejnovic, 2006; Arend *et al*., 2008; Smith, 2011)
IL-23	Acts on memory $CD4^+$ cells, promoting proliferation and differentiation in to TH1 effectors, stimulates dendritic and NK cells. Secreted by macrophages, dendritic cells.	Macrophages, memory $CD4^+$ cells, dendritic and NK cells	(Langrish *et al*., 2005; Gelderblom *et al*., 2018)

IL-1 is a co-mitogen for T lymphocytes, IL-6 is a growth factor for B lymphocytes and TNF-α propagates the T cell response via T helper cell type 1 (Th1). These three cytokines stimulate the liver to induce transcription of acute phase reactants including mannose binding protein, lipopolysaccharide binding protein, amyloid A, amyloid P and CRP (Gabay & Kushner, 1999). IL-1 possesses several biological properties that result in increased expression of inflammatory genes, such as intercellular adhesion molecule-1

(ICAM-1) on endothelial cell surfaces and inducible nitric oxide synthase (iNOS) leading to increased expression of nitric oxide (NO). ICAM-1 in particular supports infiltration of inflammatory and immunocompetent cells into the extravascular space. IL-6 induces antibody production within B cells and induces hepatic acute phase protein response (Baumann & Gauldie, 1994; Brennan & McInnes, 2008), however it is also capable of suppressing proinflammatory responses via the downregulation of TNF-α and IL-1, however both TNF-α and IL-1 are potent enhancers of IL-6 expression, suggesting a carefully orchestrated balance between them. Whilst TNF-α is an important inducer of an immune response it is also responsible for inflammation and tissue injury during abnormal physiological responses. It plays a role in Th1 immune response regulation and also induces synthesis of IL-12 and IL-18 both inducers of IFN-γ, eventually increasing $CD4^+$ T cell activation and inflammatory response. Secondary mediators induced by TNF-α include IL-1, IL-2, IL-4, IL-6, IL-10, IL-12, IL-18, IL-23 and TGF-β. TNF-α is produced by many cell types including B and T cells, macrophages, dendritic cells, basophils, eosinophils and NK cells (Bazzoni & Beutler, 1996; Edwards & Hallett, 1997; Brennan & McInnes, 2008).

Of these cytokines, IL-1, IL-6 and TNF-α are amongst many others involved within the inflammatory pathways important within the progression of rheumatoid arthritis. These cytokines include pro-inflammatory cytokines such as, IL-7 which plays a role within the synovium reducing B cell apoptosis and increasing antibody production (Lubberts *et al*., 2005), IL-12, a highly inflammatory cytokine and stimulator of both TNF-α and IFN-γ (Furst & Emery, 2014) and IL-15, produced by monocytes, fibroblasts and endothelial cells which induces cytolytic factors, induces IFN-γ and TNF-α, activates neutrophils, and is involved in T cell proliferation (Petrovic-Rackov & Pejnovic, 2006). Another two pro-inflammatory agents are IL-17 and IL-18. IL-17 is a T cell derived cytokine involved within the stimulation of iNOS and chemokine synthesis, which induces osteoclast genesis

and is found in high concentrations in RA patient synovial fluids (Kotake *et al*., 1999; Brennan & McInnes, 2008). IL-18 is involved in the increase of gene expression in IL-6, iNOS and COX-2, and also increases chemokine production within human PBMC. It works in synergy with other cytokines to stimulate production of INF-γ in T cells (Ushio *et al*., 1996; Arend *et al*., 2008). Of the anti-inflammatory cytokines, IL-10 inhibits gene expression of T-cell and macrophage proinflammatory cytokines, and reduces the function of antigen presenting cells (de Waal Malefyt, 1991; Petrovic-Rackov & Pejnovic, 2006), IL-11 down regulates IFN-γ (Kotake *et al*., 1999), and IL-13, reduces the induction of IL-1β, TNF-α and iNOS (Arend *et al*., 2008).

Working together with cytokines are the group of proteins known as chemokines. Chemokine is a shortened version of chemoattractive cytokine(s) and references a group of 40 plus secreted proteins ranging between 6-14kDa. Chemokines recruit and activate lymphocytes, monocyte/macrophages, eosinophils and neutrophils. This family of proteins include IL-8, MCP-1, MCP-2, MCP-3, RANTES and IFN-γ-inducible protein 10 (IP-10) amongst others (Szekanecz *et al*., 2010).

1.3.8 The Pentraxin Family

CRP belongs to the pentraxin family of calcium dependent ligand-binding plasma proteins, made up of long pentraxin 3 (PTX3) and short pentraxins serum amyloid protein (SAP) and CRP (Osmand *et al*., 1977). These proteins show high sequence homology, are evolutionarily highly conserved and are expressed in response to systemic inflammation, tissue damage or infection (Sjöwall & Wetterö, 2007a). The short pentraxins CRP and SAP recognise harmful pathogens and eliminate them via macrophage or complement action (Agrawal *et al*., 2009). SAP is involved in resistance to bacterial infection and is also found as a pentamer, although this has the ability to form decamers in the absence of

calcium in the conformation of two stacked pentameric rings (Hutchinson *et al*., 2000). PTX3 is produced from vascular and immune cells, SAP and CRP are produced predominantly within the liver, all in response to pro-inflammatory signals and stimuli (Hutchinson *et al*., 1994; Koenig *et al*., 1999). All three pentraxins are implicated in the inflammatory disease atherosclerosis, a condition greatly accelerated in RA patients (Bassi *et al*., 2009).

1.4 Human C-reactive Protein

1.4.1 Overview of C-reactive Protein

The human CRP molecule (115 kDa) consists of five 23 kDa, identical, nonglycosylated subunits, non-covalently associated and arranged symmetrically around a central pore known as pentameric CRP (pCRP) (Shrive *et al*., 1996). CRP was first observed in the laboratory by Oswald T. Avery, who was developing therapies against pneumococcal pneumonia (Tillett & Francis, 1930). Tillet & Francis in the early 1930's discovered a new antigen within the serum of patients with inflammatory and infectious diseases, calling it Fraction C (Tillett & Francis, 1930). They noted that this agent produced a strong precipitation reaction with C-polysaccharide (CPS) during initial acute disease stages, but fell as the patient recovered. The same reaction occurred within patients suffering from other diseases such as bacterial endocarditis and acute rheumatic fever with the three researchers terming the protein C-reactive (Tillett & Francis, 1930).

The necessity of calcium ions to enable CRP binding was established in addition to its role within a non-specific response to infection or inflammation. CRP was the first of the acute phase proteins to be described due to the sudden and dramatic rise in its serum levels in the initial phase of inflammatory disease (de Beer *et al*., 1982). As part of the acute phase inflammatory response, it forms part of the body's non-specific biochemical and physiological reaction to tissue damage through infection, inflammation or malignant neoplasia (Hirschfield & Pepys, 2003).

In healthy adults, baseline levels of CRP range from 1-3mg/l (Pepys & Hirschfield, 2003). Due to the sensitivity of CRP detection within plasma or serum, it is possible to predict inflammation within the body when levels are raised above 3 mg/L, which is considered a strong predictor of current or future disease (Pepys & Hirschfield, 2003).

However it is reported that associations between genetic polymorphisms in IL-1 and IL-6 can cause fluctuations within this baseline level, with those affected individuals linked to elevated basal levels of CRP. In addition to IL-1 and IL-6 polymorphisms, mutations within the promoter region of the CRP gene are also implicated within higher baseline CRP levels (Vickers *et al*., 2002; Pepys & Hirschfield, 2003). Our immune defence system has evolved over thousands of years, with many proteins and systems retained and conserved across species. Some of these proteins are rarely found mutated, non-functioning or absent, and therefore suggest a beneficial role within our now complex immune system. CRP falls into this category as the common genetic variants found at the CRP locus are only associated with levels of CRP translated, not to its structure or functions which are both retained. These mutations accounted for an approximate 3.5-fold change in one study (Rhodes *et al*., 2010).

During periods of tissue damage, inflammation or infection, cytokines produced at the site of pathology instigate the upregulation of CRP and other acute phase proteins, commonly within hepatocytes of the liver. The functional role of pCRP within the human body appears predominantly to be that of binding dead or damaged cells and of opsonisation, allowing removal of particles via phagocytosis together with cytokine mediated induction of the immune system, it is therefore thought to act in a protective manner, avoiding further inflammation (Meyer, 2010). However CRP is also responsible for the induction of pro-inflammatory cytokine synthesis and release from other immune system components (Ballou & Lozanski, 1992).

CRP has a role within modulation of the immune system, via interactions between itself and C1q a constituent part of the classical pathway. CRP recruits C1q to the surface of damaged cells. C1q is the recognition molecule within the larger complex C1, composed of one C1q, two C1r and two C1s molecules (Volanakis & Kaplan, 1971; Volanakis *et al*.,

1981; McGrath *et al*., 2006). The C1q molecule is composed of six copies of three polypeptide chains, A (223 residues), B (226 residues) and C (217 residues) which combine to form a globular head region (GHR) and a collagen like region (CLR) (Kishore *et al*., 2004). Whereas CRP is composed of 5 protomers forming a pentamer of 115 kDa, C1q is four times larger with a molecular mass of 460kDa (Volanakis & Kaplan, 1971; Volanakis *et al*., 1981; McGrath *et al*., 2006). It is the GHR which is suggested to interact with CRP as shown within Figure 1.1.

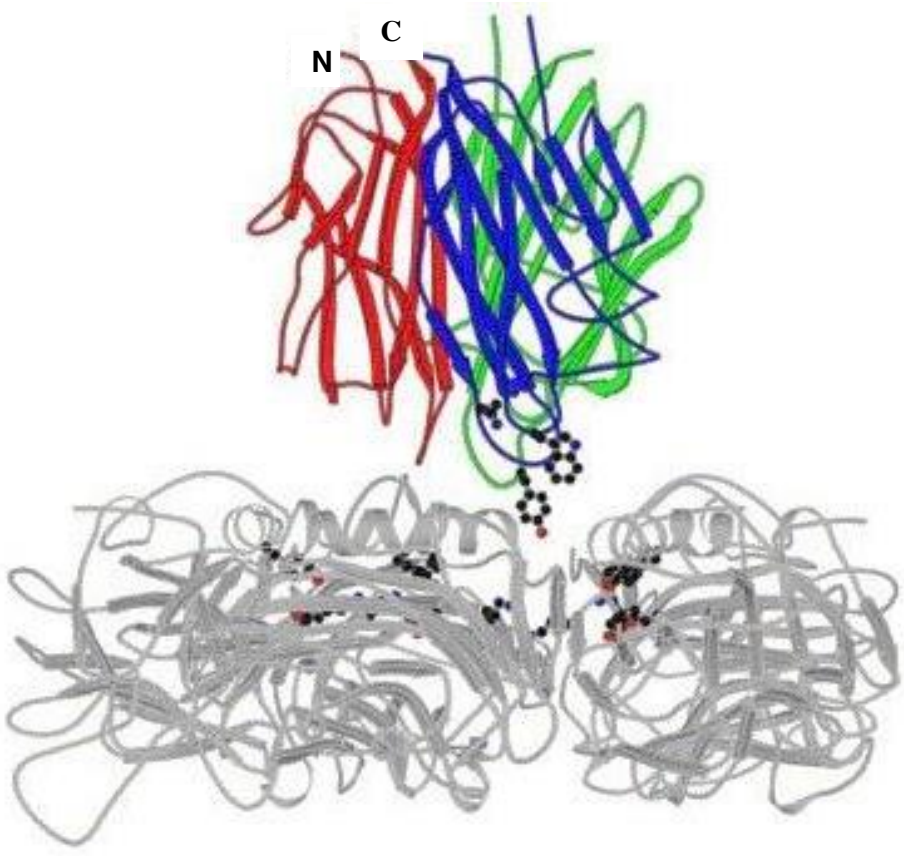


Figure 1-1 Schematic illustration of the hypothesized interaction between the globular head region (GHRs) of C1q with the effector face of pentameric CRP. GHR A shown in blue, B shown in green and C shown in red. The model was designed to explore the potential interactions between the GHR of C1q and CRP. Key residues suggested to interact between the two proteins are highlighted as ball and stick models shown (Asp112 and Tyr175 of CRP, and Tyr175B, Lyc200A and Trp147A of C1q). Image adapted from (Kishore *et al*., 2004).

With a main site of synthesis found to be within hepatic cells, serum levels rise rapidly in response to IL-6, IL-1 and TGFβ, the latter involved in upregulation of CRP gene transcription within hepatocytes (Ganapathi *et al*., 1990). Serum levels elevated above those within the normal healthy CRP range of 1-3 mg/l, often indicate an underlying diseased state, inflammation or autoimmune linked conditions such as RA. Due to the often rapid increase of serum levels in response to inflammation, upwards of 100 fold within 24-72 hours, CRP is used routinely within a clinical setting as a marker of a non-healthy inflammatory state (Pepys & Hirschfield, 2003).

Whilst the structure and suggested role of pentameric CRP has been well documented, the role, and indeed existence of, monomeric CRP (mCRP) within the human body is less certain and has been cause of discordant theories throughout the last decade. It is suggested by some to be a pathogenic form of CRP (Mihlan *et al*., 2011; Thiele *et al*., 2014; Chirco *et al*., 2016) and has been identified in co-localisation with damaged or inflamed tissue (Thiele *et al*., 2014; Chirco *et al*., 2016). Others however suggest it may not play a physiologically active role at all, because mCRP does not persist within the body as it is normally rapidly catabolised, therefore not considered to occur as a physiologically active protein (Pepys & Hirschfield, 2003).

1.4.2 Structure of Human C-reactive Protein

Each of the five identical subunits, termed protomers or monomers, contains 206 amino acids, with X-ray crystallography showing that each consist of two layered, antiparallel β sheets with a flattened jelly roll conformation similar to lectins such as concanavalin A (Shrive *et al., 1996)*. The antiparallel β sheets are illustrated within Figure 1.2 which also shows the arrangement of subunits around the central pore.

The crystal structure of CRP was solved in 1996 by Shrive *et al* and shows an internal stabilising disulphide bond. Intersubunit binding involves the interaction with salt bridges Glu101 – Lys201, Lys123 – Glue197 and Asp155 – Arg118 between the 115-123 amino acid loops in one protomer and 197-202 and 40-42 in the next (Shrive *et al*., 1996). Each protomer contains two calcium ions, involved in binding coordination of ligands such as phosphocholine (PC) (see Figure 1.3), which is expressed on the surface of dead or damaged cells. Each subunit has a recognition face consisting of a PC binding site formed by a hydrophobic pocket and two adjacent calcium ions (Shrive *et al*., 1996), and an effector face on the opposite side containing a cleft like region, extending from the central pore of the pentamer towards the middle of each protomer (Shrive *et al*., 1996; Thompson *et al*., 1999).

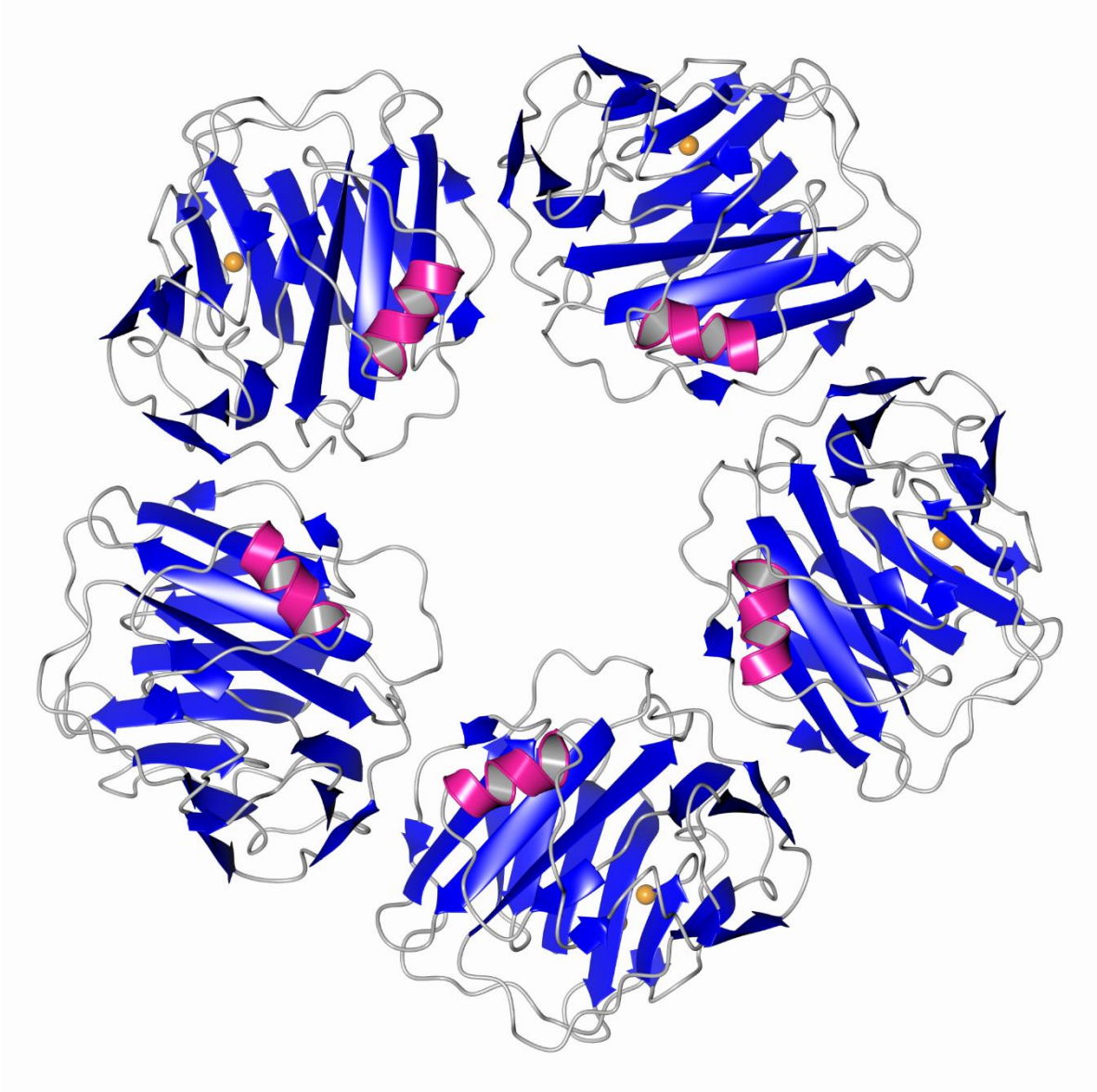


Figure 1-2 The three dimensional structure of pentameric C-reactive protein. The five protomers are arranged around a central pore, with the α-helix in pink and the β-sheets in blue. Calcium ions are illustrated in orange. C1q binding is suggested to take place on the face shown, with globular head region (GHR) of C1q positioned within the central pore. Phosphocholine binding takes place on the opposite face in close proximity to the calcium ions visible on five protomers. Image of 1GNH (Shrive *et al*., 1996) created with CCP4MG (McNicholas *et al*., 2011).

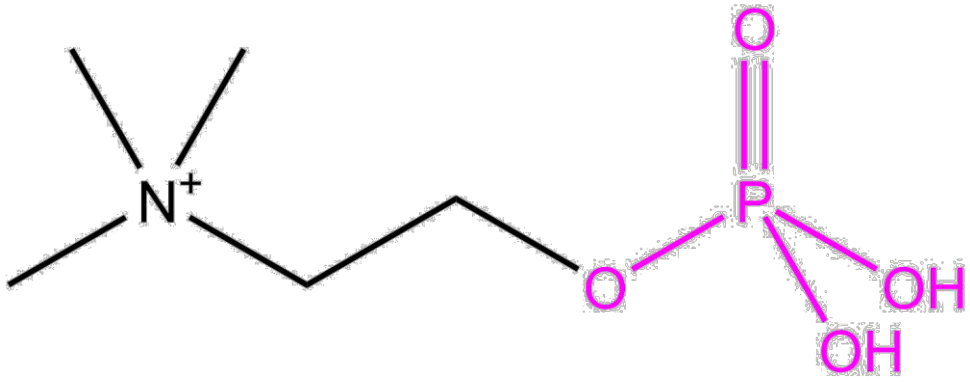


Figure 1-3 Chemical structure of phosphocholine. PC is the main ligand of CRP and a major constituent of cell membranes. PC is expressed on the surface of dead or dying cells allowing CRP to distinguish between damaged and healthy.

Each protomer contains two calcium ions, each crucial to the stability and binding ability of CRP, with an absence of calcium inducing structural changes to the pentameric conformation. When calcium is lacking the loop formed by residues 140-150 exposes inner sites vulnerable to proteolysis and destabilisation of the subunit (Ramadan *et al*., 2002) as shown in Figure 1.4. One calcium is coordinated by Asp60 together with Asn61, Glu138, Asp140 and Gln139. The second calcium is bound with residues Glu138, Asp140, Gln150, and Glu147 (Shrive *et al*., 1996, Thompson *et al*., 1999).

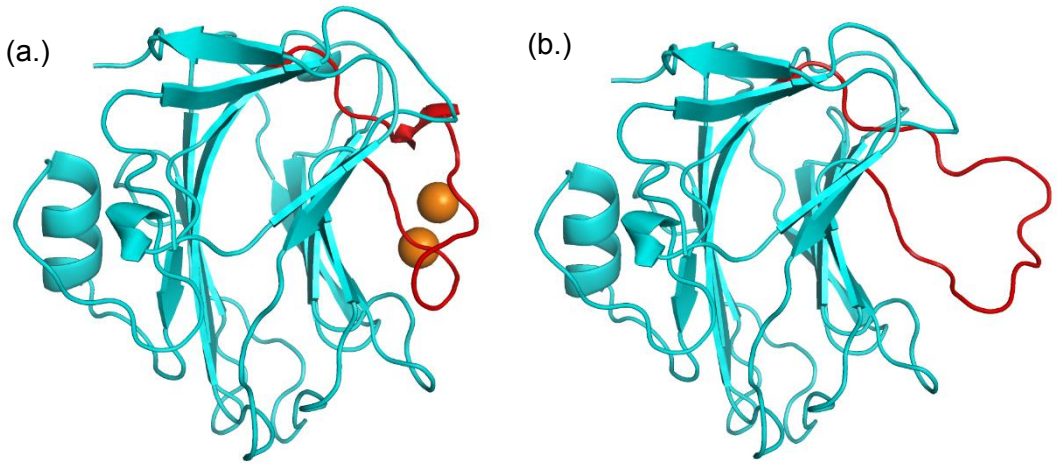


Figure 1-4 Monomeric CRP subunit with the residues 140-150 loop highlighted in red, with and without stabilising calcium ions shown in orange. (a) The loop stabilised by an interaction with calcium and (b) Relaxation of the loop in the absence of calcium allowing access to proteolytic enzymes. Image of PDB ID:1GNH (left) and 1LJ7 (right) created with PyMol (DeLano, 2002).

Phosphocholine binding is stabilised by a phosphate calcium interaction within the calcium binding site as can be seen in Figure 1.5. Two oxygen atoms of PC interact with the two calcium ions, with the third oxygen positioned towards the solvent enabling PC to interact with other molecules in the area via ester linkage. Choline is then able to position away from the calcium binding site towards the hydrophobic pocket formed by side chains from Phe66, Thr76 and Leu64 (Shrive *et al*., 1996, Thompson *et al*., 1999). A hydrophobic interaction is formed between the tertiary methyl groups of the choline moiety and the aromatic face of Phe66.

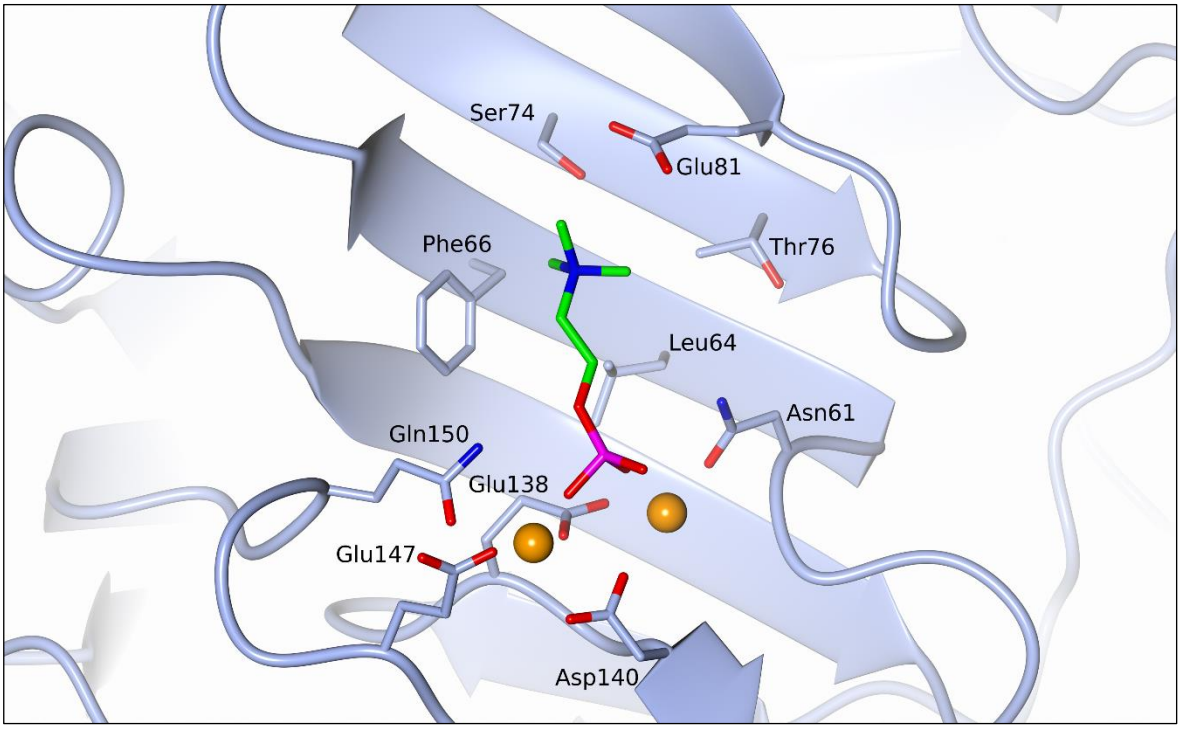


Figure 1-5 Structural model of the interaction between phosphocholine and the calcium ions on a CRP protomer. Image of PDB ID:1GNH (Shrive *et al*., 1996) created with CCP4mg (McNicholas *et al*., 2011).

The opposite side of protein from the ligand binding site lies the effector face. A cleft like region is found running from the edge of the central pore of the pentamer, to the middle of each protomer (Shrive *et al*., 1996). It is thought that this is the region of interaction between CRP and C1q, allowing activation of complement and classical pathway following CRP interaction with PC (Agrawal *et al*., 2001, Shrive *et al*., 1996, Thompson *et al*., 1999). Forming one side of the cleft is an α helix (Figure 1.2) formed from residues 168 – 176, which lies across one of the two β-sheets, with the carboxyl end forming one side of the cleft. This cleft runs from the centre of the each individual protomer to its edge at the central pentamer pore, with the opposite cleft side formed by the carboxyl termini of the protomer, forming a narrow, deep cleft which becomes shallower towards the middle of the pentamer (Shrive *et al*., 1996, Thompson *et al*., 1999). Three

residues rise form the cleft, His38, His95 and Tyr205 and the outer edge is positively charged, but the inner parts and lining of the cleft remain negative (Volanakis, 2001).

1.4.3 C-reactive Protein Synthesis

Proteins such as CRP which show high genetic uniformity through a wide variety of species, from mammals to fish, suggest their importance towards survival within those animals. CRP is an evolutionally conserved protein which forms an intrinsic part of the immune system of even the most primitive of species. The human CRP gene is located on the long arm of Chromosome 1 in the 1q23.2 region and is composed of two exons separated by one intron. The initial exon encodes the signal peptide and first two amino acids of the final expressed serum protein followed by a 278 nucleotide intron which includes a GT repeat sequence. The second exon encodes the remaining 204 amino acids, together with the stop codon (Hage & Szalai, 2007).

CRP is synthesised in response to IL-1 and IL-6 stimulation and activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and transcription factor NF-IL-6/CAAT-enhancer binding protein (C/EBP), C/EBPβ and C/EBPδ (Pepys & Hirschfield, 2003; Sjöwall & Wetterö, 2007a). The principle inducer of CRP is IL-6 whilst IL-1, glucocorticoids and other factors including complement activation products act upon IL-6 to enhance its effects (Ganapathi *et al*., 1990). The proximal region of the promoter contains two sites for IL-6-inducible CCAAT/enhancer binding proteins (C/EBP) (Li & Goldman, 1996), with two HNF-1 and one STAT3 sites located close to these sites (Zhang *et al*., 1996). Binding of C/EBPβ and δ to binding sites upstream of the promoter, results from IL-6 stimulation, in the presence of Rel protein p50. Following transcription the 206 amino acid protomers are assembled into their pentameric conformation within the endoplasmic reticulum and under physiologic conditions are retained there by two

carboxylesterases. Following the acute phase response the recorded half time for exit falls from 18h to 75mins, due to a decreased affinity for one esterase allowing the pooled CRP to be released (Yue *et al*., 1996).

CRP levels rise rapidly within 2 hours of acute inflammation/infection peaking at 48 hours (Gabay & Kushner, 1999), with a short half-life 18-19 hours, therefore providing a relatively rapid drop in levels (Pepys & Hirschfield, 2003), suggesting that increased serum levels are the result of significantly elevated expression rather than a gradual build up over a prolonged period. In human hepatoma Hep3B cells, CRP expression is induced by IL-6 via activation of transcription factors STAT3 and C/EBPβ (Agrawal *et al*., 2001). However, IL-1 has no direct action on CRP expression, but is thought to work in synergy with IL-6 (Ganapathi *et al*., 1991). A later study investigated the role of NF-κB within CRP expression and found that it regulates CRP expression in partnership with C/EBPβ on the CRP proximal promoter. Overlapping NF-κB and OCT-1 binding sites were determined to be the regulatory element. OCT-1 is a transcription factor responsible for a variety of roles in cellular transcriptional regulation as well as an activator of gene transcription. Belonging to the Pit-Oct-Unc family of homeodomain protein transcription factors (POU), OCT-1 is also able to repress transcription. Baseline CRP expression was found to increase markedly when binding of NF-κB and OCT-1 was prevented (Voleti & Agrawal, 2005).

It is suggested that during the acute phase response CRP expression is not only up-regulated, but that stocks of CRP may have previously been stored within the endoplasmic reticulum (ER), and that these may in part account for the rapid increase of plasma CRP levels (Macintyre, 1992; Yue *et al*., 1996). Although CRP is synthesised by hepatic cells at relatively low constituent levels it was suggested by Macintyre (1992) that a ‘pool’ is retained within the ER via calcium dependent binding, modulated by the flux of internal

calcium levels, allowing it to be released during the transiently reduced calcium environment found during the initial acute phase response. Macintyre (1992) suggested that there may be further functions for CRP within the ER that may be 'superseded' during an acute phase response, noting the similarities in sequence and structural homology between CRP and nucleoplasm, and the ability of CRP to bind chromatin, histones and snRNPs (Macintyre, 1992). Further research by Yue *et al*., *(*1996) clarified these findings when it became apparent that CRP interacted with two glycosylated microsomal carboxylesterases, gp60a and gp60b, within the ER. The carboxylesterases are localised within the ER lumen via COOH-terminal retention signals HIEL and HTEL, suggested to be part of the KDEL retrieval pathway (Yue *et al*., 1996). The KDEL receptor is an integral membrane protein which retrieves proteins of the ER labelled with a C-terminal KDEL sequence (Cabrera *et al*., 2003).

During an acute phase response the CRP rate of synthesis within hepatocytes is increased and the binding affinity to gp60a/b is reduced, allowing rapid release of the pooled CRP. Of the two carboxylesterases, gp60a has a lower binding affinity for CRP but is found in abundance whereas gp60b in contrast has higher affinity but is found in less abundance. This group further speculated that the sequences involved within CRP and gp60 interaction were Ala-Arg-Asn-Arg-Asn found at the beginning of high affinity gp60b and the similar Ala-Arg-Asn-Gly-Asn sequence found in the lower affinity gp60a (Yue *et al*., 1996). These sequences were proposed due to similarities with CRP binding sequences found by Du Clos *et al*, (1999), who looked at CRP binding to histones, H2A, H1 and the small nuclear ribonucleoprotein particle protein Sm-D, finding a common motif consisting of positively charged amino acids, lysine or arginine which alternate with neutral alanine or glycine (Mold *et al*., 1999).

In addition to expression within hepatocytes, it is now accepted that CRP is synthesised locally within a variety of cells and tissues, including renal tumour cells (Jabs *et al*., 2005). In their 2010 study Haider *et al* showed expression within peripheral blood mononuclear cells (PBMC). They were also able to show that induction was increased by IL-1, IL-6 and TNF-α with IL-10 reducing CRP expression in these cells (Haider *et al*., 2006). Another study investigating CRP expression in human lung epithelial cells, using cell line A549, showed that CRP was expressed following treatment with either TNF-α, carbon particles (CB) or ultra-fine carbon black particles (ufCB). It was also found that expression of CRP with CB and ufCB treatments was dependent on NFκB (Ramage & Guy, 2004). CRP expression has also been shown in human vascular smooth muscle cells (HVSMC) and human umbilical vein endothelial cells (HUVEC) (Kang *et al*., 2005).

1.4.4 Serum CRP Levels in Health and Disease

In normal, healthy adults the baseline level of CRP ranges from 1 -3 mg/l (Pepys & Hirschfield, 2003). Serum CRP has long been used within diagnosis to determine disease or risk of disease. Rheumatoid arthritis is just one such disease in which levels climb and are often sustained at a higher than normal concentration. As a marker of inflammation it is useful to determine severity and risk factor for a variety of inflammatory and autoimmune diseases, including cardiovascular disease, atherosclerosis, hypertension and diabetes (Kuller *et al*., 1996; Koenig *et al*., 1999; Hayaishi-Okano *et al*., 2002). In addition to physical diseases, depressive symptoms, frequently associated with schizophrenia have recently been associated with elevated CRP levels above 3 mg/l, with 40.4 % of 125 participants in the study found to have high CRP levels (Faugere *et al*., 2018). In fact, levels of CRP above this baseline have been suggested to be as strong indicators of disease or predicted diseased state in a wide range of conditions. In their 2010 paper Zhao *et al*

described the strong correlation between a levels of CRP ≥ 2.09 mg/l and incidence of obesity, hypertension, diabetes, cholesterol imbalance and cardiovascular disease (Zhao *et al*., 2010).

CRP levels vary with ethnic status and where the risk factor cutoff for CVD is 3 mg/l in Western populations, this may not be the case within others. It is reported that the distribution of CRP in the Asian population differs from that of the Western with a suggested 'safe' cut off of CRP ≥ 2.09 mg/l with mean values of 0.55mg/l (0.61 mg/L in males, 0.51 mg/L in females) (Zhao *et al*., 2010). Elevated CRP is closely linked with inflammatory diseases, although it is still not completely clear as to whether this is due to cause or effect.

1.4.5 Pentameric C-reactive Protein

The native conformation of CRP is in its pentameric form, with all five PC binding sites found on the same face of the pentamer. The effector face forms the opposite side of the pentamer, it is here that complement C1q binds CRP; it is presumed Fcγ receptors also interact at this face (Shrive *et al*., 1996). Structural studies investigating FcγRIIa interactions with the effector face of SAP show binding via a receptor which lies diagonally across each SAP pentamer, with contact made with ridge helices from two SAP protomers (Lu *et al*., 2008). The structural homology between SAP and CRP suggests this location is also the area of Fc binding with CRP. It is known that in order for C1q to activate complement, it must interact with more than one CRP pentamer, this may be due to the differences in scale of the two proteins and steric hinderance, C1q being considerably larger (Voleti & Agrawal, 2005). Black *et al*., (2004) suggest C1q interaction with the effector face of CRP and optimal binding take place following slight conformational changes within the CRP structure; they also propose that conformational changes occurring during C1q binding differ according to which ligand is bound (Black *et*

al., 2004), with Figure 1.6 illustrating the range of ligands CRP is known to bind. Agrawal however disagrees arguing PC ligand binding by CRP does not produce a conformational change, or signal within the protein leading to C1q initiation of complement, but that the necessity of C1q to bind multiple CRP pentamers is overcome by the aggregation of CRP bound to exposed PC (Voleti & Agrawal, 2005).

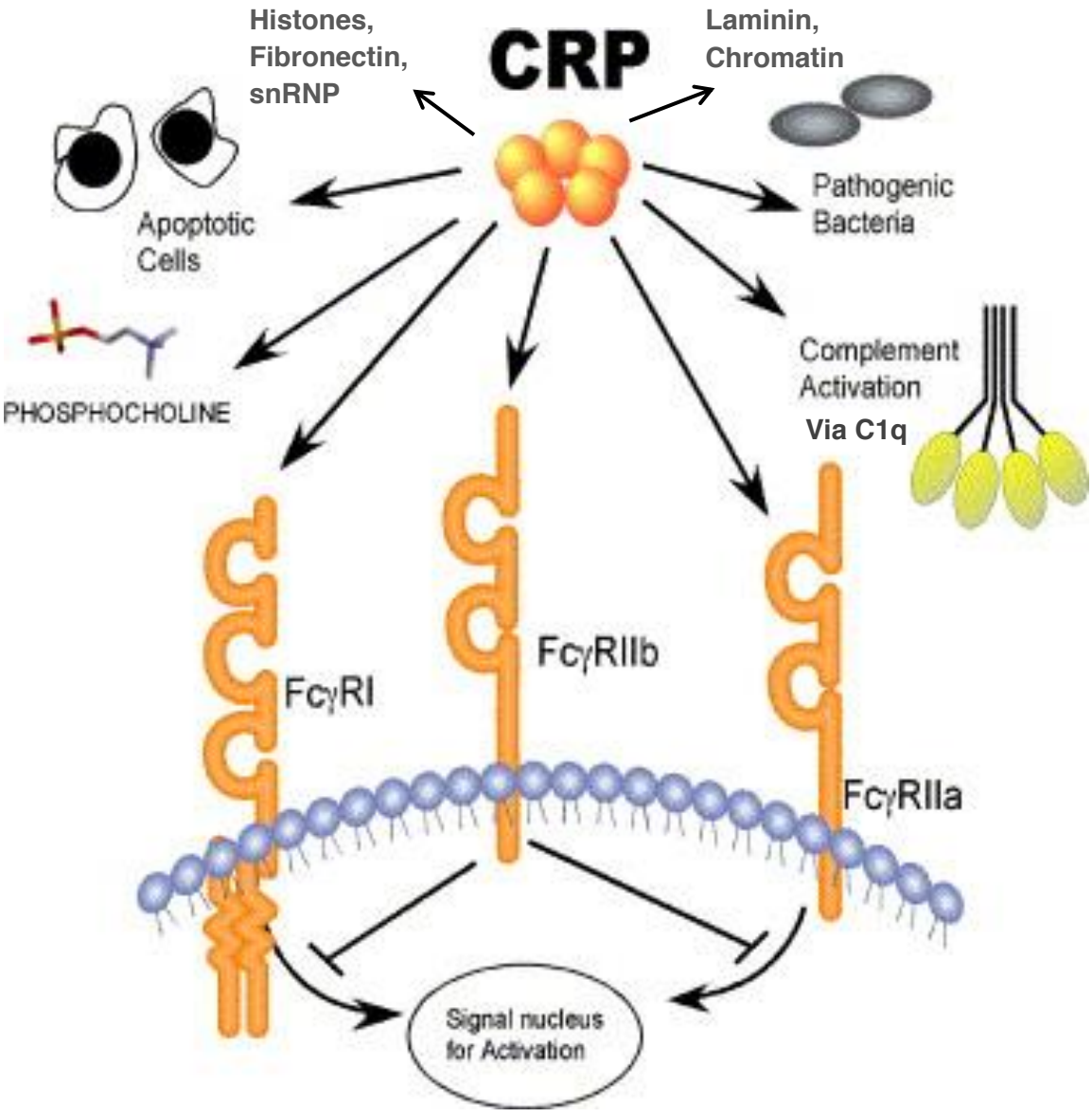


Figure 1-6 Diagram illustrating CRP ligands, including phosphocholine present on bacteria and apoptotic cells, C1q and FcγR1, FcγRI1b and FcγR1Ia. Adapted from Marnell *et al*., 2005.

1.4.6 Monomeric C-reactive Protein

Although initially dismissed (Pepys & Hirschfield, 2003), growing evidence now exists for the presence of a monomeric form of CRP (mCRP) occurring naturally within the body (Rees *et al*., 1988; Ciubotaru *et al*., 2005; Sjöwall & Wetterö, 2007a; Agrawal *et al*., 2009; Zhao *et al*., 2010),with auto-antibodies against mCRP found within the serum of SLE patients (Sjowall and Wettero, 2007). It is possible that the two conformations hold distinctly different inflammatory, stimulation and inhibition roles. Controversy still surrounds the biological existence of mCRP and the role it together with the pentameric form may play in the development of cardiovascular diseases such as atherosclerosis (Schwedler *et al*., 2005; Agrawal *et al*., 2010). The controversy surrounding the inflammatory effects of either pCRP or mCRP is compounded by the evidence suggesting commercially available anti-CRP antibodies used to identify pCRP in many studies (e.g. CRP-8 mAb, Sigma Aldrich), actually cross react with mCRP and in some cases have greater affinity to the monomeric form (Ciubotaru *et al*., 2005; Jabs *et al*., 2005; Schwedler *et al*., 2009). This knowledge may call into question some of the pro-inflammatory effects claimed for native pentameric CRP, and it is indicated that mCRP plays an active role within the inflammation associated with atherosclerosis (Ji *et al*., 2009).

Pentameric CRP is considered to be a soluble serum protein, in contrast mCRP is considered a poorly soluble, tissue based form of the protein (Ciubotaru *et al*., 2005; Agrawal *et al*., 2009; Ji *et al*., 2009), with a tendency to self-aggregate (Ciubotaru *et al*., 2005). Whilst it is possible that the native pentameric form may behave as a precursor to a proinflammatory monomeric form (Ji *et al*., 2009), little is known about the physiological origins of mCRP, although there is much speculation regarding possible local expression and dissociation mechanisms.

Monomeric subunits have been formed *in vitro* from the native pentamer via various harsh denaturing conditions such as a high urea concentration, altered pH, heat above 70ºC or low calcium levels, in the presence of a chelating agent (Kresl *et al*., 1998; Sjöwall & Wetterö, 2007a; Taylor & Van Den Berg, 2007). Whilst it is possible that the acidic conditions, pH5.5 – 7 (Zhang *et al*., 2009) and mild hypocalcemia < 2.12 mM calcium (Aderka *et al*., 1987; Zhang *et al*., 2009) of an inflammatory microenvironment or bacterial infection may allow dissociation of pCRP into the monomeric form, these *in vitro* creation methods have led to debate as to the biological relevance of this form (Pepys and Hirschfield, 2003).

In support of the existence of naturally occurring mCRP, evidence appears to confirm dissociation under physiologically relevant conditions, following binding to plasma membranes (Sjowall and Wettero, 2007), and possible *in vivo* dissociation following membrane interaction (Ji *et al*., 2009; Schwedler *et al*., 2009). Although originally believed stable and unlikely to dissociate into subunits, Ca^{2+} dependent binding of circulating pCRP to liposomes or cell membranes, was found to result in subsequent dissociation (Eisenhardt, Habersberger & Peter, 2009; Ji *et al*., 2009; Schwedler *et al*., 2009). Apoptotic monocytic THP-1 and Jurkat T cells can mediate this form of dissociation (Eisenhardt, Habersberger & Peter, 2009; Eisenhardt, Thiele, *et al*., 2009), with similar results found using activated platelets (Eisenhardt, Habersberger & Peter, 2009; Filep, 2009; Ji *et al*., 2009) seen in Figure 1.7 in which dissociation is facilitated by platelet membrane binding. In an early study investigating pCRP dissociation on monolayer membranes, (Wang & Sui, 2001) observed the specific binding of pCRP to PC containing membranes and subsequent dissociation of the pentamer into an open ring structure consisting of 2 subunits. This conformation retained C1q binding site availability and also exposed two neoepitopes (Wang & Sui, 2001).

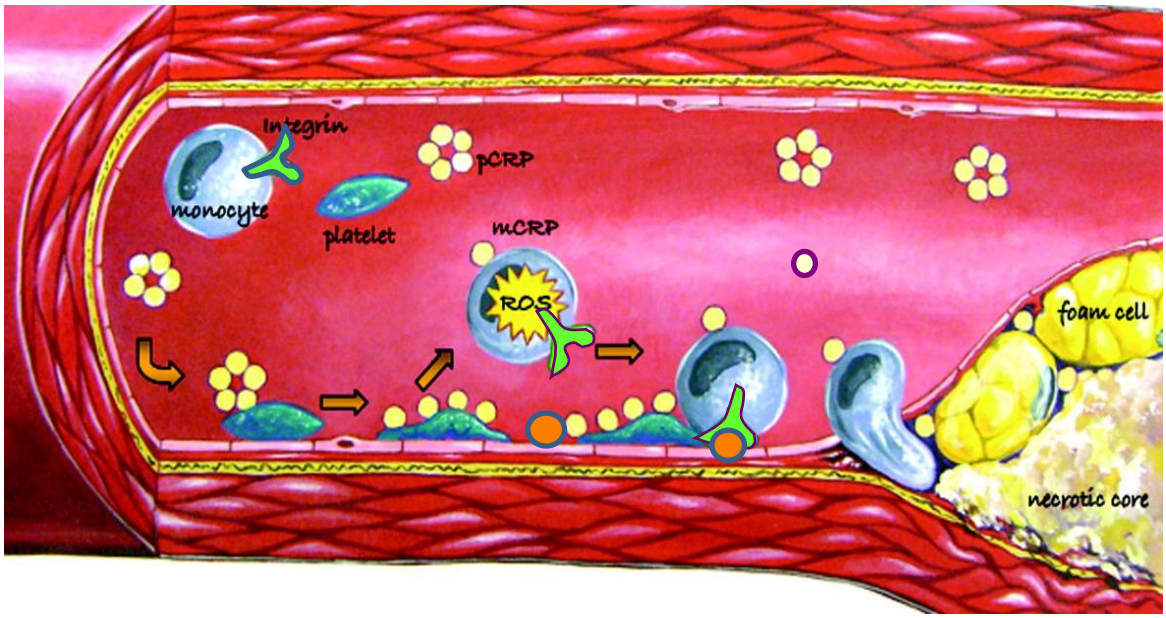


Figure 1-7 Diagram showing a suggested mechanism of pCRP dissociation mediated by activated platelets at the site of atherosclerotic plaques. Inflamed or damaged endothelium leads to the adherence of platelets which become activated exposing lipids such as lysophosphatidylcholine on the cell surface. Circulating pCRP binds to PC on adhered platelets, dissociating into mCRP. Circulating monocytes are stimulated by mCRP and integrin receptors activated (shown in green), reactive oxygen species (ROS) are released. Monocytic cells bind to receptors on endothelial cells (shown in orange) and transmigrate into the intima, forming foam cells. mCRP is then deposited within the atherosclerotic plaque. It is also possible that mCRP becomes dislodged from monocytic cell membranes becoming circulatory. Figure has been adapted from Eisenhardt *et al*., (2009).

The binding of pCRP to lysophosphatidylcholine (LPC) expressed on the surface of activated platelets and apoptotic monocytic THP-1 cells, reportedly resulted in structural changes of the subunits from predominantly β sheet, to predominantly α helix during dissociation to mCRP. This was suggested to expose inter-subunit residues 197-202 normally hidden as indicated within Figure 1.8, which are further suggested to form neoepitopes (Filep, 2009). This theory would be dependent on the breakage of a strong internal di-sulphide bond formed between the two β sheets of each protomer stabilising its conformation. It is possible that newly exposed internal and inter-subunit residues provide

the monomer with additional physiological functions, and that all CRP isoforms may have distinct bioactivities under differing pathophysiological conditions (Ji *et al*., 2007).

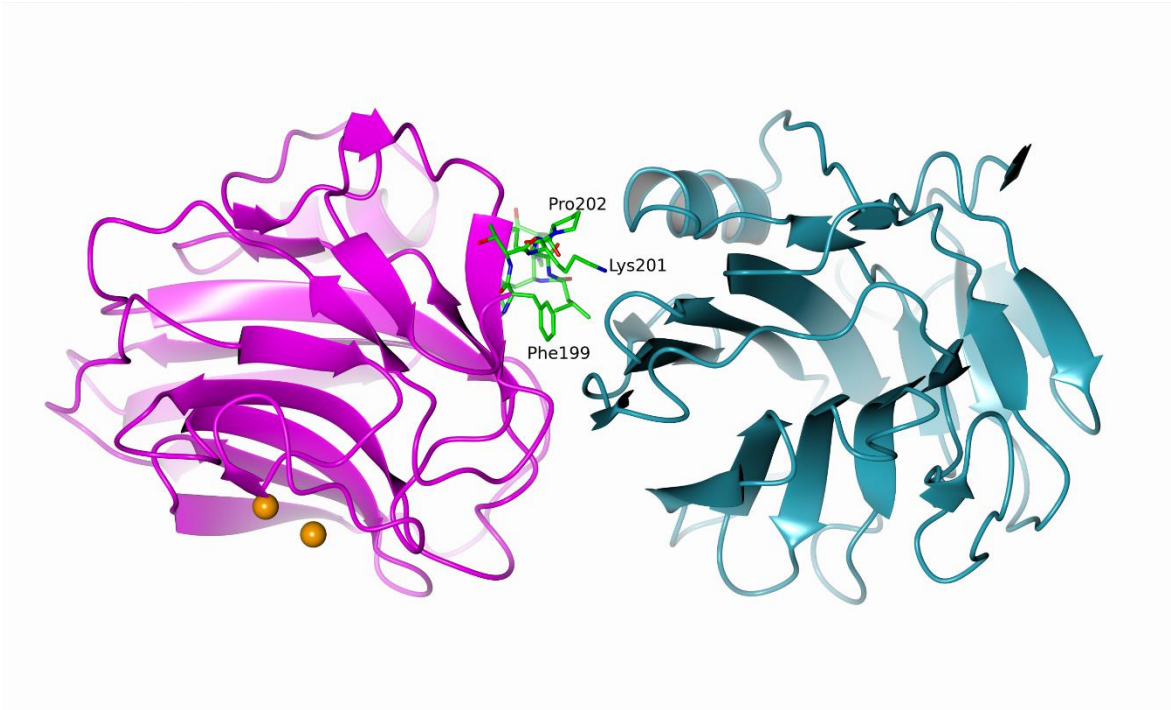


Figure 1-8 Structure illustrating the intersubunit contacts between two CRP protomers. The two calcium ions are shown in subunit 1 (pink), with their absence illustrated in protomer 2 (turquoise). Residues 199-206 within the interface believed to be exposed upon dissociation are illustrated in ball and stick. Image of PDB ID:1GNH (Shrive *et al*., 1996) created with CCP4mg (McNicholas *et al*., 2011)

During urea gradient electrophoresis, Kresl *et al*. (1998) reported a transitional conformation between pCRP and the formation of mCRP, observed at a 3M urea concentration. It was not possible to reverse these findings and re-associate the monomers. This may be due to the absence of an 18 amino acid leader sequence which forms part of the initially expressed pre-CRP protein and is cleaved during post-translational pentameric processing. It may also be possible a molecular chaperone protein is necessary during conformational folding and subunit association forming the final pentameric structure (Kresl *et al*., 1998).

A dissociated open ring conformation has been explored by Ji *et al*. (2007), with a proposed mechanism of mCRP formation. Using egg phosphatidylcholine (eggPC) / lyso-phosphatidylcholine (lysoPC) liposomes as membrane models, a reduction in the tryptophan content of CRP indicated a change of tertiary structure. Monoclonal antibody (3H12) created by the group, was used to probe for neoepitopes aa.199-206, as illustrated within Figures 1.8 & 1.9, exposed solely in mCRP, which again indicated membrane associated dissociation. The group suggests that multipoint attachment to a fluid membrane and a hydrophobic micro-environment are essential for the dissociation to take place. They propose that the membrane bound subunit ($mCRP_m$) interacts via hydrophobic insertion and although dissociated, retains a loose pentameric conformation, as evidenced by dual antigenicity to both mCRP and pCRP mAbs. The change to $mCRP_m$ also revealed greater complement activation efficiency, possibly due to avoidance of steric hindrance provided by Lys (114) and overcoming the greater size of C1q, suggested as a biological reason for the dissociation (Ji *et al*., 2007). This dissociation and membrane binding process is in broad agreement with similar studies (Wang and Sui, 2001; Ji *et al*., 2009; Filep, 2009; Sjowall and Wettero, 2007; Kresl *et al*., 1998).

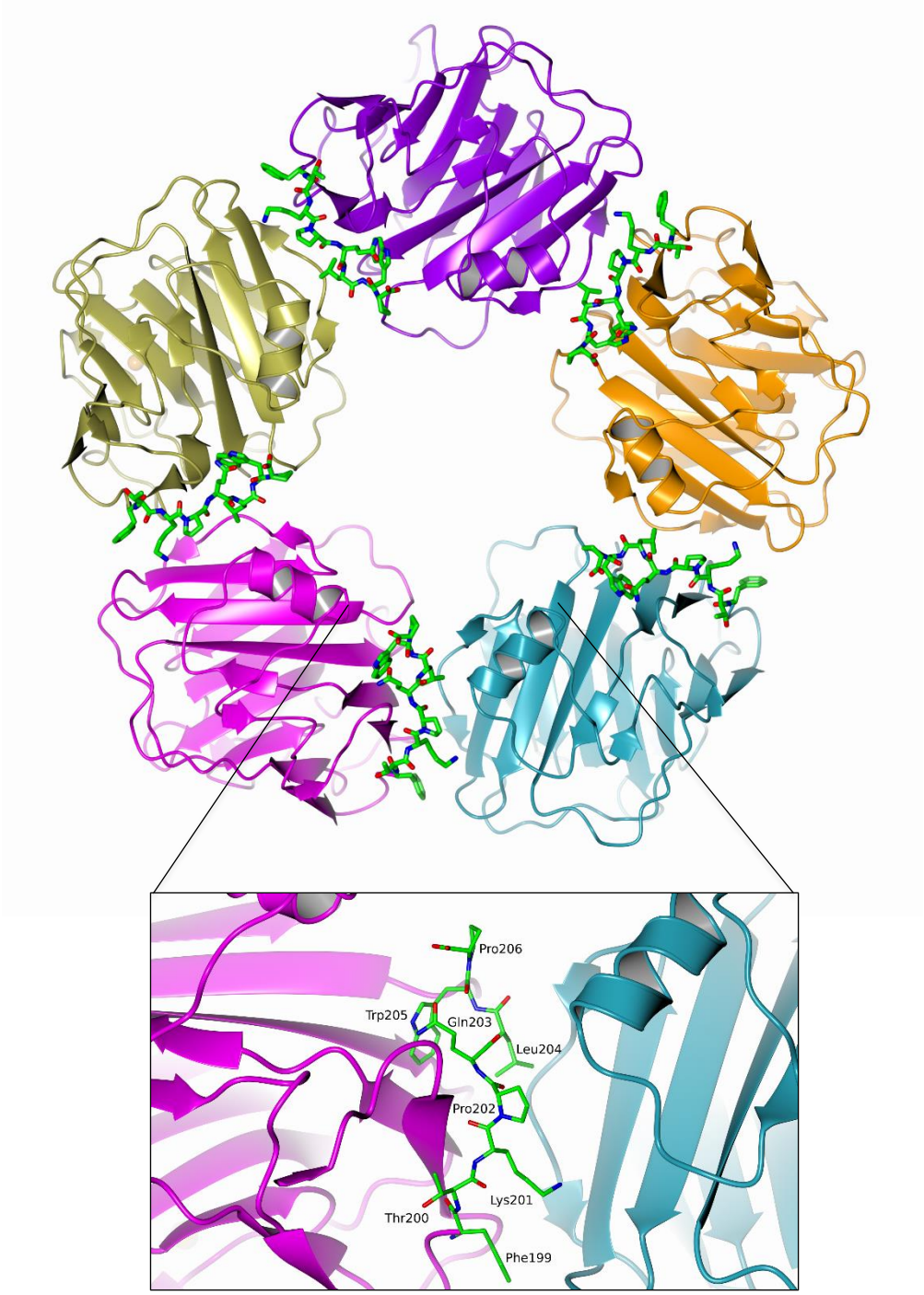


Figure 1-9 Structure of pentameric CRP with the intersubunit residues 199-206 highlighted as sticks. Residues 199-206 believed to be exposed when the pentamer dissociates, are detected by the monomer specific, monoclonal mouse anti-human mCRP (3H12) antibody. Image of PDB ID:1GNH (Shrive *et al*., 1996) created with CCP4mg (McNicholas *et al*., 2011)

The modified monomeric form of CRP shows differing binding properties to the native pentameric form, as well as unique antigenic and electrophoretic reactivities (Kresl *et al*., 1998), resulting in the change of isoelectric point (pI) from 6.4 to 5.4 (Bíró *et al*., 2007). Monomeric CRP may not specifically bind PC, as pCRP is observed to; Wu *et al*., *(*2002) reported that monomeric CRP protomers bind via nonspecific electrostatic forces to negatively charged membranes, with positively charged and neutral surfaces found to contain few monomers (Wang *et al*., 2002). It is suggested in their study that the interaction between monomers, although mainly via salt bridges is also stabilised by hydrophobic forces. The dissociation may result from the breakdown of the salt bridges and exposure of the hidden hydrophobic areas within acid or depolarized environment.

The monomeric form of CRP has now been identified within several diseases including age-related macular degeneration (Chirco *et al*., 2016), stroke and Alzheimer's disease (Strang *et al*., 2012; Slevin *et al*., 2015), atherosclerotic plaques and infarcted myocardium (Thiele *et al*., 2014), acute myocardial infarction (Wang *et al*., 2015) and antibodies against mCRP have been observed in systemic lupus erythematosus (SLE) and tubulointerstitial nephritis and uveitis (TINU) syndrome (Tan *et al*., 2008; Sjowall *et al*., 2009; Wetterö *et al*., 2009).

1.4.7 Human C-reactive Protein Ligand Interactions

CRP is documented to interact with a range of ligands in a calcium dependent manner (Figure 1.6), the pentraxin group are characterised by their interaction with phosphate containing ligands in addition to PC; these include small nuclear ribonucleoproteins, chromatin, histones, phosphoethanolamine, fibronectin, polycations and laminin (Black *et al*., 2004; Sjöwall & Wetterö, 2007a).

CRP has been shown to interact with human apoptotic cells, modified plasma lipoproteins and small ribonucleoprotein particles, in addition to pathogens within the body including bacteria, parasites and fungi. CRP detects ligands present on the surface of these microorganisms and will bind enabling an immune response (Mold *et al*., 1999; Gershov *et al*., 2000; Pepys & Hirschfield, 2001, 2003). The principle ligand of CRP is PC which is a constituent part of the polar head group of phospholipids, teichoic acids, bacterial lipopolysaccharides and galactan polysaccharides (Volanakis *et al*., 1981; Volanakis, 1982).

Cell membranes from both eukaryotic and prokaryotic cells are rich in PC, although it is inaccessible within the healthy cells of a human body where it forms part of compounds such as phosphatidylcholine. Secretory enzyme phospholipase A2 cleaves phospholipids upon damage to a cell membrane, exposing PC and therefore allowing detection and binding of CRP to damaged but not healthy cells (Volanakis & Narkates, 1983). When CRP encounters a bacterial cell it will usually bind a PC component which is part of a larger structure such as the C-polysaccharide present on *Streptococcus pneumoniae*. C-polysaccharide is a teichoic acid, a bacterial polymer of glycerol phosphate or ribitol phosphate, choline phosphate, N-acetyl galactosamine, N-acetyldiaminotrideoxyhexose and glucose residues, part of the gram positive bacterial cell wall (Gotshclich & Liu 1966, Poxton *et al*., 1978).

1.4.8 Human C-reactive Protein Receptor Binding

Receptors for the Fc regions of IgG provide important links between the humoral immune system and effector cells. Three important receptors involved within CRP cellular interactions are FcγRI, FcγRII and FcγRIII, of these receptors FcγRI (CD64) is a high affinity receptor and the remaining two low affinity. CRP shares many functions with IgG,

including the ability to activate complement and bind monocyte and neutrophil receptors with an affinity comparable to IgG. Native pentameric CRP binds normal, undamaged cells via FcγRI and RII (CD32, CD64) in a calcium independent manner via the effector face (S.-R. Ji *et al*., 2006). Further studies with COS-7 cells and pentameric CRP found binding to both FcγRI and FcγRII but not FcγRIII (Tron *et al*., 2008). It is expected that CRP associate equally with FcγRI and FcγRII, however this was not the case, binding was preferential to the high affinity receptor. Possible explanation suggests that CRP is more able to bind if clustered to FcγRII. FcγRI has three extracellular Ig like domains, whereas FcγRII and FcγRIII have two, suggesting reduced affinity (Tron *et al*., 2008). The Fc fraction of human IgG binds each receptor, however it was found that mouse IgG1 isotype does not. Xing *et al*., *(*2008) found similar CRP / FcγRI interactions *in vivo*.

As the number of Ig classes and isotypes increased during evolution so did the number of isotype specific receptors (Akula *et al*., 2014). The four IgG isotypes are accompanied by the four major types of Fc receptor, FcγRI, FcγRII, FcγRIII, FcγRIV, each with variable affinity dependent on IgG isotype, these interactions are shown in Table 1.3. The Ig binding subunit is the α chain of the receptor and in humans all IgG and IgE receptors have two extracellular Ig like domains with the exception of FcγRI which has three (Akula *et al*., 2014). All IgG and E receptors have a transmembrane region anchoring to the membrane except FcγRIIIB, which is a glycosylphospatidylinositol (GPI) anchored receptor. Both IgG and each FcγR gene are found on Chromosome 1, with FcγRII and FcγRIII in close proximity to the IgG gene location at 1q21.2 (Akula *et al*., 2014) and CRP gene location of 1q23.2. It is suggested by Srinivas Akula *et al*., *(*2014) that the Ig receptors may originate from a common ancestor. The gene loci of 1q21-23 are subject to frequent chromosomal aberration within hematological malignancy with FcγRIIB thought to be affected (Callanan *et al*., 2000).

Ji *et al*., *(*2006) found that association of pCRP with human aortic endothelial cells (HAECs) expressing these receptors also resulted in the conformational structural change of pCRP to $mCRP_m$ found in monolayer studies. Unexpectedly the binding was found to be calcium dependent rather than the calcium-independent association between FcγRs and pCRP. They concluded that the binding of, and conformational changes to, pCRP in association with these cell membranes, may occur via FcγR independent binding of the recognition face to the PC head group of sphingomyelin, within specialised lipid raft domains, rather than the C1q associated effector face (S.-R. Ji *et al*., 2006). FcγRIII (CD16) has been identified as the receptor for neutrophil binding of mCRP (Heuertz *et al*., 2005), however in a further study, mCRP was shown to bind U937 macrophage cells which do not express CD16. This binding was also shown to be predominantly independent of protein receptors CD32 or CD64, a further indication of FcγR independent membrane binding (Zhao *et al*., 2010).

Table 1.3 Summary table showing Fcγ receptor binding affinity, expression/function and signalling motifs.

FC Receptor	Binding Affinity	Expression / Function	ITAM / ITIM Signalling Motif	Source
FcγRI / CD64	High IgG1=3>4 High pCRP	**Expression:** Macrophages, neutrophils, eosinophils, dendritic cells **Function:** Enhances effector responses at inflammatory sites, Immune complex capture by dendritic cells.	γ chain ITAM	Getahun and Cambier, 2015; Ji *et al*., 2006; Marnell *et al*., 2005;Tron *et al*., 2008.
FcγRIIA / CD32	Low IgG3>1>2 Low pCRP	**Expression:** Macrophages, neutrophils, mast cells, eosinophils, platelets, dendritic cells **Function:** Activation - Effector cell activation by immune complex, cytotoxic Ab	α chain ITAM	Getahun and Cambier, 2015; Ji *et al*., 2006; Marnell *et al*., 2005;Tron *et al*., 2008.
FcγRIIB / CD32	Low IgG3>1>2>4 Low pCRP	**Expression:** Macrophages, neutrophils, mast cells, eosinophils, dendritic cells **Function:** Inhibition - Set threshold for effector cell activation by Fc, B cell repression, maintain tolerance.	α chain ITIM	Getahun and Cambier, 2015; Ji *et al*., 2006; Marnell *et al*., 2005;Tron *et al*., 2008.
FcγRIIIA/ CD16	Medium IgG High mCRP	**Expression:** Macrophages, mast cells, basophils, NK cells, dendritic cells **Function:** Activation – Dominant pathway for IgG activation of effector cell, Arthus reaction, immune complex capture by dendritic cells.	γ chain ITAM	Akula, Mohammadamin and Hellman, 2014; Getahun and Cambier, 2015; Heuertz *et al*., 2005; Marnell *et al*., 2005.
FcγRIIIB	Low IgG1=3>2=4 High mCRP	**Expression:** Neutrophils **Function:** Decoy –focus immune complex to PMN, synergize with FcγRIIA	α chain ITAM, ITIM	Akula, Mohammadamin and Hellman, 2014; Getahun and Cambier, 2015; Heuertz *et al*., 2005; Marnell *et al*., 2005.

1.4.9 C-reactive Protein as a Clinical Biomarker

In a clinical setting a fast, easy and often automated system based on the ELISA concept is often employed. This provides ease of use and sensitivity based on the combination of antigen and antibody complexes to determine CRP concentrations (Algarra *et al*., 2013). However although these systems accurately detect levels of CRP, they do not seek to identify whether it is of pentameric or monomeric form. Of the analytical methods used some are more sensitive than others and able to detect minor changes from healthy levels, others indicate the large changes occurring through inflammation. The most sensitive methods for measurement of CRP are nephelometric light scattering assays, which measure the increase in scattered light occurring via interaction with antigen-antibody complexes. This method allows highly sensitive determination of low level of CRP which may help assess the risk of future disease development (Bassuk *et al*., 2004; Koivunen & Krogsrud, 2006). Other analytic methods include immunosorbent assays, immunoturbidity, surface plasmon resonance, latex agglutination and chemiluminescence (Gella *et al*., 1991; Lechuga, 2007; Laiwattanapaisal *et al*., 2008; Department of Biology Davidson College, 2014). These methods when used appropriately allow accurate comparison of CRP levels between research and clinical settings internationally. However results will depend upon the specificity of antibodies used within the detection assays and therefore levels of CRP determined may potentially be a combination of both monomer and pentamer.

1.4.10 U937 Cellular Expression of CRP

Macrophages are nucleated cells which differentiate from monocytes. It has previously been shown that macrophages are capable of local CRP expression (Kawai & Akira, 2010; Wang *et al*., 2011). Immortal monocytic cell line U937 is successfully used as an *in vitro*

CRP macrophage expression model. These cells differentiate upon the addition of phorbol 12-myristate 13-acetate (PMA), expressing a macrophage like morphology and have been shown to express monomeric rather than pentameric CRP (Ciubotaru *et al*., 2005). In Cuibotaru *et al*'s study, U937 CRP expression following PMA differentiation was initially unaffected by the addition of lipopolysaccharide following 24 hours, however expression increased significantly in a time dependent manner peaking at 48h and with limited increase between 48 and 72 hours. No associated increase in mRNA expression was found suggesting LPS modulates CRP synthesis at the translational level rather than at the level of transcription. Inhibition studies found that increased expression was mediated via IL-6 and IL-1β (Ciubotaru *et al*., 2005).

1.5 Interactions Between C-reactive Protein and Immune Components

1.5.1 Monocytes and Macrophages

CRP plays a key role within the recruitment and modulation of several immune components in addition to its activation of the inflammatory response via binding of dead or damaged cells. During the inflammatory response a site of infection is flooded with monocytic cells which mature into macrophages. CRP is suggested to play a part in the recruitment of monocytes, their maturation to macrophages and further regulation of these cells via Fcγ receptors. Low affinity CRP binding to phagocytic cells was revealed by Ballou & Cleveland in 1990, via flow cytometry studies, which occurred in a calcium dependent manner (Ballou & Cleveland, 1990). Using a macrophage like cell line (Mono Mac 6) Tron *et al* in 2008 illustrated CRP binding to Fcγ receptors I and II, but preferentially to FcγRI. Further studies with COS-7 cells and pCRP found binding to both FcγRI and FcγRII but not FcγRIII (Tron *et al*., 2008). Further it appears CRP is capable of inhibiting the maturation of macrophages via Fc receptor binding to the macrophage cellular surface (Zhang *et al*., 2011). This inhibition was prevented by IgG binding, presumably via counter blocking of the Fc receptor sites (Zhao *et al*., 2010).

CRP interactions with macrophage cells have several correlations with those between the macrophage and IgG, including macrophage cytokine production in response to CRP or IgG of TNFα, IL-1β and IL-6, together with complement activation, cytokine secretions and opsonisation. CRP also facilitates phagocytosis of C-polysaccharide (CPS) coated cells and induction of coagulation via the release of tissue factor (Cermak *et al*., 1993; Stein *et al*., 2000; Torzewski *et al*., 2000). The role of monocytes and macrophages within the immune response involves phagocytosing harmful pathogens together with

recruitment of other immune cells to the area and CRP plays a role in their activation, regulation and enhancement within a coordinated acute phase response.

1.5.2 Lymphocytes

An interaction between lymphocytes and CRP was first observed in 1937, with lymphocytes observed in a later study to bind CRP within children suffering from rheumatic fever (Williams *et al*., 1978). Another early report indicated that CRP bound preferentially to T lymphocytes and inhibited their capacity to form spontaneous rosettes with sheep erythrocytes and also inhibited their responses to allogenic cells in a mixed lymphocyte culture. Mortensen *et al*., *(*1975), also found that CRP did not bind B lymphocytes nor did it alter B cell function (Mortensen *et al*., 1975). Ballou and Lozanski (1992) also observed CRP bound to lymphocytes and reported that the CRP originated from two distinct locations, the first produced primarily by liver hepatic cells, which then bound to lymphocyte cell membranes in a calcium dependent manner and the second expressed by the lymphocyte itself and inserted into the cell membrane (Ballou & Lozanski, 1992). They further found that in the healthy adult population 2.5% of lymphocytes expressed and inserted CRP within their membranes and a further 1.5% of lymphocytes were found with CRP of hepatic origin bound in a calcium dependent manner to their membranes. The percentage of lymphocytes with CRP of lymphatic origin was increased in RA patients, whilst CRP of hepatocyte origin remained at the same level, except in cases where serum CRP concentration exceeded 50µg/ml.

The interaction between lymphocytes and CRP takes place through Fc receptors in a similar way to those observed between CRP and monocytic cells (Gewurz *et al*., 1982). Early studies suggested CRP added to leucocyte suspension was mitogenic at low

concentrations but actually toxic at higher than 10μg/ml and that *in vitro* studies suggested CRP inhibited growth of human melanoma cells in the presence of lymphocytes.

1.5.3 Platelets

The primary role of platelets is that of wound repair; through activation of the coagulation cascade with hemostasis their best characterised function (Monroe *et al*., 2002; Jenne & Kubes, 2015). Platelets are activated by platelet activating factor (PAF), a phospholipid mediator of inflammation, released early in inflammation by a variety of cell types including platelets, macrophages, neutrophils and endothelial cells (Chao & Olson, 1993). PAF has been implicated within the pathogenesis of autoimmune conditions and inflammatory diseases (Leong *et al*., 2007; Sato *et al*., 2014). The luminal walls of blood vessels are coated by glycocalyx layers, which separate the platelets from contact with endothelium or basal layers. Should the platelet breach this protective layer, platelets would become active and clot formation begins to fill the gap (Sato *et al*., 2014). Upon platelet activation, platelet derived microparticles (PMPs) are released from the plasma membrane and are associated to RA disease activity (Knijff-Dutmer *et al*., 2002). Interaction between CRP and PAF may enhance PAF induced inflammatory activity *in vivo* as shown in the study by Sato *et al*., *(*2014), although alternative research indicates CRP may play a protective role in PAF-induced platelet aggregation, via CRP binding to PAF and platelet surface phospholipids (Kohayakawa & Inoue, 1986; Nagpurkar *et al*., 1988).

1.6 The Complement System and C-reactive Protein

The complement pathway consists of more than 30 plasma and membrane bound proteins and is an essential tool in the body's defense mechanism against infectious diseases, playing an important role in the inflammatory response (Volanakis, 1982; Sarma & Ward, 2011; Nonaka, 2014). Complement may be activated by either the classical, alternate or mannose binding lectin pathways; CRP activates the classical pathway. Activation of the classical pathway is dependent upon the presence of either IgM or IgG within the immune complex, and involves the C1 complex via the C1q subunit (Volanakis *et al*., 1981; Mold *et al*., 1999; Matsushita & Fujita, 2001; Dunkelberger & Song, 2010). Both pathways stimulate cellular and humoral interactions such as phagocytosis and support B-cell differentiation enabling activation of adaptive immune systems. C1q recognises targets such as immune complexes, apoptotic and necrotic cells, amyloids and micro-organisms which lead to complement activation (Agrawal & Volanakis, 1994; Gaboriaud *et al*., 2003; McGrath *et al*., 2006). Antibody binding to C1q induces conformational changes allowing activation of C1r and C1s serine proteases, leading to C3 convertase formation (Chen *et al*., 2010). C1q itself is formed from six subunits each containing a C-terminal globular head and N-terminal collagenous tail, held together in a bouquet formation, together with C1s and C1r. It is the globular head group of C1q that binds a pathogen and activates the complement cascade via the cleavage of C4 by C1 forming C4b2a also known as C3 convertase (Wallis *et al*., 2010); it is also the globular head region which interacts with CRP.

Various changes in levels of complement components have been linked to RA, including a deficiency in C1q which has been linked to both RA and SLE (Chen *et al*.,

2010; Wallis *et al*., 2010). Table 1.4 details other changes to complement activity identified within RA.

Table 1.4 Summary table of complement activity found within Rheumatoid Arthritis.

Complement Component	**Deficiency / Increase in RA**	**Source**
C1q	Deficiency linked to RA and SLE	(Chen *et al*., 2010; Wallis *et al*., 2010),
Complement activity	Significantly lower in RA joint fluid than non-inflammatory arthritis	(Chen *et al*., 2010)
Soluble complement activation fragments	Significantly increased in RA joint fluid	(Chen *et al*., 2010)
Complement production	Enhanced local complement production within synovial tissue	(Neumann *et al*., 2002; Chen *et al*., 2010)
C3 and MAC	Presence within synovial tissue	(Neumann *et al*., 2002; Chen *et al*., 2010)
C3a and C5a	Increased levels within synovial fluid and serum	(Neumann *et al*., 2002; Okroj *et al*., 2007; Chen *et al*., 2010; Bradford *et al*., 2016)

Excessive complement activation has therefore been implicated in RA, leading to the suggestion that complement control agents able to regulate excess complement activity may have beneficial uses (Mizuno *et al*., 2001). Complement is capable of recognising and attacking self-cells, and control mechanisms exist to regulate and restrict activity to target pathogens. However it is possible that in the case of excessive complement activation, host regulatory mechanisms are exceeded, leading to harmful inflammatory pathways (Chen *et al*., 2010). It has been suggested that excessive complement activation may propagate inflammation in RA, but that a suppression or deficiency of C1q may actually be involved in RA induction (Chen *et al*., 2010).

Complement factor H is a major inhibitor of the alternate complement pathway, involved within the regulation of complement activation in host cells, plasma, tissue and importantly at sites of inflammation (Molins *et al*., 2016). Factor H is a serum glycoprotein expressed by a variety of cells and is involved within accelerated decay of C3 convertase

(C3bBb), essentially regulating the alternative pathway of complement ensuring that complement action is directed towards pathogens rather than self-tissue. Over activity may result in reduced complement action towards pathogenic cells and underactivity results in increased complement activity on healthy cells (Ferreira *et al*., 2010). Factor H is known to interact with CRP, studies show both pentameric and monomeric involvement, with two binding sites for pCRP identified (Molins *et al*., 2016). Further research also shows factor H interactions with of mCRP within SLE. Auto-antibodies directed against mCRP amino acids 35-47 were found within lupus patients to be associated with more severe renal damage. This finding suggests mCRP may be protective in nature via a reduction in factor H function, and that auto-antibody binding of mCRP may prevent the ability of mCPR to bind to and therefore modulate factor H function (Q. Li *et al*., 2017). However this group found factor H binding of mCRP, reduced its proinflammatory effects (Molins *et al*., 2016). It is unclear how the interaction between factor H and mCRP modulates the immune response; it may be inconsistent across different disease state.

1.7 C-reactive Protein and Disease

1.7.1 C-reactive Protein and Cardiovascular Disease

Cardiovascular disease (CVD) was the most common cause of death in the UK for women in 2012 (28% of female deaths), however cancer was the biggest killer for males. Prevalence of coronary heart disease is highest in the North of England (4.5% in North East) and Scotland (4.3%), with about three times more men than women suffering from myocardial infarction. In 2012/13 the NHS in England spent £6.8 billion on CVD, therefore presenting a significant burden on public health funding (Bhatnagar *et al*., 2015).

Atherosclerosis is a major factor in the development of CVD, distinguishable from the lesions in arteries throughout the body, with characteristic inflammation (Hanson &

Libby 2006). Over time atherosclerotic plaques form leading to complications through rupture, myocardial infarction or stroke, dependent upon the location of the plaque (Libby, 2006). Deposition of CRP within the arterial wall is a feature of atherosclerotic lesions, with CRP implicated within the release of inflammatory markers including ICAM, VCAM-1 and pro-inflammatory cytokines (Hirschfield & Pepys, 2003; Sun *et al*., 2005; Zhang *et al*., 2012). CRP binds to LDL and oxidised or partially degraded LDL aiding the formation of foam cells and atherosclerotic plaque creation (Hirschfield & Pepys, 2003; Zhang *et al*., 2012). It is therefore suggested that CRP may play a pro-inflammatory role within CVD through expression of cell adhesion molecules, instigation of complement and release of inflammatory cytokines (Zhang *et al*., 2012).

1.7.2 C-reactive Protein and Systemic Lupus Erythematosus

CRP levels are raised in response to inflammation in both atherosclerosis and RA; however another autoimmune inflammatory disease, systemic lupus erythematosus (SLE) shows a different CRP profile (Bell *et al*., 1998). Pentameric CRP levels remain low in the disease, however auto-antibodies to mCRP have been found in up to 78% of SLE patients (Bell *et al*., 1998). It is as yet unclear why CRP levels remain low in this disease. SLE is an autoimmune disease in which the body mistakenly attacks healthy tissue. Unlike RA which is predominantly confined to joints, SLE is systemic affecting skin, joints, kidneys, other organs and even the brain. Like RA the condition is more prevalent in women than men however unlike RA it affects more people of Hispanic, African or Asian descent than any other ethnic group (Gonzalez *et al*., 2013). This disparity between ethnic groups is thought to be a combination of both genetic and non-genetic factors (Gonzalez *et al*., 2013). Genetic factors play a strong role in SLE with concordance between identical twins and

increased risk and frequency of disease among first degree relatives, with multiple genes responsible (Mok & Lau, 2003).

Anti-mCRP serum levels are found to be correlated to SLE patients during flare, with Sjowall and Wettero (2007) observing SLE disease flares in seven of ten patients who also had high mCRP auto-antibodies, a greater correlation was found between patients with active lupus nephritis who were all positive for the auto-antibodies (Sjöwall & Wetterö, 2007b). This finding was mirrored within a further study by Jakusko *et al*., *(*2017) who also found that the correlation between mCRP directed self-antibodies in SLE is higher in patients with lupus nephritis as compared to SLE patients without renal involvement (Jakuszko *et al*., 2017). Animal models have demonstrated CRP presence in lupus nephritis renal immune deposits (Szalai *et al*., 2002), with further reports suggesting co-localisation of auto-antibodies targeted against mCRP leading to amplification of the classic pathway activation of complement (Trouw *et al*., 2004).

Anti-mCRP is not only correlated to SLE disease but also to IgG levels. In their 2001 study, Minatani *et al*. (2001) found serum levels of IgG auto-antibodies directed against mCRP raised in 32% of SLE patients and 29% of RA patients, interestingly, within SLE patients these levels were directly correlated with serum IgG levels. This group suggested an immunopathogenic role for mCRP within rheumatic diseases (Minatani *et al*., 2001). Whilst levels of SLE disease, IgG and mCRP auto-antibodies all correspond, no such correlation was found between levels of serum CRP and anti-mCRP IgG; however previous studies have not tested for serum mCRP as it has been suggested to be tissue bound rather than soluble (Tan *et al*., 2008) Tan *et al* also propose that levels of CRP are low in SLE due to decreased pentameric expression or accelerated conversion of pCRP to mCRP, they further suggest that levels of auto-antibodies against mCRP may be due to

partial secretion of mCRP by local immune cells (Iwamoto *et al*., 1994; Duong *et al*., 1997).

1.7.3 C-reactive Protein and Alzheimer's Disease

Several studies have linked CRP levels to Alzheimer's disease (AD), with neuropathological studies showing CRP associated to neurofibrillary tangles and senile plaques taken from AD brain tissue (Iwamoto *et al*., 1994; Duong *et al*., 1997).

The characteristic changes seen at autopsy show neuritic plaques, neuronal loss, amyloid angiopathy and neurofibrillary tangles (Cummings *et al*., 1998). As in RA and SLE, female gender is suggested to increase the risk of development, with an onset of between 40 and 90 years old. Symptoms include impaired cognitive function, such as language and perceptual recognition (Cummings *et al*., 1998).

Elevated CRP levels in early life imply a risk of AD in later life which was observed within a 25 year follow up on elevated midlife CRP levels within a longitudinal study revealed an increased risk of AD in addition to vascular dementia (Schmidt *et al*., 2002). However studies have also shown that CRP levels appear to be lower within AD sufferers than age matched controls (O'Bryant *et al*., 2010). A later follow up study showed that although these levels were lower within the AD patients, those amongst the study who had elevated CRP also suffered greater levels of poor cogitative function and a reduction in survival time (Nilsson *et al*., 2011).

1.7.4 C-reactive Protein and Diabetes

Diabetes mellitus is a metabolic disease characterised by hyperglycemia and associated damage to different organs including kidneys, eyes, nerves heart and blood vessels. The condition can be characterised into three groups, type 1 diabetes resulting from autoimmune destruction to the β-cells of the pancreas leading to insulin deficiency,

type 2 diabetes manifesting in insulin resistance and impaired insulin secretion and gestational diabetes mellitus diagnosed within the second to third trimester of pregnancy (American Diabetes Association, 2010). Diabetes is a known independent risk factor of atherosclerosis and is associated with elevated levels of coronary heart disease and coronary atherosclerosis (Schmidt *et al*., 1999), with studies showing that patients have elevated CRP when compared to controls. CRP has also been identified within the tubule tissue of diabetic patients. Kidney biopsies showed that enhanced tubular mCRP staining was associated with renal decline. Histologic changes were also significantly correlated to mCRP staining (Schwedler *et al*., 2003).

Type 2 diabetes is also associated with an elevated prevalence of cardiovascular disease. Baseline changes to CRP levels are suggested to be an indicator of cardiovascular risk in type 2 diabetics. However the prognostic value of CRP within this condition is disputed amongst groups (Jager *et al*., 1999; Schulze *et al*., 2004; Bruno *et al*., 2009; Schöttker *et al*., 2013). A study by Pradhan et al in 2001, considered the involvement of IL-6 and CRP in the development of type 2 diabetes in a cohort of 27,628 women who were not originally diabetics, of those 188 went on to develop diabetes. In the diabetic group, levels of both IL-6 and CRP were significantly raised compared to age matched controls, suggesting CRP and IL-6 levels were useful in predicting future development of diabetes (Pradhan *et al*., 2001). It is suggested that low grade inflammation indicated by CRP may over time lead to the manifestation of type 2 diabetes (Pradhan *et al*., 2001; Ford, 2003; Tabak *et al*., 2010). An additional study also found that elevated serum CRP levels enhanced the risk of diabetes development (Dehghan *et al*., 2007), suggesting that although the mechanism is as yet unknown, the association between inflammation, CRP and diabetes may be beneficial in the prediction of later disease.

1.8 Rheumatoid Arthritis

1.8.1 Overview

A well balanced, healthy body possesses many tools at its disposal to enable maintenance of a non-diseased state. However, our modern lifestyle has resulted in an increasing level of disease, including cancers, diabetes, heart disease and autoimmune diseases such as RA and SLE. It has become difficult to determine whether the incidence of disease is greater, or we have become better at detecting and diagnosing it, or if we simply live longer and no longer escape diseases associated with old age by untimely death. RA is a disease of the immune system - an autoimmune disease whereby the normally healthy body begins to attack and destroy itself. Many theories have been proposed towards the misdirection of immune components, but no single one fully explains it. RA is a common disease with a prevalence of about 1% of the world's population and an annual incidence of approximately three in 10,000 adults (Spector, 1990). The economic burden of RA in the United States was estimated at up to $14 billion annually in 1998 (Callahan, 1998), with that cost estimated to be almost £8 billion in the UK by 2010 (NRAS, 2010). The financial burden is not just that of treatment cost but in overall cost to the economy including productivity due to incapacity.

This chronic and disabling inflammatory condition leads to joint destruction if intervention does not occur early in disease. Characterised by synovitis, the joint destruction is associated with a hyperplastic synovial membrane with infiltration of synoviocytes, immune cells and inflammatory cells, including macrophages, lymphocytes, dendritic and plasma cells (Firestein, 2003). The consequences of this condition include joint damage, commonly to the small joints of the hands and feet, joint stiffness, pain and bone destruction (Choy & Panayi, 2001).

1.8.2 Clinical Features of Rheumatoid Arthritis

Destruction of synovial cartilage and bone are the hallmarks of RA (Scott *et al*., 2010), although the severity of symptoms varies greatly between individuals (Goronzy & Weyand, 2009). Long term prognosis is poor with a substantial number of patients, more than 40% after 10 years (Goronzy & Weyand, 2009), and 80% after 20 years (Choy & Panayi, 2001) eventually suffering some level of disability resulting from joint destruction. The joint pain and destruction is a result of synovial hyperplasia, and influx of inflammatory cells, of which CD4+ T cells form a large part (Choy and Panayi, 2001). Early symptoms can include low grade fever, fatigue, early morning stiffness, together with pain and swelling to the joints (Boilard *et al*., 2012).

An autoimmune disease, RA begins with what is termed the pre-articular phase, in which auto-antibodies are generated against host cells rather than foreign entities. During this phase the self-antigen and IgG antibody complex activates the innate immune system (Fazal *et al*., 2018). Dendritic cells bind the complex and trigger differentiation and growth of Th1 cells, together with Th17 and regulatory T cells. B cells are activated by the differentiated Th1 cells, which then go on to produce auto-antibodies such as rheumatoid factor (RF) and anti-cyclic citrullinated peptide (anti-CCP) (Fazal *et al*., 2018). Pathophysiology of RA results from the involvement of both T and B cells together with involvement of pro-inflammatory cytokines TNFα, IL-1 and IL-6, with activated $CD4^+$ T cells contributing to the pathogenesis of the condition (Rivellese *et al*., 2017). The activation of the autoimmune response then leads to the characteristic destruction of bone, synovium and cartilage.

IL-1 and TNF-α stimulate both the recruitment of neutrophils to the site of joint damage and the expression of adhesion molecules on local endothelial cells. Neutrophil release of both proteases and elastase lead to the degradation of the proteoglycan layer and

exposure of chondrocytes. IL-1, TNF-α and activated CD4+ T cells stimulate synovial fibroblast and chondrocytes to express matrix metalloproteinases, which may in turn be responsible for the enzymatic joint damage in RA (Choy and Panayi, 2001). The synovial membrane or synovium is a coating to the joint which is protected by lubricating synovial fluid. A normal joint has a synovial membrane, called the intima, consisting of a thin layer of one to three synoviocytes depth, in contrast to an affected joint in which synoviocytes form a thick synovial membrane leading to the hyperplastic synovial lining (Middleton *et al*., 2004; Strand *et al*., 2009). The intima has an outer lining of two synovial cell types, termed A and B intima cells. Type A are macrophages and type B are fibroblast like synoviocytes which are secretory cells releasing components such as hyaluronic acid and lubricin (Fazal *et al*., 2018). The hyperplastic synovium together with a cocktail of immune components such as neutrophils, osteoclast, fibroblast, dendritic, macrophage, T lymphocytes, B lymphocytes and plasma cells, lead to the destructive pannus formation and eventual joint destruction (Lundy *et al*., 2007), as seen in Figure 1.10, which shows the influx of immune components within an RA joint in comparison to a healthy normal joint.

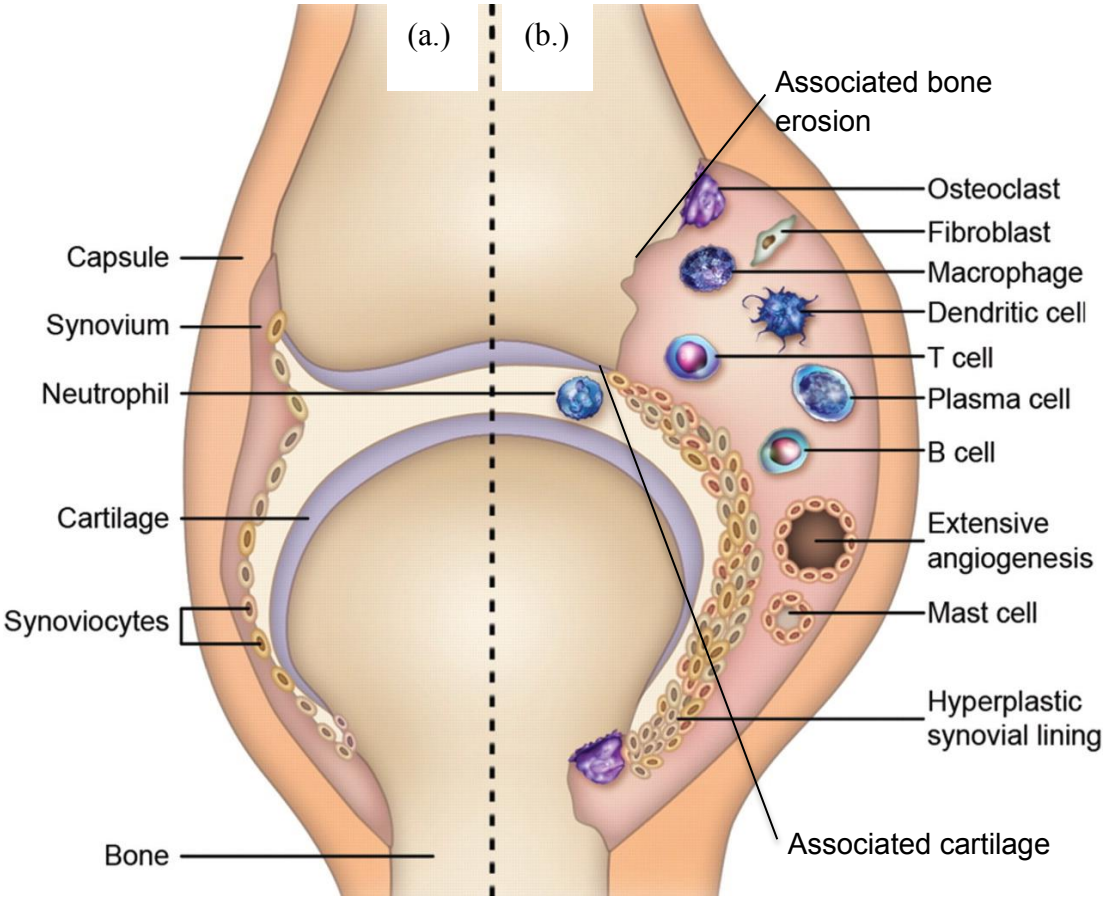


Figure 1-10 Illustration showing the effects of rheumatoid arthritis within a normal and healthy joint. (a.) showing normal bone and cartilage formation (b.) showing the influx of immune components leading to bone and cartilage destruction. Adapted from (Smolen & Steiner, 2003)

Bone destruction occurs due to a modulation of the normal process of bone remodeling which is ongoing in healthy joints. Osteoclast cells resorb bone whilst osteoblast form it in a process held in careful balance, however in RA patients this process is disturbed. Osteoclasts regulate mineral accumulation in the human body by releasing calcium and other minerals from the tubercular and cortical bone into the blood stream, with the associated bone damage repaired and replaced by osteoblast cells (Smolen and Steiner, 2003). In RA the balance between osteoclast and osteoblast differentiation and maturation is affected by proinflammatory cytokines, leading to constant osteoclast differentiation. Joint destruction results from matured osteoclast binding to the bone matrix

and secretion of hydrochloric acid and proteolytic enzyme cathepsin K (Gravallese *et al*., 2000; Boyce & Xing, 2007). A recent paper by Jia *et al*., (2018) described the effects of mCRP within an RA mouse model, with the monomeric, but not pentameric conformation, actively modulating osteoclast differentiation through NF-κB and phospholipase C signaling. Interestingly mCRP can bind RANKL, the major driver of osteoclast differentiation, and prevent its activities. The binding and inhibition of RANKL are mediated by the cholesterol binding sequence of mCRP (Jia *et al*., 2018).

A further component of RA pathogenesis is cartilage degradation. Articular cartilage is composed of the extracellular matrix containing type II collagen, aggrecans and proteoglycans. The proinflammatory cytokines within the inflamed joint stimulate synovial fibroblasts to release an enzyme capable of degrading the cartilage called matrix metalloproteinase (MMPs) (Fazal *et al*., 2018). This results in degradation of cartilage and the eventual rubbing of bones together leading to bone polishing, osteophyte and bone spur formation (Ainola *et al*., 2005).

RA patients suffer comorbidity with diseases such as atherosclerosis and coronary heart disease, one factor attributed to the excess mortality observed in RA patients (Myasoedova *et al*., 2010). It has been suggested that the chronic inflammatory state found in RA contributes to not only an increase in cardiovascular mortality, but to an accelerated rate of atherosclerosis (Gabriel, 2008; Wu *et al*., 2013). An association between RA inflammation and atherosclerosis has been documented, with RA considered an important independent risk factor for its development. The progression of atherosclerosis is not only accelerated in RA sufferers but occurs prematurely, and is accompanied by increased cardiovascular mortality within the group (Montecucco & Mach, 2009; Yuri Gasparyan *et al*., 2010).

The importance of endothelial cell dysfunction and foam cell formation is identified in the progression of both RA and atherosclerosis. Foam cells are known to play a significant part in atherosclerotic plaque build-up and are also found within the synovial membrane of RA patients (Winyard *et al*., 1993). The study by Winyard *et al*. (1993) showed foam cell formation surrounding blood vessels within intimal connective tissue and in association with fibrin deposits, indicating the presence of oxLDL. These results were not found within control synovial membranes (Winyard *et al*., 1993).

It is suggested that endothelial cells allow entry of low-density lipoproteins (LDL) to the intima, via spaces between them, leading to the oxidization of LDL to oxLDL in RA patients. In turn oxLDL activates endothelial cells and leads to the up-regulation of adhesion molecules and release of chemokines, in turn recruiting leucocytes to the now forming atherosclerotic plaque. Circulating monocytes and macrophages attracted to the plaques engulf oxLDL and convert to foam cells (Montecucco and Mach, 2009). An increase in oxLDL levels together with a decrease in levels of high density lipoproteins (HDL), often considered to exert anti-inflammatory effects, has also been shown in RA patients (Montecucco and Mach, 2009).

Macrophages are an important group of cells mediating RA disease progression, with high numbers being found in both the synovial membrane and at the site of pannus formation (Mulherin *et al*., 1996; Kinne *et al*., 2000). Within atherosclerotic plaques, macrophage activation via Toll like receptors (TLR) results in the release of proinflammatory cytokines IL-1, IL-6, TNF and matrix metalloproteinase (MMP). IL-1 and Il-6 are both inducers of hepatocyte CRP expression (Lundberg and Hansson, 2010). Macrophages generated from U937 monocyte cell line not only express CRP but in its monomeric rather than pentameric form (Ciubotaru *et al*., 2005). Synovial fibroblasts mediated cartilage degradation when co-cultured with U937 macrophage cells in an IL-1, 6

and TNFα dependent manner in a study by Scott *et al*., *(*Scott *et al*., 1997), linking mCRP, IL-1, 6, TNFα and RA.

1.8.3 Etiological Aspects

The cause of RA is poorly understood, a combination of environmental and genetic factors may be involved. Interesting clues as to the etiology of the disease have been considered by Entezami *et al*. in their 2012 paper in which they consider various theories of 'Old World or New World' disease (Entezami *et al*., 2011). RA was less prevalent in previous centuries suggesting RA may be a relatively new disease within Europe and North Africa. Where similar diseases such as osteoarthritis, gout and ankylosing spondylitis have been found in archaeological skeletons, no evidence for RA has been found. However, Hippocrates described a form of arthritis with remarkably similar symptoms to RA, with similar observations found in writings by a range of other ancient scribes, including Caesar's physician Scribonius, Emperor Constantines IX's adviser and Greek physician Soranus, these are all anecdotal rather than confirmed RA cases (Entezami *et al*.2012). The first modern day description was by Frenchman Augustin Jacob Landre-Beavais in 1800, who examined clients with severe joint pain, which affected women more than men and poor rather than wealthy. Landre-Beavais suggested incorrectly that the disease was a relative of gout. In 1859 Alfred Garrod an English physician termed the disease 'Rheumatic Gout', with his son Archibald continuing his work giving the condition its name 'Rheumatoid Arthritis'. Archibald proposed it a disease of ancient origin due to his observations of ancient bones examined from graves around the world (Entezami *et al*. 2012).

Signs of RA have been found in ancient Native American skeletons dating over one thousand years ago, with some of the oldest paleopathological specimens displaying RA

found in the Americas (Halberg, 1991; Entezami *et al*., 2011). Native American-Indian populations have the highest recorded occurrence of RA, with a prevalence of 5.3% noted for the Pima Indians (Del Puente *et al*., 1989). This has led to further suggestions that RA may have crossed that Atlantic after Columbus returned from the Americas in 1492 (Entezami *et al*.,2012).

It has also been suggested that an environmental trigger may be responsible for RA development, with microbial pathogens such as bacteria and viruses including parvovirus B19, rubella and Epsein-Barr all suggested (Sawada & Takei, 2005; Entezami *et al*., 2011). However RA patients may just be more susceptible to disease and therefore these infections are not related to cause and are merely coincidence. Another potential environmental trigger proposed is smoking, which is linked to RA incidence in RA patients who have the HLA-DR SE alleles, which links to suggestions that RA was introduced to Europe upon Columbus' return, together with tobacco. However only a third of RA individuals carry the HLA-DR SE gene and not all smoke (Entezami *et al*., 2012). Nicotine has been implicated in the pathogenesis of RA (Mattey *et al*., 2002) and is also a known independent risk factor for the development of cardiovascular diseases including atherosclerosis (Mao *et al*., 2012). An interesting study linking nicotine and CRP investigated U937 cells and the effect of nicotine on CRP expression. This study showed a significant increase in CRP mRNA and protein expression 6 times that of the control following, 24 hours incubation with 10^{-5}M nicotine (Mao *et al*., 2012).

An association between RA and oral disease/periodontitis is also apparent, with similar prevalence across the population (5%) of each. The inflammatory cascade is regulated by TNF-α in both RA and periodontitis. *Porphyromonas gingivalis* is common within periodontitis and has also been identified in synovial fluid. In addition to this, *P. gingivalis* is able to citrullinate host peptides by proteolytic cleavage at Arg-X peptide

bonds by arginine gingipains. Therefore it may be able to induce autoimmune responses in RA through development of anticyclic citrullinated peptide antibodies (Persson, 2012) suggesting that infection may play a role in loss of self-tolerance to self-antigens in RA pathogenesis.

RA generally manifests between age 30 – 65 (Majithia *et al*., 2007) and the average life expectancy was only 30 years in 1800 when it was first described in France, suggesting people may well have died before the onset of the disease (Riley, 2005). It is likely that RA did exist in ancient times; however few people survived long enough for it to become a problem. There is a strong genetic link between RA sufferers and various RA associated genes including HLA-DRB1, PAD14, PTPN22, TNFAIP3, STAT4 and CCR6 with variable risk associations to RA and differences between disease development and progression within identical twins, suggesting an interaction between an environmental component and the regulation of these genes (Gerlag *et al*., 2016; Paul *et al*., 2017). Epigenetic studies have therefore become a key area of research.

1.8.4 Rheumatoid Arthritis Epidemiology

In socio-economic terms, RA is the most common and most important of the inflammatory rheumatic diseases, with a prevalence of 0.5-1% of the population worldwide, estimated to increase by ~22% between 2005 and 2025 due to the ageing population. The median age of onset of RA is 58 years, with the relatively high prevalence, irreversible joint damage and widespread occurrence of co-morbidities determining the huge societal impact of this disease (Gerlag *et al*., 2016). There are differences between different ethnic groups across the world with a high prevalence of RA reported in native American Indian populations, the Pima Indians (5.3%) and Chippewa Indians (6.8%), compared to low occurrences reported in populations from China and Japan (0.2-0.3%)

(Silman & Pearson, 2002). This data suggests a genetic role within the risk of disease, however studies show a reduced familial recurrence risk in RA when compared to other autoimmune diseases. The greatest genetic risk factor is presence of the HLA-DR gene variant in the major histocompatibility locus (MHC), with the strongest susceptibility factor found to be in HLA DRB1 alleles and HLA DRB1*0404 allele (Silman & Pearson, 2002). There is a second association with the tyrosine phosphatase PTPN22 gene, which is a gain of function polymorphism reducing T-cell activation response to antigens. The impact of this polymorphism is thought to be a reduced capacity of the thymocytes to negatively select self-antigens and is present in several autoimmune diseases (Bottini *et al*., 2006).

1.8.5 Rheumatoid Arthritis Classification Criteria

Whilst there is currently no cure for RA, early diagnosis and treatment are key to achieving a low disease activity state. A DAS score of less than 2.6 signifies remission or significantly reduced symptoms; 2.6-3.2, low disease; 3.2-5.1 shows moderate disease activity, and may indicate a change of therapy is required and above 5.1 shows severe disease. The level above 5.1 is the threshold for being considered for biologic treatment as per NICE guidelines. Seropositive patients, who have either RF or anti-CCP have a greater likelihood of developing more serious disease than those who are seronegative (nras.org). (https://www.nras.org.uk/data/files/Publications/DAS%20patient%20guide.pdf).

Table 1.5, Summary table of classification criteria for RA, a score of ≥6/10 confirms diagnosis of RA. Table adapted from Humphreys, Verstappen *et al*., 2012 and Nras.org

Diagnosis Test	Details	Score
Disease Activity Score (DAS)	28 joint assessed for number of swollen or tender	
	1 large joint	0
	2-10 large joints	1
	1-3 small joints (with or without involvement of large joints)	2
	4-10 small joints (with or without involvement of large joints)	3
	>10 joints (at least 1 small joint)	5
Serology	Negative RF/CPA	0
	Low positive RF/low positive ACPA	2
	High positive RF/ high positive ACPA	3
Acute Phase Reactants	Normal CRP & erythrocyte sedimentation rate (ESR)	0
	Abnormal CRP & erythrocyte sedimentation rate (ESR)	1
Duration of symptoms	< 6 weeks	0
	≥ 6 weeks	1

1.8.6 Clinical use of C-reactive Protein in Association to Rheumatoid Arthritis

Once a patient has been diagnoses as having RA, regular testing is important in order to ensure the disease is being managed appropriately, with CRP levels just one of the indicators used to assess inflammation and disease activity. Chronic inflammatory conditions such as RA are characterised by high plasma levels of CRP, especially during periods of active flare. In healthy individuals the median, baseline concentration of CRP is 0.8mg/l, which following inflammatory stimulus resulting from cellular or tissue injury may increase over 1000 fold, from levels below 50µg/l to over 500mg/l (Kresl *et al*., 1998; Koenig *et al*., 1999; Hirschfield & Pepys, 2003). These increases are rapid, with levels reaching excess of 5 mg/l after 6 hours, and peaking at 48hours. CRP is useful as not only is it elevated in RA patients (Yue *et al*., 1996; Rindfleisch & Muller, 2005; Rhodes *et al*., 2011), but can also be a predictive factor of future RA diagnosis (van Zeben & Breedveld, 1996). RA has long been considered an autoimmune disease, with the production of auto-antibodies such as rheumatoid factor (RF) strongly associated with it (Montecucco and

Mach, 2009, Peters *et al*., 2008). First described in 1964, Kunkel and Williams recorded the production of IgG auto-antibody involvement in RA, describing them as rheumatoid factors (Kunkel and Williams, 1964).

Studies show that CRP is involved within the activation of complement in RA, and levels were also found to correlate with disease activity in a study by Molenaar *et al*., (2001). In their study considering interactions between CRP and complement components in RA, plasma levels of C3d-CRP and C4d-CRP were elevated in the majority of patients assessed, with significantly elevated levels of CRP complexes in those patients with active RA, as opposed to those with inactive RA. Levels of C3d-CRP and C4d-CRP also correlated with DAS28 scores (Molenaar *et al*., 2001). A further study confirmed this finding when investigating the actions of Infliximab a key treatment for RA. Infliximab, decreased plasma CRP levels by blocking TNF, and was also found to reduce complement activity, with levels of plasma C3 and C4 reduced, as were CRP-complement complexes and CRP levels (Familian *et al*., 2005).

In addition to high levels of CRP within RA patients, several studies have shown that auto-antibodies to CRP occur in RA patient blood, although at lower levels to those found in SLE. Antibodies to CRP were detected in 23% of patients with rheumatoid arthritis in a study by (Rosenau & Schur, 2006). No correlation was found between CRP and anti-CRP levels, although it was suggested that these auto-antibodies could be directed against modified rather than pCRP. Although it is possible the antibodies increase antigenicity of CRP binding to apoptotic cells during inflammation, it is unknown if anti-CRP is significant in RA (Rosenau and Schur, 2006). The frequency of RA patients with high anti-mCRP auto-antibody levels was again reported in Minatani *et al*'s study (2001), this time with 29% of patients presenting with anti-CRP auto-antibodies. This group also

concluded that mCRP rather than pCRP was the main target of these antibodies (Minatani *et al*., 2001).

1.8.7 Cells of the Synovium in Rheumatoid Arthritis

RA is characterised by systemic and synovial tissue chronic inflammation, with bone and cartilage erosion and destruction. Several cells play key roles within this destructive pathway, including dendritic cells, synovial fibroblasts, osteoclast, osteoblast, macrophages and both T and B lymphocytes as shown in Table 1.6.

Table 1.6 Summary table of cells of the synovium of rheumatoid arthritis, with function and role in RA.

Cell	Function	Role in Rheumatoid Arthritis	Source
Dendritic cells	Major antigen presenting cells, important activators and regulators of the immune system. Prime naïve Tc and Th enhancing Tc cell cytotoxicity. Stimulate differentiation and maturation of T cells via Toll like receptor ligands. Remove autoreactive lymphocytes.	Dendritic cells can prime autoimmune responses in lymphoid organs and therefore enhance development of the pathology seen in RA. Take part in antigen processing and presentation within synovial tissue and fluid, releasing inflammatory mediators.	(Banchereau & Steinman, 1998; Lanzavecchia & Sallusto, 2000; Lutzky *et al*., 2007)
Synovial Fibroblasts (SF)	Composed of synovial macrophage and type B synoviocytes. 1-3 cell thick in healthy synovium.	Within RA synovium >3 cell thick. Feature of hyperplastic synovium, involved within infiltration and degradation of bone and cartilage. Bind and invade cartilage expressing adhesion molecules and matrix degrading pro-inflammatory mediators. Release vascular endothelial growth factor (VEGF), IL-8, IL-15, monocyte chemoattractant protein (MCP-2). Bone and cartilage degradation results from matrix metalloproteinases (MMPs) and cathepsins released by SF. Resistant to apoptosis.	(Firestein *et al*., 1995; Müller-Ladner *et al*., 1996; Pierer *et al*., 2004; Abad *et al*., 2006).

Endothelial Cells	Cell of vascular system lining interior surface of blood vessels and lymphatic vessels. Form interface between blood or lymph and the vessel wall, controls passage of leucocytes to and from bloodstream. Vasoconstriction / vasodilation and control of blood pressure	Phenotypic changes – apoptoic, leaky, angiogenic or activated in RA. Allow migration of leucocytes into joint tissue. Increased expression of adhesion molecules, cytokines, proteases. Proliferate as part of angiogenesis, bringing oxygen and nutrients to pannus. Leucocyte adhesion and migration via ICAMs.	(Butcher *et al*., 1999; Kulka *et al*., 1955, Schulze-Koops *et al*., 1995;Walsh *et al*., 1999, Webber *et al*., 2000)
Osteoclasts	Multinucleated cell with role in resorbing and remodelling bone. Work in partnership with osteoblast to maintain bone mass in adults. Osteoclasts may recruit osteoblasts to the site of bone remodeling through sphingosine 1-phosphate (S1P) and bone morphogenic protein 6 (BMP6) with bone formation stimulated through enhanced activation of Wnt (secreted glycoproteins) /BMP pathways	Pro-inflammatory cytokine signals modulate SIP, BMP6 and Wnt/BMP pathways.	(Haynes *et al*., 2001; Atkins *et al*., 2003; McInnes & Schett, 2007; Pederson *et al*., 2008; Rachner *et al*., 2011)
Osteoblast	Bone formation. Direct osteoclast differentiation through RANK, RANKL and osteoprotegrin (OPG). Balance with osteoclast carefully orchestrated in normal joints.	SIP, BMP6 and Wnt/BMP pathways modulated by pro-inflammatory cytokines affecting bone repair.	(Haynes *et al*., 2001; Atkins *et al*., 2003; McInnes & Schett, 2007; Pederson *et al*., 2008; Rachner *et al*., 2011)
Chondrocytes	Cell of the adult cartilage maintaining cartilaginous matrix (secreting mainly collagen and proteoglycan). .Maintain balance between degradation and synthesis of matrix components. Synthesise glycosaminoglycan. .	Cartilage destruction during interaction between cartilage and synovial pannus where SF attach and release proteinases capable of digesting cartilage matrix components. Elevated MMPs in synovial fluid continues cartilage degradation, MMP-1, MMP-10 and MMP-3 are produced by synovium and chondrocytes.	(Maroudas *et al*., 1998; Verzijl *et al*., 2000; Shikhman *et al*., 2004; Ainola *et al*., 2005; Li *et al*., 2006; Müller-Ladner *et al*., 2007)
Lymphocytes	White blood cell including natural killer cells (cell-mediated, cytotoxic innate	Aggregated T and B cells found close to synovial membrane due to cytokine	(Maroudas *et al*., 1998; Verzijl *et al*., 2000; Shikhman *et*

	immunity), T cells (cell-mediated, cytotoxic adaptive immunity), and B cells (humoral, antibody-driven adaptive immunity).	and chemokine release. B cells produce rheumatoid factor (RF) and other autoantibodies. ROS and hypoxic background in synovium supports T cells which can resist apoptosis and lose expression of CD25, CD28, CD27 and CD40L, but have enhanced expression of inflammatory cytokines including IFN-γ and TNF-α. Th17 cells mediate pannus growth, osteoclastgenesis and synovial neoangiogenesis within RA, producing IL-17, IL-21 and IL-22 and pannus growth. Imbalance of ratio between Treg and other T cells implicated in RA.	*al*., 2004; Ainola *et al*., 2005; Li *et al*., 2006; Mauri & Ehrenstein, 2007; Müller-Ladner *et al*., 2007; Pickens *et al*., 2010)

1.8.7.1 Dendritic Cells

Dendritic cells are major antigen presenting cells and are important activators and regulators of the immune system, orchestrating immune response by ingesting and presenting antigens to T cells (Banchereau and Steinman, 1998; Bell *et al*., 2017). These cells prime naïve Tc and Th and enhance Tc cell cytotoxicity, following antigenic uptake the dendritic cell migrates to the lymphoid organs and stimulates differentiation and maturation of T cells via the uptake of the antigen via Toll-like receptor ligands (Lanzavecchia & Sallusto, 2000; Liu, 2015; Schraml & Reis e Sousa, 2015). Dendritic cells also remove autoreactive lymphocytes and increase the regulatory T cell population (Lutzky *et al*., 2007), playing an important role in self-tolerance (Bell *et al*., 2017). Within RA, dendritic cells can prime autoimmune responses in lymphoid organs and therefore enhance development of the pathology seen in RA (Ludewig *et al*., 1998; Suwandi *et al*., 2015), as illustrated in Figure 1.11. They also take part in antigen processing and

presentation within synovial tissue and fluid, allowing them to take up and process antigens present within the area of inflammation, and releasing inflammatory mediators, therefore contributing to disease perpetuation (Ludewig *et al*., 1998). Dendritic cells mature in response to proinflammatory cytokines including IL-6, IL-1 and TNF-α as well as tissue factors such a hyaluronic fragments, heat shock proteins, heparin sulphate, the action of migration across endothelial cells within inflamed tissue and T cell derived signals (CD154) (Lutzky *et al*., 2007; Schraml & Reis e Sousa, 2015).

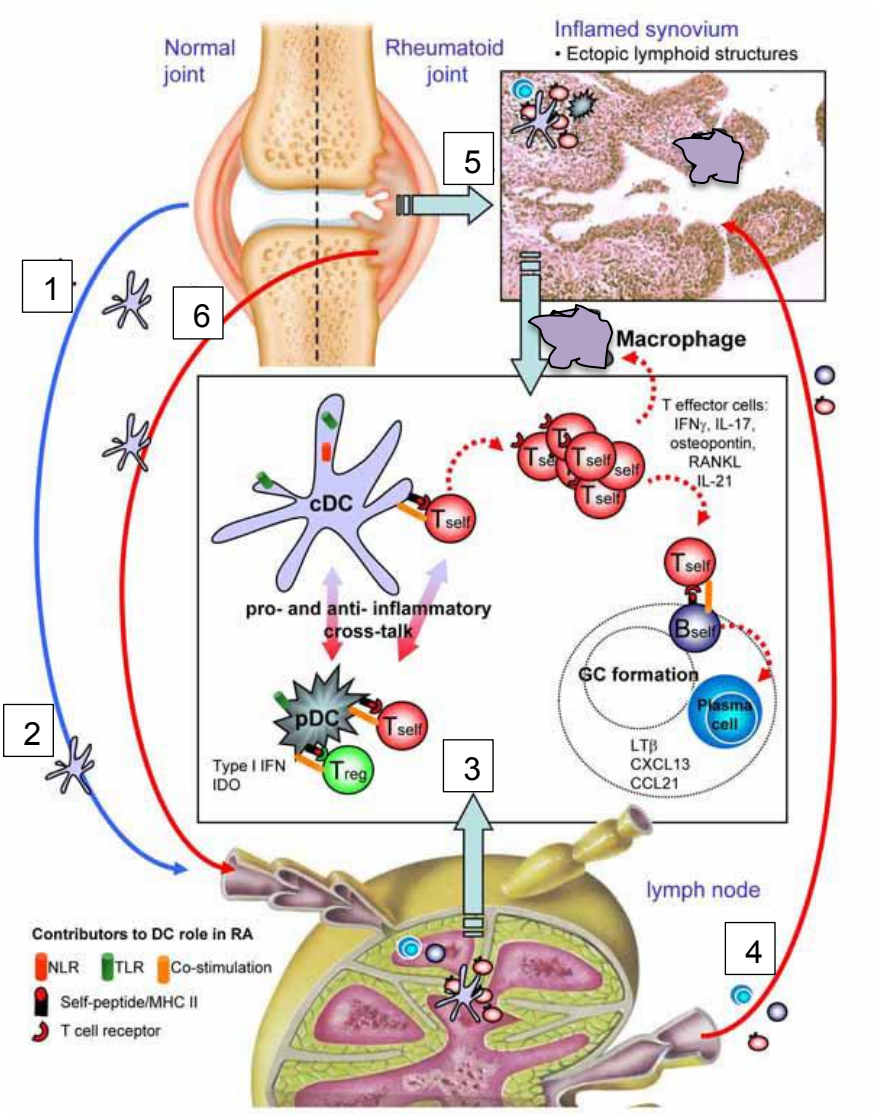


Figure 1-11 Diagram illustrating the role of dendritic cells within RA.(1) migration of conventional dendritic cells (cDCs) presenting antigens in the lymph nodes (LN), normally maintaining tolerance (2), however abnormal activation of cDCs through innate / pattern recognition receptors following mechanical damage or infection could lead to proliferation of autoreactive T cells (3). Presentation of self-antigens by plasmacytoid dendritic cells (pDCs) leads to two possible outcomes, inflammation through the production of type I interferon and the regulation of autoreactive T cell function via regulatory T cells / secretion of indoleamine 2,3-dioxygenase (IDO). Within the lymph node self-restricted B cells activity is supported enabling class switching and plasma cell differentiation, leading to high levels of self-antibodies. Self-restricted T effector cells recirculate into inflamed joints and tissue, encountering APCs (4). T cells re-activated by these DCs support macrophage activation, expression of costimulatory molecules i.e. RANKL leading to further joint pathology (5). The cycle is perpetuated with increased antigen released from the damaged joint within the lymph node (6), eventually allowing the progression of disease within the tissue. Adapted from Benson, (Benson *et al*., 2010).

1.8.7.2 Synovial Fibroblasts

A further class of cells found at the site of inflammation within RA, are synovial fibroblasts (SF). This group is composed of two cell types, the synovial macrophage and the type B synoviocytes, also termed fibroblast-like synoviocytes, which are derived from mesenchyme populations. SF cells are a feature of the hyperplastic synovium which is involved in the infiltration and degradation of adjacent bone and cartilage. The SF actively bind and invade cartilage expressing adhesion molecules and matrix degrading and proinflammatory mediators (Müller-Ladner *et al*., 1996). In the pre-inflammatory phase of RA it is thought that activation of the innate immune system leads to the upregulation of effector molecules in SFs, with infectious and non-infectious agents proposed to be potential triggers (Müller-Ladner *et al*., 2007). SFs are stimulated via TLRs which in turn release vascular endothelial growth factor (VEGF), IL-8 and IL-15. Interestingly, cytokines IL-1 and TNF-α enhance expression of TLR2 in SFs, perpetuating the inflammatory cycle (Pierer *et al*., 2004).

1.8.7.3 Endothelial Cells

Endothelial cells are also active participants within RA and undergo phenotypic changes characterised as apoptotic, leaky, angiogenic or activated. During leucocyte migration within the inflammatory sites, selectins mediate leukocyte tethering and rolling, resulting in leucocyte adhesion via integrins and ligands on endothelial cells, such as ICAMs (Butcher *et al*., 1999). Anti-ICAM-1 treatment in RA patients results in a reduction of disease activity and blocks trafficking of T cells, suggesting great functional importance of adhesion molecules in human RA (Schulze-Koops *et al*., 1995). Infliximab is an anti-TNF treatment which decreases E-selectin and ICAM-1 levels, suggesting their reduction may reduce migration of leukocytes into RA joints (Van Schouwenburg *et al*., 2013).

1.8.7.4 Osteoclasts and Osteoblasts

Osteoclasts are multinucleated cells of hematopoietic origin and have a primary role in resorbing cells and remodeling bone. These are giant cells which are actually a fusion product of up to 20 single cells and enable shaping of bones and remodeling of the skeleton from birth to old age (Schett, 2007). Bone mass in adults is maintained through a close partnership between osteoclast and osteoblasts, mediated through a variety of hormones, cytokines, growth factors and matrix proteins. Bone resorption by osteoclasts requires a signal from either osteoblast or from cells lining the bone itself, with osteoblasts directing osteoclast differentiation through pathways including RANK, RANKL and osteoprotegerin (OPG) (Khosla, 2001). In RA, proinflammatory cytokine signals can modulate this pathway and may adjust the process in which osteoblasts repair bone. OPG is a soluble decoy receptor for RANKL, which blocks the pro-osteoclastogenic activity of RANKL. In RA synovial tissue there is an increase in the ratio of RANKL:OPG suggesting that pro-osteoclastogenic condition dominate the inflammatory environment of the RA joint (Haynes *et al*., 2001). As osteoblasts mature their expression of RANKL reduces and is replaced with an increase in their expression of OPG, creating an environment that supports bone formation (Atkins *et al*., 2003). It is clear that the balance of osteoblast and osteoclast is carefully orchestrated within normal joints and that this balance is corrupted within RA through alterations to the complex signalling pathways involved together with the proinflammatory microenvironment of the RA joint.

1.8.7.5 Chondrocytes

Adult cartilage is populated solely with a specialised cell called a chondrocyte, from the Greek chondros – cartilage and kytos – cell. These cells maintain the cartilaginous matrix which is composed of a combination of collagen and proteoglycans and are derived

from mesenchymal stem cells. The collagen network is made up of types II, IX and XI collagens, providing tensile strength and aids retention of proteoglycans (Verzijl *et al*., 2000) Compressive resistance is provided by the aggregating proteoglycan aggrecan, which attaches to hyaluronic acid polymers. Chondrocytes maintain a balance between the degradation and synthesis of matrix components, with a half-life in excess of 100 years for type II collagen (Verzijl *et al*., 2000), and three to 24 years for aggrecan (Maroudas *et al*., 1998). They are surrounded by a type VI collagen microfibril cellular matrix, which maintains chondrocyte membrane attachment via interactions with hyaluronic acid, bi-glycan and decorin (Verzijl *et al*., 2000).

Glycosaminoglycan, a component of aggrecan, together with other cartilage matrix components are synthesised by chondrocytes during periods of low turnover. Glucose is the main energy supply for chondrocytes and is also a precursor for glycosaminoglycan, with glucose transport facilitated by the GLUT family of transport proteins (GLUT3, 8, 1 and 6) (Shikhman *et al*., 2004). Cartilage destruction occurs in areas of interaction between the cartilage and the synovial pannus where evidence shows attachment of synovial fibroblasts, which can release proteinases capable of digesting the cartilage matrix components (Edwards, 2000). Degraded cartilage matrix components can be considered potential autoantigens within RA synovial inflammation.

1.8.7.6 Lymphocytes

Within RA inflammatory sites, ectopical germinal centre like structures can be seen, formed from aggregates of B and T cells and found in close proximity to the synovial membrane. It is likely that this gathering of lymphocytes is due to the variety of immune cells present within the synovium, including dendritic cells, and fibroblast like synoviocytes. These cells release factors such as B cell activating factor of the TNF family (BAFF) and chemokine ligands CXCL12 and CXCL13, leading to the organisation,

trafficking and survival of B cells (Tan *et al*., 2003). B cells support an autoimmune response following interaction with T cells, processing and presenting antigens via major histocompatibility complex class II and further producing cytokines. Genetic susceptibility to RA is associated to DRB1 genes encoding HLA-DR4 and HLA-DR1 (Newton *et al*., 2004).

The predominant population of antigen presenting cells in later immune responses are the B cell, although dendritic cells also play an important role in priming naïve T cells. Interestingly, B cells producing rheumatoid factor (RF), those that bind the complexes via membrane immunoglobulins, are effective in presenting immune complexes to T cells, irrespective of the antigen within an antigen-antibody complex (Roosnek *et al*., 1991) suggesting any antigen within a complex could be presented to and activate a T cell. Antibody secreting B cells may also produce auto-antibodies which form immune complexes therefore creating further activation of the immune system. T cells are assisted by mature B cells which also activate differentiation of effector T cells. Both T cells and B cells are capable of producing proinflammatory cytokines, with a subset of B cells secreting IL-10 a regulatory cytokine, capable of downregulating the immune response (Mauri and Ehrenstein, 2007).

T cells found within the synovium express a variety of cell surface antigens indicating prior antigen experience, including markers of effector memory cells such as expression of chemokine receptors and integrins and markers confirming differentiation, migratory experience and effector function amongst others (Cope, 2008). Pro-inflammatory Th17 cells produce IL-17, IL-21 and IL-22, with the process of conversion from naïve T cell to Th17 requiring the presence of IL-6, TGF-β, IL-21, IL-1 and IL-23 (Sheu *et al*., 2010). IL-17 binding to target cells leads to release of proinflammatory

mediators including cytokines, chemokines and MMPs, supporting pannus growth, synovial neoangiogenesis and osteoclastogenesis (Pickens *et al*., 2010).

1.8.8 Cytokine Interactions within Rheumatoid Arthritis

Cytokines are a diverse group of proteins including lymphokines, interleukins, monokines, colony stimulating factors, and chemokines and act through heterodimeric or heterotrimeric receptors. Imbalances within the cytokine network may lead to inflammation. In early RA synovial T_H cells express IL-2, IL-4, IL-13, IL-17, and IL-15, this changes as RA progresses with established RA synovial T_H cells expressing IFNγ, TNF and IL-10 with little IL-2, IL-4, IL-5 and IL-13 (Cope, 2008). The synovial tissue within inflamed RA joints, is infiltrated with activated macrophages and leukocytes and is rich in pro-inflammatory cytokines such as TNFα, IL-6, IL-1 and IL-17 together with growth factors such as M-CSF, capable of impacting bone remodeling within the RA microenvironment (Lorenzo *et al*., 2008). IL-1 and TNF-α are perhaps the main pro-inflammatory cytokines involved in RA, with TNFα mediating the inflammatory response, promoting the activation of synovial fibroblast whilst IL-1 is involved with cartilage and bone destruction (Vervoordeldonk & Tak, 2002), as seen in Figure 1.12. Neutralisation of TNF-α, decreases the production of IL-1, IL-6 and Granulocyte-macrophage colony-stimulating factor (GM-CSF) (Vervoordeldonk & Tak, 2002). TNF-α is a target of many RA biological drugs as illustrated in Table 1.7.

Table 1.7 Summary table of cytokines involved within RA pathogenesis, their function, originating cell and drug targeted by.

Cytokine	Function in Disease / Secreted by	Cytokine Inhibiting Drugs	Source
IL-1 (IL-1α,IL-1β)	Co-mitogen for T lymphocytes. Stimulate liver inducing transcription of acute phase proteins (APP). Leads to increase expression of inflammatory genes. Secreted by monocytes, macrophages, B cells, dendritic cells, endothelial cells.	Anakinra	Tracey and Cerami, 1994; Brennan and McInnes, 2008; Dinarello, 2009; Gabby and Kushner, 1999; Baumann and Gauldie, 1994; Siebert *et al*., 2015
IL-6	Growth factor for B cells. Stimulate liver inducing transcription of APP. Induces antibody production within B cells. Downregulation of TNF-α, IL-1. Secreted by monocytes, macrophages, T_H2 cells, bone marrow cells.	Tocilizumab	Tracey and Cerami, 1994; Brennan and McInnes, 2008; Dinarello, 2009; Gabby and Kushner, 1999; Baumann and Gauldie, 1994; Siebert *et al*., 2015
TNF-α	Propagates T cell response via T helper cell type 1 (Th1). Stimulate liver inducing transcription of APP. Pro-inflammatory, induces cytokine production, cell proliferation and apoptosis. Secreted by macrophages, NK cells, $CD4^+$ lymphocytes, adipocytes, basophils, eosinophils, dendritic cells.	Infliximab, adalimumab, golimumab, certolizumab and etanercept	Tracey and Cerami, 1994; Brennan and McInnes, 2008; Dinarello, 2009; Gabby and Kushner, 1999; Baumann and Gauldie, 1994; Linlin Chen, Huidan Deng, Hengmin Cui, Jing Fang, 2017; Siebert *et al*., 2015
IL-7	Pro-inflammatory, role within synovium reducing B cell apoptosis, increasing antibody production. Induces differentiation into progenitor B and T cells. Secreted by bone marrow and		Lubberts, Koenders and van den Berg, 2005

	thymic stromal cells.		
IL-10	Anti-inflammatory. Inhibits gene expression of T cell and macrophage pro-inflammatory cytokines, reducing function of antigen presenting cells. Secreted by T_H2 cells, T cells, B cells		Linlin Chen, Huidan Deng, Hengmin Cui, Jing Fang, 2017; de Wall Malefyt, 1991; Petrovic-Rackov and Pejnovic, 2006
IL-15	Stimulates intestinal epithelium growth, T cell proliferation, co-mitogen for B cell activation and proliferation. Promotion of CD8 T cell survival, osteoclastogenesis and bone destruction. Secreted by T cells and mononuclear phagocytes.		Joseph Lorenzo, 2016; S Saeed and PA Revell, 2001; Guangwei Cui et al, 2014
IL-17	Involved within stimulation of iNOS and chemokine synthesis, inducer of osteoclasts genesis and RANKL expression. Secreted by T cells, including Th17	Secukinumab (Phase III trial), several other Phase I & II trials ongoing	Kotake et al., 1999; Brennan McInnes, 2008; Duvallet et al 2011; Wei Jin and Chen Doud, 2013; Siebert *et al*., 2015

The ratio of Th1, Th17 and Treg lymphocytes is altered in the peripheral blood of RA patients (Pawłowska *et al*., 2014), which can be assumed creates an imbalance of cytokines released. Th1 cytokines favour T-cell mediated cellular immunity, cytotoxicity and monocyte activation, which leads to amplified production of pro-inflammatory cytokines IL-2 and IFNγ (van den Berg & Miossec, 2009). The Th2 cytokines, IL-4, IL-5, IL-6, IL-10, and IL-13 are involved within the stimulation of antibody mediated immune responses and Th17 cytokines are involved in the elimination of fungal and extracellular pathogens (Chizzolini *et al*., 2009). Macrophages produce proinflammatory TNFα, IL-6, IL-1,IL-15, IL-23, IL-18 and IL-12; T cells produce anti-inflammatory IL-4, IL-10, TGFβ, and IL-6 which has both pro and anti-inflammatory properties, protective IL-22 and pro

inflammatory IL-21, IL-17 and IFNγ; with fibroblast producing proinflammatory IL-18, IL-6, IL-8 and IL-15 (Brzustewicz & Bryl, 2015). Cytokines are a key target of RA drugs with many (discussed in section 1.9.10), designed to knock out aspects of the proinflammatory pathways by blocking these signal molecules.

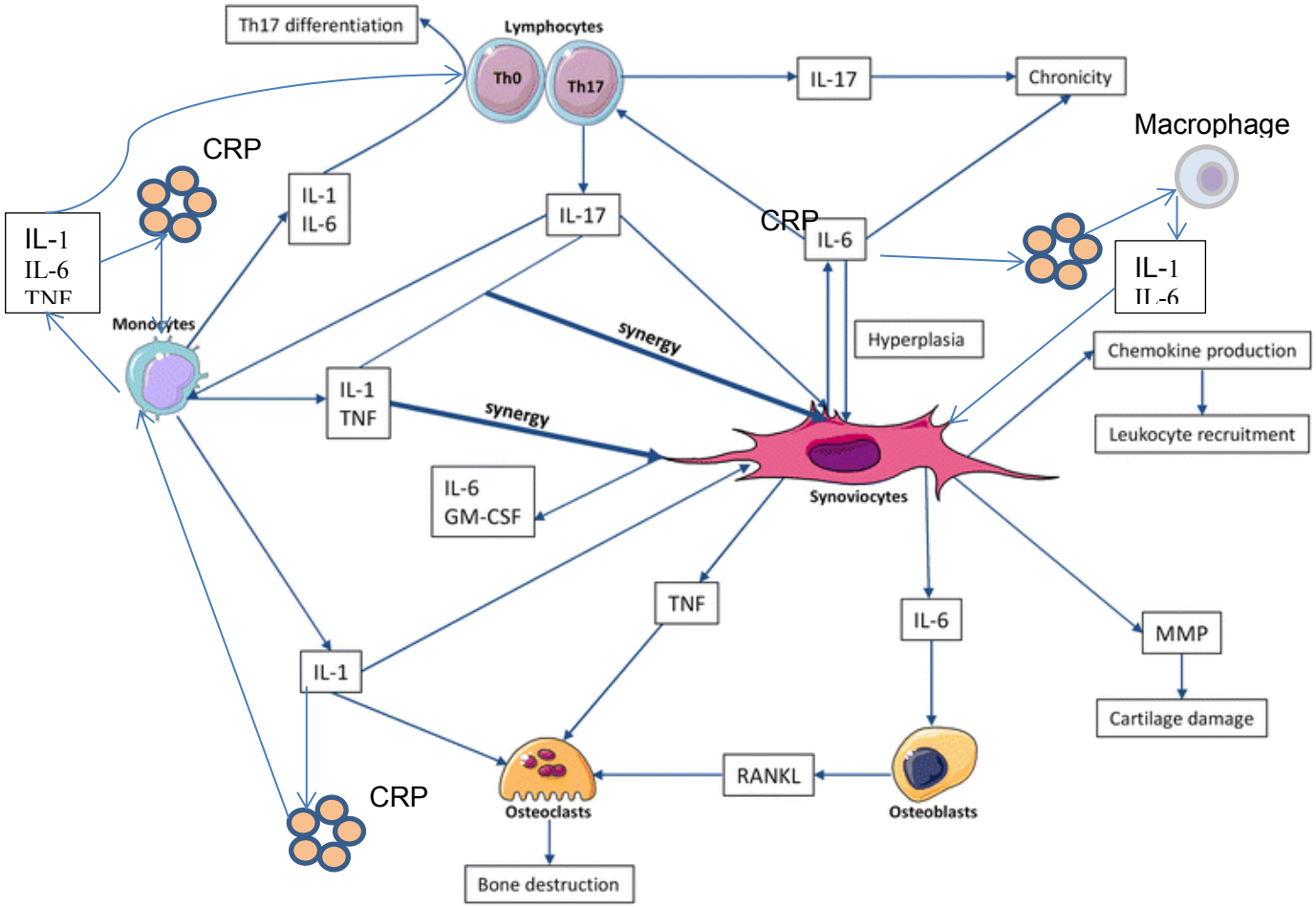


Figure 1-12 Diagram illustrating cytokines pathways involved within RA and interactions with CRP. Monocytic cells secrete IL-1 and IL-6 upregulating CRP expression within hepatocytes; in turn CRP binding activates further macrophage/monocyte expression of IL-1 and IL-6. This inflammatory cascade enhances TH17 differentiation/release of IL-17 leading to chronicity. Synoviocyte expression of Il-6 upregulates CRP, again leading to further IL-1 and IL-6 expression and cyclical pro- inflammation cytokine production (Sproston and Ashworth 2018; Noack and Miossec). Figure adapted from Noack and Miossec, 2017

1.8.9 Auto-antibodies within Rheumatoid Arthritis

RA has long been considered an autoimmune disease, with the production of auto-antibodies such as rheumatoid factor (RF) strongly associated with it (Montecucco and Mach, 2009, Peters *et al*., 2008). First observed in 1964, Kunkel and Williams described the production of IgG auto-antibody involvement in RA, describing them as rheumatoid factors (Kunkel and Williams, 1964). RF consists of between 70–80% of IgM-class auto-antibodies against the Fc (fragment crystallisable) region of IgG, which then binds to IgG within RA joints, leading to immune complex deposition (Haque *et al*., 2014). The level of RF in serum has been correlated to disease severity, with seropositive RA patients suffering more aggressive disease with a worse prognosis, with the presence of RF detected several years before arthritis begins (van Zeben & Breedveld, 1996). Auto-antibodies have also been associated with increased risk of atherosclerosis, Peters *et al*, (2008) showing oxLDL auto-antibody association with cardiovascular disease in RA (Peters *et al*., 2008). It is possible that auto-antibodies produced during disease progression in RA patients, could also lead to an increase in atherosclerosis (Montecucco and Mach, 2009).

Antibodies to citrullinated protein antigens (ACPAs) or anti-cyclic citrullinated peptide (anti-CCPs) are highly specific and naturally occurring antibodies, recognising citrullinated proteins and peptides and are found in 98% of RA patients (Weyand & Goronzy, 2006). Citrullinated proteins arise from the post translational modification of arginine to citrulline by the enzyme peptidyl arginine deaminase (PAD) (Kim, 2013). By decreasing the net positive charge, citrullination can alter the structure of proteins, potentially enabling adjusted intermolecular interactions (Feist & Burmester, 2006; Egerer *et al*., 2009). Citrullination is a normally occurring process, however overproduction can

lead to inflammation induced by formation of immunocomplexes and proteins undergoing post translational citrullination such as fibrin, a cleavage product of fibinogen, may be recognised as an antigen (Weyand and Goronzy, 2006). Citrullinated proteins bind to the HLA-DBR1 molecule, leading Th cell conversion to Th17 cells (H.R. Kim, 2013), with the ratio of Th1, Th17 and Treg lymphocytes altered in the peripheral blood of RA patients.

In addition to anti-CCP and RF, anti-carbamylated protein (anti-CarP), anti-collagen type II (anti-CII) (Gerlag *et al*., 2016) and anti-acetylated protein antibodies (Juarez *et al*., 2015), have been found within RA serum, with the level of anti-CarP antibodies significantly increased in RA patients compared with healthy controls. Carbamlyation is a non-enzymatic post translational modification binding cyanate to molecules containing a primary thiol or amine group, forming carbamyl groups (Shi *et al*., 2013). Anti-CII is associated with elevated CRP, erythrocyte sedimentation rate (ESR), DAS28 and DAS28CRP at diagnosis and up to six months after and predicts a favorable outcome as opposed to patients seropositive for anti-CCPs (Manivel *et al*., 2017). Acetylation is a reversible enzymatic process where acetyl groups are added to free amines of lysine residues. Juarez *et al*., *(*2015) observed both IgG and IgA auto-antibodies directed against acetylated vimentin in RA patient serum, together with both IgG and IgA antibodies against citrullinated and carbamylated vimentin (Juarez *et al*.*;* 2015). Both RF and anti-CCPs are used within clinical settings to determine RA and the presence of both predicts the development of RA in patients with early arthritis (Rantapaa-Dahlqvist *et al*., 2003). It is clear that auto-antibodies of IgG, IgM and IgA classes are present within RA serum directed against a variety of proteins, many of which are the result of post translational changes.

1.8.10 Therapeutic Treatments for Rheumatoid Arthritis

Whilst there is no cure for RA, several therapies and modern advances in treatments are making the disease more manageable. There are a variety of different treatment options available to a clinician when attempting to contain the initial effects of RA. The first 12 weeks after early symptoms occur is regarded as an optimal treatment period and the best outcomes are attained when diagnosis is early and treatment is aggressive, preventing the onset of bone loss, joint destruction and pain. Generally RA treatments reduce inflammation by either blocking immune mediators or by modulating immune cell function or numbers (Pisetsky, 2017). Disease Modifying Anti-Rheumatic Drugs (DMARDs), reduce the progression of damage and can either be synthetic, small molecules or biologics. Conventional synthetic DMARDs have been the foundation of RA treatment for many years with methotrexate (MTX) being the most commonly prescribed. MTX is a form of folate which has an increased binding affinity for dihydrofolate reductase (DHFR) compared to its parent molecule (Guo *et al*., 2018). The actions of MTX include folate antagonism, adenosine signaling, and downregulation of adhesion molecule expression, eicosanoids and MMPs. Another synthetic DMARD, Sulfasalazine, is an anti-inflammatory and immunosuppressant drug which is thought to suppress leukotriene production and chemotaxis, IL-1, IL-6, TNFα and NF-kB activation. Chloroquine/hydroxychloroquine, also a synthetic DMARD, has immunomodulatory effects, interacting with Toll-like receptors, monocyte-derived pro-inflammatory cytokines, T-cell responses, neutrophils and cartilage metabolism and degradation. All three have side effects ranging from pulmonary damage to gastrointestinal tract, skin, central nervous system and hematologic adverse effects (Guo *et al*., 2018).

Over the last decade several new drugs have been brought to market that work in a different way to the traditional DMARDs, among these are the group known as biologics,

or biological DMARDs. Biologics include monoclonal antibodies e.g. infliximab or soluble receptors such as etanercept (Pisetsky, 2017). The different treatments available within each group are shown in Table 1.8. This group can be divided into classes dependent upon their actions, with adalimumab, etanercept, infliximab, golimumab and certolizumab all inhibiting TNFα, therefore reducing phagocytosis and pro-inflammatory cytokines, chemoattractant, adhesion molecules, Treg cell function and the function of osteoclasts, leucocytes, endothelial and synovial fibroblasts.

Table 1.8 Disease Modifying Anti-Rheumatic Drugs (DMARDs) classes, information taken from from Pisetsky 2017.

Classes of DMARD	
Conventional synthetic	Methotrexate, sulfasalazine, hydroxychloroquine, leflunomide, azathioprine
Biologic	TNF blockers (adalimumab, etanercept, infliximab, golimumab, certolizumab); T cell co-stimulatory blocker (abatacept); anti-IL-6 receptor (tocilizumab; anti-CD-20 (rituximab)
Targeted synthetic	Tofacitinib

B-cell targeting drugs include rituximab which depletes B cells with potential mechanisms including complement mediated cell lysis, antigen presentation, B cell apoptosis and Fc receptor gamma-mediated antibody-dependent cytotoxicity and phagocytosis. Another group target T cells, such as abatacept which interacts with CD28/CTLA4 signaling pathways (Guo *et al*., 2018). This group also produces side effects within patients, which without perhaps unsurprisingly include a greater risk to infection, together with increases in serum cholesterol, neutropenia, malignancy, hypertension, and abnormal liver function (Guo *et al*., 2018). The last group are small molecule DMARDs

which exploit the use of Janus Kinase (JAK) and signal transducer and activator of transcription (STAT) pathways by cytokines. The small molecules such as Jakinibs are able to therapeutically block these pathways, proving effective within disease treatment. Tofacitinib is a small molecule DMARD which targets signal transduction pathways preferentially inhibiting JAK-3 and JAK-1 over JAK-2 (Venkatesha *et al*., 2015).

Early aggressive therapy is designed to reduce inflammation and prevent damage, methotrexate is frequently the initial drug used and can when necessary be combined with glucocorticoids, however these present many patients with significant toxicity and side effects (Durez *et al*., 2007). An alternative therapy uses TNF blockers in association with methotrexate, resulting in a rapid reduction in inflammation and pain, however this combination is expensive and therefore reserved for patients who cannot be treated alternatively (Jalal *et al*., 2016).

While biologic treatments tend to be more effective, they are also expensive. Triple therapy, the combination of conventional DMARDs for instance hydroxychloroquine and sulfasalazine in addition to methotrexate, with glucocorticoids when needed is also found to be effective, and less expensive. However once again the side effects associated with glucocorticoids must be a consideration (Jalal *et al*., 2016). CRP levels are reduced in patients who take traditional DMARDs such as sulfasalazine and or methotrexate.(Stenger, 1998; Aletaha & Smolen, 2002).

1.9 Research Aims

This pilot study was designed to investigate two separate aims.

(i) The first was to clarify whether monomeric CRP was present within RA patient serum and if levels could be quantified.

a. This will involve the production of *in vitro* produced mCRP in addition to the analysis of patient serum samples to determine presence of the *in vivo* form. Varying methods of dissociation are to be used in order to dissociate the pentameric human CRP molecule into its individual protomers. This will be purified from the pentamer using size exclusion chromatography in order to prepare high levels of concentrated stock solution suitable for immunological studies.

b. It is also necessary to assess the stability of the monomer and likelihood of re-association within the stock solution during storage. The presence of monomeric CRP within serum may have important implications for both clinical practice and within research. It is important to understand whether pro or anti-inflammatory functions of CRP have been confused due to the conformation detected, and how the presence of two forms of the protein, with possibly opposing roles within the immune response may affect the patient and their treatment.

(ii) Further aims of the research were to investigate the existence of antibodies directed against both monomeric and pentameric CRP, of classes IgG, IgA and IgM. This will greatly enhance our understanding of not only RA but inflammatory diseases and the role that CRP isoforms play within it.

Our research hypothesis was that mCRP would be found within RA serum and would correlate in levels to that of any pCRP quantified as a result, potentially, of pentamer dissociation. It was further hypothesized that auto-antibodies to mCRP would be found in class IgG but not in IgM or IgA, and that auto-antibodies to pCRP will not be found, the hypothesis is based on previous research which has not found either IgM or IgA autoantiboides against mCRP or any autoantibodies directed against pCRP.

Chapter 2 - Materials and Methods

2.1 Overview

Native, pentameric C-reactive protein (pCRP) is stable within normal physiological conditions. However evidence suggests that pCRP can in certain circumstances dissociate into separate protomers, termed monomers. This isoform of CRP has been described as monomeric CRP and therefore will be referred to as mCRP from here onwards. Whilst pCRP is freely soluble, mCRP displays reduced solubility and a change in isoelectric point suggesting a change to the protein structure. The biological relevance of mCRP is unclear, however it is suggested to play a proinflammatory role within the human body (Ciubotaru *et al*., 2005; S.-R. Ji *et al*., 2006; Eisenhardt, Habersberger, Murphy, *et al*., 2009; Thiele *et al*., 2015; Jia *et al*., 2018).

RA is an autoimmune condition which resembles SLE in some aspects, but differs in levels of CRP and in anti-CRP auto-antibodies present. SLE presents with relatively normal levels of CRP, but with levels of anti-mCRP IgGs correlating to disease activity. RA is characterised by high levels of circulating pCRP and although auto-antibodies to citrullinated proteins and peptides together with RFs are present within seropositive serum, few studies have investigated anti-CRP auto-antibodies. Of those that have, none have sought to determine either IgA or IgM class antibodies directed against mCRP or pCRP. Therefore ELISA protocols were developed in order to determine the presence of mCRP and associated CRP auto-antibodies.

This chapter will describe the methodology and techniques developed in order to produce *in vitro* mCRP and to enable detection of human serum mCRP and associated mCRP and pCRP IgG, IgA and IgM auto-antibodies. It will then provide an overview of

the materials and methods used within each experiment. Production of mCRP experimental design is based upon existing literature which was further optimised within our research lab as detailed within chapter 3, with an aim to develop a methodology enabling generation of reproducible mCRP *in vitro* within conditions as close to physiologically practical as possible. Enzyme linked immunosorbent assay (ELISA) protocols were developed in order to overcome interactions between patient serum and constituent reagents, ensuring stability and accurate determination of proteins, the development of which are further detailed within chapters 4.

2.2 Techniques

The production of monomeric C-reactive protein was first described by Potempa *et al*., in 1983 wherein they described the methods employed in order to dissociate the pentameric protein into separate monomers. The process described included heat treatment and the alteration of pH to acidic conditions or dissociation through high concentrations of Urea (up to 8M) (Potempa *et al*., 1983). The following techniques were used following dissociation trials within our study to determine the level of dissociation and size of the resulting subunits, together with their stability over time and likelihood of re-association. Also described are the ELISA and western blot techniques adapted in order to detect CRP and antibodies within human serum samples.

2.2.1 Gel Filtration Size Exclusion Chromatography

Gel filtration size exclusion chromatography (SEC) is a simple method allowing the separation and purification of biological macromolecules such as proteins, polysaccharides and nucleic acids by size. Size exclusion chromatography is a generic name given to liquid chromatography separation and can be used to represent any of the following techniques, gel permeation chromatography, gel filtration chromatography, steric exclusion chromatography, exclusion chromatography or gel chromatography. Using SEC a sample can be processed and successfully purified within a variety of conditions and works on a simple basis. A column of cross-linked copolymers often of either styrene or divinylbenzene is prepared with a narrow range of pore size (Wilson & Walker, 2010). Buffer systems can be adapted to allow buffer exchange and the process can be undertaken at a range of accurately controlled temperatures or pH, and can take place in the presence of harsh chemicals including urea allowing sample purification. The system relies on the ability to pass a sample through a gel filtration medium which is closely packed within a

column. The medium consists of chemically inert and physically stable spheres such as Sephadex, formed from dextran beads which are packed together forming a cross linked porous matrix and can be formed from a variety of components chosen to meet specific pore size requirements. The liquid within the matrix is known as the stationary phase and is equilibrium with the liquid outside of the matrix known as the mobile phase. The various types of filtration medium are dependent on the sample components and separation desired. Large analytes are unable to enter the pores and therefore pass through the interstitial space between the particles and are eluted first. Smaller analytes are suspended within the buffer (mobile phase) and move through the particles and are slowed, eluting at a rate which correlates to their size. Elution volumes are determined relative to their molecular mass and as such a calibration of any system should be completed prior to use (Wilson & Walker, 2010). Calibration graphs, constructed from the elution volumes of known standard weight molecular proteins can be used to determine the molecular weight of unknown proteins within samples, based on their elution volumes (Amersham & Amersham, 2003).

A sample of between 0.1-2% of the column volume is applied onto the column via a syringe and once applied both the sample and buffer begin to migrate through the system and column. The flow rate is optimised allowing increased resolution of the resulting elution profile whilst conserving post purification protein yields. The buffer continuously passes through the system and column with larger molecules being eluted off first into fraction collection tubes. Whilst samples are eluted off the ultra-violet absorbance is measured allowing an elution profile to be collected. The applied protein will be completely eluted over one column volume of buffer passing through the column (Amersham & Amersham, 2003).

The flowrate for applying a sample must be optimised and must not exceed 1.6 ml/minute for the column used within these experiments, although the flow rate with

different columns may be faster or slower than this. Between usage columns are stored in 20% ethanol to prevent bacterial growth. Prior to use the column is flushed through with one column volume of degassed and deionised water at the maximum column flowrate (1.6 ml/min) in order to remove all ethanol. This is followed by one column volume of degassed buffer, used to equilibrate the system prior to use. Successful equilibration of the system can be monitored via observation of the UV absorbance reading, ensuring it is at baseline before use (Amersham & Amersham, 2003).

It is advised not to run samples such as blood or serum through the system due to their composition which may block the size exclusion column, reducing the accuracy of sample determination and necessitating rigorous cleaning between samples (Amersham & Amersham, 2003). It is also essential that contaminants such as dust, particulate matter or precipitation of salts are not introduced to the system by filtering all samples and buffers in a 0.2µm filter as it is easier to avoid contamination than remove it from the system. Degassing is another important step as air within the system and mobile phase may affect the pumps and detector (Amersham & Amersham, 2003).

2.2.2 Concentration of C-reactive Protein Samples

Eluted samples were concentrated using a Vivaspin Centrifugal Concentrator, with a 10kDa molecular cut off, purchased from Fisher Scientific. The centrifugal concentrator was first primed with deionised water and spun at 1,000 x g at 4^{o}C for 10 minutes. Following the priming step the protein sample was then spun at 1,000 x g at 4^{o}C for 15 minutes or until the required concentration had been achieved.

2.2.3 Polyacrylamide Gel Electrophoresis

Gel electrophoresis is a relatively simple and rapid technique which allows identification of sample contents. The technique involves the separation of bio

macromolecules according to their electrophoretic mobility through a gel under an electric field. Polyacrylamide gels are used within protein separation whereas agarose is used within deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) separation. Acrylamide is polymerised to polyacrylamide in the presence of bis-acrylamide (N,N'-Methylenebisacrylamide) which is used as a cross linking agent (Wilson & Walker, 2010). Ammonium persulfate (APS), in combination with tetramethylethylenediamine (TEMED) provide free radicals which instigate polymerization between bis-acrylamide and acrylamide monomers. Dissolving APS in water allows formation of free radicals and TEMED acts as a catalyst accelerating the polymerisation reaction due to its ability to carry electrons (Hames and Rickwood, 1998). Bis-acrylamide subunits cross-link with elongated acrylamide polymer chains and form a matrix of acrylamide chains.

The solidity/fluidity of the gel is determined by the ratio of acrylamide to bis-acrylamide and the matrix pore size governed by the amount of acrylamide used within the gel and degree of cross-linkage (Hames and Rickwood, 1998). The polyacrylamide forms a cross linking polymer and is described in terms of total percentage of acrylamide or *bis*-acrylamide present, with higher percentages used to refine smaller sized proteins and lower percentages, larger proteins. The suggested range for acrylamide gels is between 5-20%, however in practice a high percentage gel would be unable to resolve a larger molecular weight protein due to the small pore size formed (Hames & Rickwood, 1998).

Proteins possess ionisable groups which exist in solution as either cations (+) or anions (-) and therefore under the influence of an electric charge migrate towards either a cathode (-) or anode (+) dependent upon the protein's overall net charge. Electrophoresis allows the separation of proteins via the use of a power pack, electrophoresis unit and slab gel, usually formed from acrylamide as previously described. The complete acrylamide gel is formed within a gel cartridge, from both resolving and stacking gels which are poured in

two layers between two clamped glass plates. The resolving gel is first poured and allowed to polymerize before a lower concentration stacking gel is added, with comb allowing the formation of sample 'wells'. It is within the stacking gel wells that samples are loaded and proteins are then separated according to their properties within the resolving gel (Hames and Rickwood, 1998).

Upon polymerization the gel is loaded into the electrophoresis tank within appropriate buffer and the comb is removed. Samples can then be loaded into the individual wells left by removal of the comb and a current applied to the tank. The choice of buffer pH determines the mobility of the proteins and therefore separation, as does the percentage acrylamide used within the gel. The resolving gel may be formed from between 3-30% polyacrylamide whereas the stacking gel is varied little and usually between 4-5%. The stacking gel allows the protein to concentrate in a sharp band and when the current is applied enter the resolving gel at the same speed (Wilson & Walker, 2010).

To enable samples to run through the gel they are mixed with a sample buffer prior to loading. The buffer components depend upon the type of gel electrophoresis system that is being performed. Within a native gel system the sample buffer contains bromophenol blue, a dye that allows visualisation of the dye front whilst the gel is running, together with glycerol, providing density ensuring samples sink to the bottom of the loading wells. Once the gel has completed running and the dye front is either at the bottom of the gel cartridge or has just run off the bottom, the gel is removed from the unit and released from the between the gel plates. At this point the gel may be visualised by staining using a Coomassie dye based product, silver staining or be used within western blot detection of specific proteins of interest. Coomassie dye binds to the basic and hydrophobic residues of proteins and can detect as little as 10ng per band within a minigel, whereas silver staining can detect proteins to ≤ 0.5 ng of protein per band (Hames and Rickwood, 1998).

2.2.4 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Usually proteins are separated by PAGE in the presence of a detergent such as Sodium dodecyl sulfate (SDS) and under denaturing (heat) and reducing or nonreducing conditions. Sodium dodecyl sulfate polyacrylamide gel electrophoresis usually denoted SDS PAGE is perhaps the most commonly used electrophoresis method for analysis of proteins within a sample. It is capable of determining the molecular weight of proteins due to its ability to separate proteins by size, when used in conjunction with a molecular weight marker ladder. Proteins are denatured to their constituent polypeptides allowing identification of the size and number of polypeptide chains within the sample.

Within an SDS PAGE system the sample is mixed with a sample buffer prior to loading which contains the addition of an anionic detergent, SDS and β-mercaptoethanol. The addition of SDS provides the protein with an overall negative charge and denatures the protein. Proteins are heated to 95^{o}C for five minutes which allows further denaturing to take place. The addition of β-mercaptoethanol present within the sample buffer reduces disulphide bridges present within the protein. Heating, the addition of β-mercaptoethanol and binding of SDS act together to denature the protein, allowing relaxation of the tertiary conformation, resulting in linear polypeptide chains. The linear chain bound with SDS molecules takes on an overall negative charge and the proteins are drawn towards the anode, with smaller particles travelling faster than larger. A protein formed from multiple subunits will be separated into bands correlating to the size of its subunits. This method is therefore unsuitable within the determination of which conformation of CRP may be present (pentameric or monomeric) within a sample, but will accurately show the size of individual protomers (Hames and Rickwood, 1998).

2.2.5 Native Polyacrylamide Electrophoresis

Native PAGE are non-reducing and non-denaturing, allowing the detection of proteins in their physiological conformation with preserved secondary structure. The mobility of the protein through the gel is dependent upon the charge of each protein, determined by the primary amino acid sequence (isoelectric point) and the pH used within electrophoresis. The molecular mass separation of a reducing and denaturing SDS PAGE may only allow determination of one band whereas native PAGE may reveal many; however it is less accurate in determination of molecular weight due to the dependence upon protein charge and isoelectric point. SDS PAGE uses discontinuous chloride and glycine ion fronts which allow polypeptides to stack and move through the gel by charge to mass ratio. Native PAGE uses this same principle but the charge to mass ratio becomes subject to variability between different proteins. The protein moves through the gel according to both mass via the sieving effect and electrophoretic mobility (Lord, 2003; Young *et al*., 2012).

In some assays the native PAGE protocol was adapted to ensure monomeric CRP was able to run through the gel and to allow visualisation of the band. The conventional method was adapted to include $1/20^{th}$ the normal amount of SDS to both the running and the sample buffer in accordance with the method used by (Taylor & Van Den Berg, 2007). In their method SDS was omitted from the gel itself. Taylor *et al* found that in native PAGE conditions, mCRP could not be detected and it was suggested this may be due to insolubility problems. They found that by omitting the sample heating step with the lowered amount of SDS, both pCRP and mCRP were easily distinguishable. Both isoforms ran within a tight band, whilst pCRP produced a smear when normal SDS conditions were applied. This method was therefore used within mCRP detection assays.

2.2.6 Gradient Gel Polyacrylamide Electrophoresis

Gradient gels allow a larger range of protein molecular weight sizes to be run on the same gel due to the greater resolving range. Where both SDS and native PAGE use a combination of resolving and stacking gels, gradient gels utilise a gradient of acrylamide with the lowest concentration at the top and the highest concentration at the bottom. The gradient gel is formed via a gradient mixer and poured between glass plates in a similar fashion to both native and SDS gels forming a concentration range between 5 and 25%.

2.2.7 Western Blotting

The process of 'blotting' in molecular biology is a method of transferring proteins, DNA or RNA onto a membrane. The sample being transferred dictates the type of blotting performed, western blotting involves the transfer of protein, northern blotting involves RNA and southern blotting, DNA. Western blotting detects protein transferred to a membrane following gel electrophoresis using specific antibodies to probe for the desired protein. The technique was developed within the Friedrich Miescher Institute by Harry Towbin (Towbin *et al*., 1979) and is now used routinely within research and clinical settings.

An SDS or native PAGE gel is first used to separate the proteins as described within sections 2.2.4, 2.2.5 and 2.2.6 and the proteins transferred onto a membrane. Typically membranes used within western blotting are either nitrocellulose or polyvinylidene difluoride (PVDF). Nitrocellulose is a cellulose based membrane in which the cellulose is treated with nitric acid and allowed to spread over a thin film. The membrane is negatively charged resulting from the esterification of each glucose unit within the cellulose polymer with three nitrate groups. Proteins bind to the membrane by electrostatic and hydrophobic interactions. Nitrocellulose has high retention of, and affinity

for proteins, this together with its ease of use and cost make it a widely used membrane. PVDF is produced by the polymerisation of vinylidene difluoride producing a membrane and is also widely used in western blotting due to its greater strength and amino acid binding capacity when compared to nitrocellulose. PVDF membranes require hydration in methanol prior to use due to its hydrophobic surface, this additional step ensures effective transfer and binding of macromolecules (Towbin *et al*., 1979; Komatsu, 2015).

Proteins separated out by PAGE are transferred to the membrane following the application of an electrical current, which pulls the proteins from the gel onto the membrane as they migrate towards the cathode (+) (Towbin *et al*., 1979; Komatsu, 2015).

Membranes used within western blot efficiently and effectively bind all proteins and therefore steps must be taken to ensure interactions between the detection antibody used and the membrane itself are prevented. Therefore effective blocking must be undertaken to ensure avoidance of non-specific binding. The membrane is blocked following transfer with either bovine serum albumin or non-fat dried milk powder, which bind to areas of the membrane where the target protein has not bound (Towbin *et al*., 1979; Komatsu, 2015).

A specific antibody is then applied to 'probe' the membrane for the desired protein, termed the primary or detection antibody. A monoclonal antibody is generated following exposure of an antigen to a host species, therefore ensuring specificity to the target protein, or fragment of protein. The primary antibody is incubated with the membrane for varying time periods dependent on protocol before removal and the membrane washed to remove residual unbound primary antibody. A secondary antibody is then applied, this antibody will recognise the Fc fragment of the primary antibody and is enzyme conjugated for later visualisation. The enzyme is often either horseradish peroxidase (HRP) or alkaline phosphatase (AP). It is important to ensure antibodies are species specific, therefore if the

primary detection antibody has been raised in a mouse, the secondary antibody will be chosen to recognise a mouse antibody. Once the secondary antibody has been removed and the membrane carefully washed the enzyme substrate is added, with various substrates available determined by cost and sensitivity required (Murphy *et al*., 2008). HRP can be used to cleave chemiluminescent agents, the reaction resulting in light that can be detected by a charged coupled device (CCD) photodetector, captured digitally by camera (Gruner *et al*., 2002). More cost effective detection methods can be used including 4-chloronapthol stain with hydrogen peroxide, which is simple to use and results in a deep purple stain upon contact to the bound enzyme conjugated antibody. This can be photographed easily and does not require specialist equipment. However, there is a trade between cost and ease of use and sensitivity, where cheaper assays provide reduced detection levels (Murphy *et al*., 2008).

2.2.8 Enzyme Linked Immunosorbent Assay

2.2.8.1 Sandwich Enzyme Linked Immunosorbent Assay

An ELISA system is based upon the principle of immunoassays whereby specific antibody binding of an antigen, such as a protein, peptide, hormone or antibody, is detected within a fluid sample immobilised upon a solid phase carrier, in this case a 96 well ELISA plate. An enzyme acts as the reporter label, allowing measurement of the analyte within the sample. The enzymes now routinely used as conjugates to the detection antibodies are often either HRP, AP or urease (Winston *et al*., 2001). The ELISA technique was developed from a radioimmunoassay (RIA) by the research group of Peter Perlmann and Eva Engvall at Stockholm University in Sweden and first published in 1971 (Engvall and Perlmann, 1971).

The solid phase carrier used within an ELISA can be categorised as either high-capacity or low-capacity, with advantages and disadvantages to each. High-capacity materials include cellulose, nitrocellulose and cyanogen bromide-activated Sepharose, which are useful for impure preparations of antigen, but involve greater difficulty within wash steps and can produce high background signals. Low capacity materials include polystyrene, polyvinyl and polypropylene, all of which are easy to use and wash, with lower levels of background signal, however they can provide challenge when binding some antigens such as haptens and polysaccharides which do not adsorb to plastic surfaces as readily as proteins (Coutinho *et al*., 1995).

An antigen may be coated directly to the ELISA 96 well microplate by incubating it in a bicarbonate buffer pH 9.6 (Coutinho *et al*., 1995). Within a sandwich ELISA an initial coating antibody is used which allows specific binding of the target antigen, applied within a sample, this antibody is also added to the plate in a bicarbonate buffer pH 9.6. Figure 2.1 illustrates the sequence of binding with each component of the ELISA system.

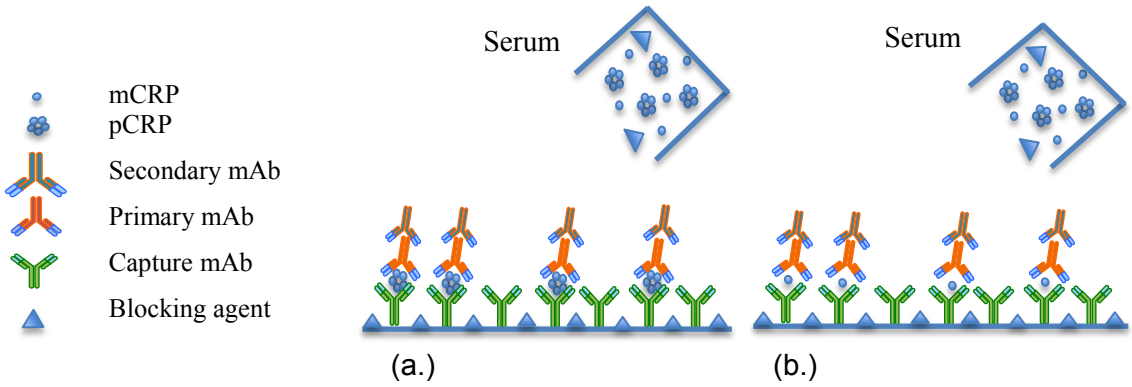


Figure 2-1 Diagram of a sandwich ELISA for the detection of a.) pCRP and b.) mCRP. The diagram shows the capture antibody specific to each form of CRP and bound CRP. This is followed by a specific detection and secondary antibodies.

Once the capture antibody (or antigen) has bound, following a wash step, all vacant binding sites are blocked with a protein such as BSA, preventing nonspecific binding of further antibodies or antigens to the plastic surface (Kemeny, 1991). The antigen (within

sample) is added to the plate and incubated, allowing binding to the captured antibody, a wash step removes unbound sample. A primary detection antibody is then added to the plate at an optimised dilution and incubated, allowing binding to the specific target antigen, as seen in Figure 2.1. This antibody is then detected by an enzyme-conjugated secondary antibody, allowing amplification of the signal and visualisation once the substrate is introduced to the antibody/antigen complex. The substrate is incubated for approximately 30 minutes and stopped using either sulfuric acid or sodium hydroxide and results in a measurable colour change, which is either visible of fluorescent (Gan & Patel, 2013). The ELISA plate can then be read on a plate reader and quantification of the antigen calculated by the inclusion of a known concentration of protein dilutions acting as a calibration.

A key step to successful ELISA is the wash step which takes place between the additions of each component of the ELISA onto the plate. A phosphate buffered saline solution is used with the addition of a detergent such as tween allowing effective removal of all residual unbound sample or antibody from the plate (Gan and Patel, 2013).

Whilst the ELISA is sensitive, able to detect smaller amounts of protein than the western blot and quantifiable, it is unable to determine the molecular weight of an antigen and there is a possibility of false positives occurring due to the sensitivity of the technique (Murphy *et al*., 2008; Gan & Patel, 2013). There are several variations to the basic ELISA method, with sandwich ELISA being just one of them. The choice can be dependent upon whether an antibody or antigen is the target of interest. The simplest is a direct ELISA in which the antigen is bound directly to the plate and a conjugated antibody is used to determine its presence, an indirect ELISA relies upon an antigen bound to the plate but both a primary and then secondary antibody used within the detection steps. A sandwich ELISA is two to five times more sensitive that a direct or indirect ELISA and more specific due to the use of two antibodies within the detection of the antigen (Murphy *et al*., 2008).

2.2.8.2 Competitive Enzyme Linked Immunosorbent Assay

A competitive or inhibition ELISA describes an assay in which the measurement of a target antigen or antibody relies upon the ability of a substance to interfere with normal antibody binding, therefore reducing the resulting chromogenic or fluorescent signal. The system can be based upon all types of ELISA from direct to sandwich and is sometimes also described as a blocking ELISA.

A direct ELISA used to detect an antigen within a competitive system involves an antigen bound directly to the solid phase, for instance a 96 well ELISA plate. The sample is pre-incubated with blocking solution to prevent non-specific binding and added to a labelled antibody specific for the bound (and target) antigen. If the antigen within the sample is the same as that bound to the plate, it will be bound by the antibody forming an antigen-antibody complex and the antibody will therefore be unable to bind the antigen immobilised in the well. Unbound labelled antibody will bind the immobilised antigen and all other antibodies will be washed away. The amount of competition is dependent on the number of molecules of test and solid phase antigens, where there is no antigen present within the test sample, the labelled antibody is fully available to bind the solid phase antigen. Where large amounts of antigen are present within the sample a reduction in antibody binding will occur and therefore a reduction in detection signal proportional to the concentration will result (Walker, 2001).

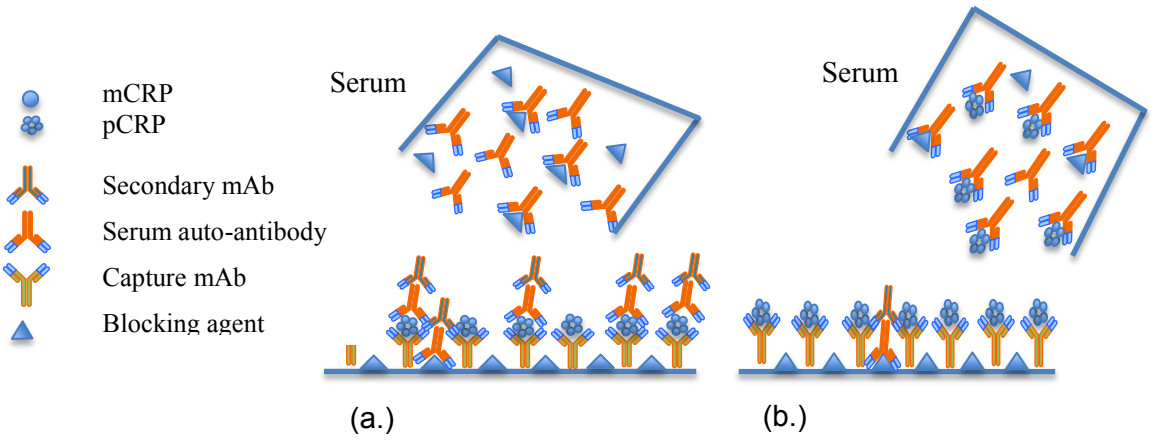
When a competitive ELISA is used to detect a specific antibody within solution, a similar system to that of antigen detection can be used. The competitive aspect is between specific detection antibodies and antibodies within the test sample. Following the addition of specific antibodies directed against the same antigen as those sought, to the sample containing the test antibody, the solution is added to antigen pre-bound to a 96 well plate. Sample antibodies compete to bind the antigen with the specific added antibodies.

Antibodies that do not detect the solid phase antigen are not able to bind and therefore washed away. Anti-species specific antibodies are then added and will only bind to the added antibodies which cannot be the same species as those within the test sample. For instance if detecting anti-human CRP antibodies, a serum sample would be premixed with mouse anti-human CRP antibodies. Human CRP would be bound to the plate and the mixture of mouse anti-CRP antibodies and serum sample added to the solid phase. A proportion of the antigen would be bound by antibodies present within the serum and therefore prevent binding of a secondary conjugated anti-mouse IgG, reducing the colour signal upon addition of substrate. A sandwich ELISA can also be used within the competitive ELISA system, however care must be taken to ensure no interaction occurs between the capture and detection antibodies (Walker, 2001).

An alternative antigen detection system employs a competitive binding principle facilitated by the addition of the original antigen. The primary antibody is incubated with the antigen containing sample, forming antigen-antibody complexes if antigen is present. This solution is added to a 96-well plate which is pre-coated with the same antigen. Antibody-antigen complexes are unable to bind the immobilised antigen and are washed from the plate, with the degree of competition proportional to the amount of antigen in the sample. The greater the amount of antigen present, the fewer antibodies will be able to bind and the secondary antibody conjugate will elicit reduced signal as compared to a control in which antibody only is added to the immobilised antigen. This system is also effective when used to identify antibodies within a sample; this type of ELISA is illustrated in Figures 2.2 and 2.3 for anti-pCRP and anti-mCRP detection respectively. In this situation the antigen is once again bound to the plate, either directly or via a capture antibody. The antibody containing sample is pre-incubated with antigen for 2 hours at room temperature of overnight at 4^{o}C. The antibodies present will once again form antigen-antibody

complexes and be unable to bind to the solid phase bound antigen. Pure sample introduced to wells containing bound antigen will be able to bind the antigen, however nonspecific binding may also occur against blocking and capture agents. The reduction of resulting signal measured from secondary conjugated antibody binding is proportional to the antibody-antigen complexes formed. It is therefore important that added antigen is in excess of that bound to the wells (Walker, 2001).

Figure 2-2 Diagram showing the competitive ELISA system used to determine auto-antibody presence directed against pCRP within patient samples a.) A specific pCRP capture agent is bound to the plate followed by pCRP. The patient sample is pre-incubated with blocking solution prior to incubation upon the plate, followed by specific detection and then conjugated secondary antibodies. b.) A specific pCRP capture agent is bound to the plate, followed by pCRP. The patient sample is pre-incubated within blocking solution and pCRP, forming complexes between autoantibodies and pCRP, preventing binding of autoantibodies to pCRP captured on the ELISA plate. The reduction in absorbance found between plate a.) and b.) is proportional to patient auto-antibody presence.



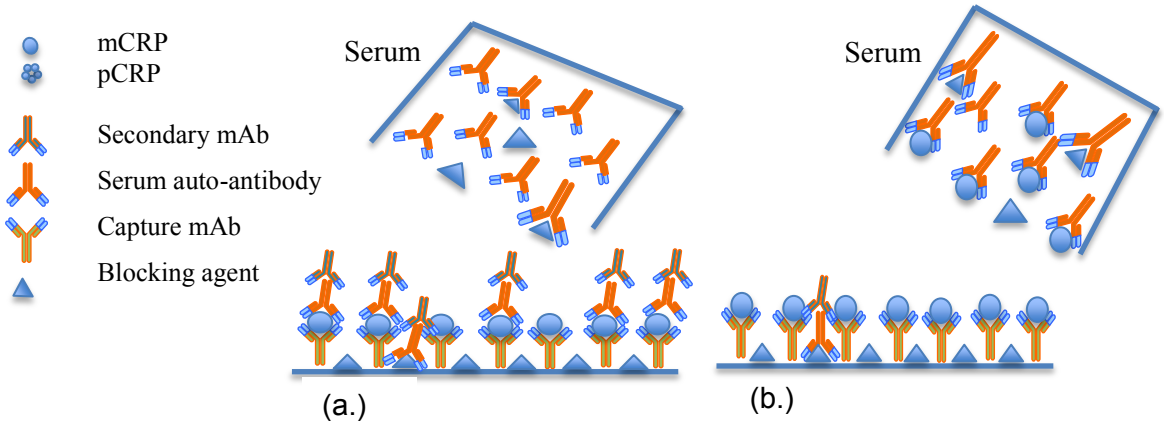


Figure 2-3 Diagram showing the competitive ELISA used to determine auto-antibody presence directed against mCRP within patient samples a.) A specific mCRP capture antibody is bound to the plate followed by mCRP. The patient sample pre-incubated with blocking solution prior to incubation upon the plate, followed by specific detection and then conjugated secondary antibodies. b.) A specific mCRP capture antibody is bound to the plate, followed by mCRP. The patient sample is pre incubated within blocking solution and mCRP, forming complexes between auto-antibodies and mCRP and preventing binding of auto-antibodies to mCRP captured on the ELISA plate. The reduction in absorbance found between plate a.) and b.) is proportional to patient auto-antibody presence.

2.3 Materials and Methods

2.3.1 Ethical Application

Prior to experimental collection of patient samples and any experimental procedure took place both peer review and ethical research committee approval was sought. This oversight ensured the soundness of the study aims and assessed risk to both participants and researcher.

The Joint Keele Independent Peer Review Committee reviewed the study under their peer review system, involving a local research community who review and scrutinise quality of the research together with the experimental context, methodology and aims. The review panel also assess the feasibility and scientific validity of the study. The application for this research study was approved in August 2012.

Following Peer Review approval an application to the Integrated Research Application System (IRAS) was submitted. This system ensures that the study risks are acknowledged and that all participants are provided with clear and detailed information about the study, allowing them to make an informed decision whether to take part or not. Participant Informed consent would be given after explanation of the study aims, sample collection, storage of samples and actions in the event of participant withdrawal. IRAS was used within the preparation of applications to both the Research Ethics committee and the NHS Research & Development offices. This application was formally approved in November 2012. The project was approved by: NRES Committee North West – Lancaster; REC reference 12/NW/0818; IRAS reference 100079 and NHS Research Governance Facilitator permission for research confirmed April 2013.

The full title of the research project was:

'Investigations into the possible involvement of C-Reactive Protein (CRP) in the progression of rheumatoid arthritis

The project was submitted as a student study. The chief investigator (CI) was Dr. D. Mattey. Other members of the study included Jennifer Moran (PhD student), Dr. A. K. Strive (Co-supervisor) and Professor T. G. Greenhough (Advisor). The principle question was:

'Is monomeric CRP found within the serum of patients with a confirmed diagnosis of rheumatoid arthritis?'

Identification of patients took place at the Haywood Rheumatology Hospital, Burslem, Stoke on Trent. A criteria for participant eligibility for the study was adhered to:

I. Patients with confirmed diagnosis of RA according to internationally recognised criteria, attending routine monitoring clinic (Haywood Hospital) and due to undergo routine blood testing as part of their clinical care
II. Male or female over the age of 18 years.

Study exclusion criteria were:

I. Participants under the age of 18
II. Any patient with a confirmed diagnosis of HIV, Hepatitis B or C
III. Participants unable to provide or understand informed consent

Participants taking part within this study were all in or out patients of the Haywood Rheumatology Hospital. Initially participants were identified by a member of the clinical care team or a research nurse and given a verbal explanation of the study. All participants interested in taking part in the study were provided with a patient information sheet (PIS) by a member of the clinical team (see Appendix 1), which outlined the study aims, risks and benefits. Participants were given time to decide if they wished to participate and informed that an extra blood sample would be taken at the time they were due to undergo their routine blood test. They were not required to undergo any additional clinical procedures and could withdraw from the study at any time. Further, it was explained that any information generated by the study was anonymised and therefore a particular participant would gain no direct benefit.

Participants happy to take part within the study, should the research team be fully satisfied that they had fully understood the information discussed, were provided with a patient consent form (PCF, see Appendix 2). Patients were then asked to sign and date the consent form, initialing boxes confirming full understanding of the studies ramifications. These forms were then stored at the Haywood in accordance with NHS procedures, with a copy placed on each patient file. Only members of the clinical care team would be provided with access to the PIS and PCF. Participants were informed that should they wish to withdraw from the study at any point they would be removed and their samples destroyed.

2.3.2 Acquisition and Preparation of Human Serum Samples

Upon recruitment of a patient to the study and a signed and dated consent form provided, a qualified phlebotomist at the Haywood Rheumatology Hospital drew venous blood, following their internal clinical care protocols. The samples were collected within a

red topped Becton Dickinson (BD) Vacutainer blood handling tube without anticoagulant and allowed to clot for up to 30 minutes. The sample was centrifuged at 2,000g at room temperature (22^{o}C) for 10 minutes allowing separation of the serum and red blood cell clot.

The serum (supernatant) was carefully removed by Pasteur pipette and stored in 0.5ml aliquots within cryo tubes at -20^{o}C until further analysis was performed. Freeze thaw cycles were avoided by the use of stored duplicate 0.5ml sample tubes. Control samples were collected from a pool of volunteers working within UHNS and the Haywood Rheumatology Hospital; all provided consent and confirmed no previous identification of inflammatory disease.

Each sample was given an anonymised sample number (e.g. CRPRA001 or CRPC001) and kept separately to identification documents which were stored in a locked cabinet at the hospital. Detailed list of samples held together with date of collection, storage and disposal were kept throughout the study. All information generated throughout the study was stored on a password-protected computer, with only named study researchers having access to it.

2.3.3 Calibration of the Size Exclusion Chromatography Column

Before running any samples through the column it was necessary to calibrate it in order to accurately determine protein sizes via the reported elution volumes. The GE Healthcare AKTA explorer 100, Fast Protein Liquid Chromatography (FLPC) system with a HiLoad 16/60 Superdex 200pg Column was used within this study. The operating software UNICORN 5.11. allows estimation of protein amounts via recorded UV absorbance at 280nm and measured elution volume, allowing the accurate reporting of an elution profile correlated to molecular weight (Amersham Biosciences, 2003).

The calibration was performed using a Gel Filtration Calibration Kit (GE Healthcare), with the included molecular weight markers detailed within Table 2.1. The void volume was determined using Blue Dextran. Samples were reconstituted from lyophilisate to 20mg/ml in a stock solution of 150mM NaCl, 50mM Tris, and 0.01% sodium azide, adjusted to pH 7.20, The stock protein solutions were further diluted to working final concentrations as indicated in Table 2.1.

Table 2.1 Table of protein standards and their respective molecular weights and final concentrations used within the calibration protocol of the AKTA 100, HiLoad 16/60, Superdex 200pg FPLC system.

Protein Standard	Molecular Weight (Da)	Final Concentration (mg/ml)
Ferritin	440,000	0.3
Aldolase	158,000	4
Conalbumin	75,000	3
Ovalbumin	44,000	4
Ribonuclease A	13,700	3

Buffers and solutions used for storage and preparation of the column are detailed in Table 2.2. The column is stored in 20% ethanol and three column volumes (120ml/column volume) deionised H_2O were used to remove ethanol storage buffer from the system. The system of tubing and column were then equilibrated using three column volumes of elution buffer (50mM Tris, 150mM NaCl, 0.01% sodium azide). All buffers used within the system were first filtered using a 0.2μm cellulose acetate filter (Sartorius Stedim Biotech) and degassed. To avoid the risk of protein precipitation or elution peak contamination two separate FPLC runs were performed, the first containing Ferritin, Conalbumin and Ribonuclease A, and the second, Aldolase and Ovalbumin. The total volume of the loaded

sample is recommended to be between 0.1% to 2% of the total column volume (0.12 – 2.4 ml) in order to achieve good resolution, therefore a sample volume of 500µl was added to the column (GE Healthcare UK Ltd, 2015). Prepared molecular weight samples were loaded onto the column via a 2ml sample injection loop and a flow rate of 1ml/min used. Absorbance was measured at 280nm and sample fractions of 5ml collected to a total volume of 120ml, equating to one column volume.

Table 2.2 Table listing buffers and solutions used within the equilibration of the HiLoad 16/60 Superdex 200pg size exclusion chromatography column.

Buffer/Solution	Components	pH
Wash solution	Deionised H_2O	7.0
Elution Buffer	50mM Tris, 150mM NaCl, 0.01% Sodium Azide	7.2
Storage Solution	20% Ethanol	7.0

2.3.4 Purification of Pentameric and Monomeric C-reactive Protein

Determination of the molecular weight of CRP samples was achieved by the use of an AKTA explorer 100, Fast Protein Liquid Chromatography (FPLC) system (GE Healthcare). The resulting elution volumes were used to assess dissociation of pCRP to mCRP by comparing the resulting trace to that of the calibration prepared in section 2.3.3, with buffers and solutions detailed within Table 2.3. The column was pre-equilibrated using one column volume, 120ml, of filtered deionised water followed by two column volumes of calcium free elution buffer (20mM Tris, 280mM NaCl, pH 8.0). Initial trials involved 500µl of treated CRP sample ~ 200µg/ml injected onto the 2ml sample loop and loaded onto the HiLoad 16/60 Superdex 200 pg column (GE Healthcare, 2011). A flow rate of 1ml per minute using calcium free elution buffer was used in all assays. Fractions

were collected in separate 5ml elution volumes within 20ml universal tubes. This method was used to determine molecular weight and purity for the native pentamer and following dissociation trials involving treatment by change of pH to either pH6 or pH4, heat or urea treated sample, as described in chapter three. It was further used to determine possible re-association of monomeric CRP samples. Optimum dissociation conditions were assessed and further experiments continued with 100µl CRP and 400µl buffer to a final concentration of 2M urea.

Table 2.3 Table listing the buffers and solutions used within the elution of CRP preparations.

Buffer/Solution	Components	pH
Wash solution	Deionised H_2O	7.0
CRP Storage Buffer	20mM Tris, 280mM NaCl, 0.01% Sodium Azide	8.0
Storage Solution	20% Ethanol	7.0

2.3.5 Concentration and Storage of pCRP and mCRP Protein Samples

The chosen protein elution fraction was concentrated using a Sartorius™ Vivaspin™ 20 Centrifugal Concentrator, 10kDa molecular weight cut off (Fisher Scientific). The sample was centrifuged at 1,000 x g for between 10-20 minutes at 4^{o}C, until the desired volume/concentration was reached. This allowed the buffer to pass through the filter whilst the protein is captured and concentrated. Prior to use the Vivaspin concentrator was spun with solely deionised water for 10 minutes at 1,000g (4^{o}C), ensuring the membrane was primed for optimum protein recovery. The sample was then stored within the CRP storage buffer (Table 2.3) at 4^{o}C until required for further analysis.

Native pentameric C-reactive protein (pCRP) was purchased from SCRIPPS laboratories (C0129), at a concentration of 2.43mg/ml (confirmed to be >99% pure by SDS PAGE analysis). The protein was supplied within a standard storage buffer composed of 20mM Tris, 280mM NaCl, 5mM $CaCl_2$, 0.01% sodium azide, pH 8.0 and was stored at 4^{o}C until needed.

2.3.6 Native Polyacrylamide Gel Electrophoresis

Native PAGE was used to assess the purity and conformation of CRP eluted from the size exclusion column, to determine antibody specificity assays and to confirm mCRP and pCRP within patient serum samples as determined by ELISA. The native gels were typically prepared with a 12.5% acrylamide resolving gel and a 4% acrylamide stacking gel. TEMED and 30% acrylamide/bis-acrylamide were purchased from Sigma Aldrich, APS was prepared to a 10% w/v solution immediately prior to use. Stock acrylamide/bisacrylamide was stored at -20^{o}C and thawed prior to use. Alternatively a precast gel was used with up to 30µl of combined sample/buffer added to each well, dependent on sample concentration, within a pre-cast BioRad native gel (12%). Each gel contained positive lanes containing 1µg each of pCRP or mCRP.

The conventional Laemmli method was adapted within mCRP detection assays to include an addition of 1/20th the normal SDS amount within both the sample and running buffers as per Taylor *et al* 2007 (detailed within section 2.2.5). Samples were added to a two times non-reducing native sample buffer with an addition of 1/20th the amount of SDS added within a normal Laemmli system buffer, at a 1:1 ratio (as described within Table 2.4) and detailed within section 2.2.5. A maximum of 20µl was loaded into each well dependent upon protein concentration. Glycine, glycerol and Tris were all purchased from Sigma.

All gels were run in a BioRad mini PROTEIN II cell (BioRad), connected to a Powerpac 300, (BioRad). Gels were run for 20minutes at 100V followed by a further 30 minutes at 150V in 500ml of running buffer (see Table 2.4), until the dye front left the bottom of the gel. The gel was then removed from the electrophoresis tank and carefully released from the gel casing, and resolving/stacking gels separated if required. Gels were visualised using Coomassie Brilliant Blue (Biorad), or used within western blot analysis. Gels stained with Brilliant Blue were allowed to develop over 20 minutes and then rinsed in deionised water before being captured on a Syngene GBOX image detector. Brilliant Blue has a detection limit of approximately 1μg protein, therefore sufficient protein must be loaded to ensure visualisation.

Table 2.4 A table detailing the components required for the preparation and casting of either native PAGE or the amended PAGE technique including 1/20th SDS within both sample and running buffers.

Component	**Reagents**	**Volume/Amount**
12% Resolving Gel	30% Acrylamide/bisacrylamide	3.33ml
	Deionised H_2O	2.58ml
	Tris 1.5M pH 8.8	2.0ml
	100% Temed	6µl
	10% APS	60µl
4% Stacking Gel	30% Acrylamide/bisacrylamide	0.67ml
	Deionised H_2O	3.0ml
	Tris 0.5M pH 6.8	1.25ml
	100% Temed	6µl
	10% APS	60µl
Sample Buffer (Native)	Tris 0.5M pH 6.8	1.25ml
	Deionised H_2O	5.55ml
	Glycerol v/v	2.5ml
	Bromophenol Blue 0.5% w/v	0.2ml
Sample Buffer (1/20th SDS technique)	As above Tris, Glycerol, Bromphenol Blue,	
	SDS 10% w/v	0.1ml
	Deionised H_2O	5.45ml
Running Buffer pH 8.3 Native (500ml)	Tris	1.515g
	Glycine	7.21g
	SDS 10% w/v (1/20th SDS technique only)	0.25ml

2.3.7 Western Blotting of C-reactive Protein

Protein samples of interest were analysed by western blot following separation by native PAGE as described in section 2.3.6. The acrylamide gel was allowed to equilibrate within transfer buffer (as shown in Table 2.5), for up to 30 minutes whilst a 0.2μm nitrocellulose membrane, 0.2 μm pore size, 8.5 cm x 13.5 cm (ThermoFisher Scientific), filter paper and fibre pads were soaked in transfer buffer for 10 minutes. Once equilibrated a 'sandwich' was made of the fibre pads, filter paper, nitrocellulose membrane and gel and clamped within the cassette, whilst at all times keeping the components beneath the buffer and ensuring no trapped air bubbles remain. It is important that the gel is directed towards the anode and the membrane towards the cathode. The cassette plus a pre frozen ice pack preventing overheating, were placed within a Mini Trans-Blot Electrophoretic Transfer Cell (BioRad) which was filled with 500ml transfer buffer. A magnetic stirrer was added to ensure even temperature and ion concentration. The transfer module was placed upon a plate stirrer and connected to a PowerPac 300 (BioRad) and run at before running at 30mA overnight at 4^{o}C or at 80V for two hours at 4^{o}C.

Table 2.5 A table showing reagents required for the western blotting transfer buffer.

Buffer/Solution	**Reagents**	**Volume/Amount**
Transfer Buffer	Tris	24mM
	Glycine	194mM
	Deionised H^2O	500ml

Each cassette was removed from the transfer unit and a pencil used to carefully mark the wells and perimeter of the gel onto the membrane. The membrane was separated from residual gel before immersion in blocking solution (Table 2.6) overnight at 4^{o}C upon a mechanical rocker. The blocking solution was then removed by pouring away and the

membrane washed five times for five minutes each in BLOTTO (Table 2.6). Once thoroughly washed the primary antibody was added. The majority of antibody dilutions took place in BLOTTO, however serum was pre-incubated (diluted) within blocking solution overnight within auto-antibody studies, antibodies used within western blotting are detailed within Table 2.7. Membranes were then incubated for either 2 hours or overnight upon a rocker at 4^{o}C. Once again a wash step took place with five times five minute washes within BLOTTO before the addition of the conjugated secondary antibody, at various concentrations dependent of sample used. The membrane was incubated for 1 hour at 4^{o}C before once more washing five times five minutes.

Table 2.6 A table showing reagents required for the preparation of western blotting solutions.

Buffer/Solution	Reagents	Amount/Volume
Tris-HCL buffered Saline (TBS) pH 7.4 or PB	Sodium Chloride	150mM
	Tris	50mM
Blocking Solution	TBS	40ml
	Marvel Dried Milk	1g
BLOTTO	TBS	200ml
	Marvel Dried Milk	2g
	Triton-X-100	4ml
ECL Reagent	Clarity peroxidase reagent	7ml
	Clarity western luminol/enhancer reagent	7ml

The membrane was then washed six further times for five minutes in BLOTTO before visualisation via chemiluminescence was achieved. A high sensitivity reagent was applied to the nitrocellulose membrane which was then left to incubate whilst rocking for five further minutes. BioRad, Clarity Western Enzyme Chemiluminescence Substrate

(ECL), was chosen to visualise any detectable bands resulting from HRP conjugated antibody binding. Each membrane required a mix of 7ml each Clarity peroxide and Clarity western luminol/enhancer reagent to be pre mixed immediately before and added to the membrane whilst gently rocking to ensure movement of the reagents against the nitrocellulose membrane. Once incubated for a minimum of five minutes the membrane was wrapped in Saran Wrap, protein side up, ensuring it at no point dried out. It was then possible to view the membrane on a FlourChem M system image detector at an exposure time of up to 40 minutes dependent on the intensity of the band and protein concentration detected.

Table 2.7 A table providing details of antibodies used within western blot analysis, supplier and concentration.

Technique	Antibody	Supplier	Target	Concentration / Dilution	Species
Western Blotting	Detection Ab 3H12	L. Potempa	mCRP	1:50	Mouse
	Detection Ab 1D6	L. Potempa	pCRP	1:50	Mouse
	Detection Ab Clone 8	Sigma C1688	mCRP / pCRP	4.55µg/ml	Mouse
	Secondary Ab	Sigma A9044	Mouse IgG	35.3µg/ml	Rabbit
	Conjugated anti-human IgG	Sigma A8667	Human IgG	0.47µg/ml	Goat
	Conjugated anti-human IgA	Invitrogen AH10104	Human IgA	4.5ng/ml	Goat
	Anti-human IgM	Sigma I6385	Human IgM	40µg/ml	Mouse
	Secondary Ab	Sigma A5420	Goat IgG	186ng/ml	Rabbit

Within auto-antibody detection assays the above method was amended. Patient serum samples were diluted 1:100 in blocking solution and incubated overnight to avoid non-specific binding to the blocking step. The membrane was also blocked overnight and carefully sectioned following washing. The diluted patient or control serum was then used as a primary detection antibody and incubated overnight upon a rocker at 4^{o}C. A mCRP /

pCRP control lane was probed with anti-mCRP primary antibody 3H12 diluted 1:50 in BLOTTO or Clone 8 (Sigma) diluted 1:1000 in BLOTTO. The secondary antibody, either goat anti-human IgG and IgA or mouse anti-human IgM, diluted in BLOTTO was incubated, followed by a wash step as previously described (antibody concentrations detailed in Table 2.7). Lastly rabbit anti-mouse IgG was added to the IgM assay at a concentration of 1:30,000 for 1 hour at room temperature before once more washing, followed by development as previously described with an exposure time of up to 40 minutes dependent on the intensity of the band and protein concentration detected

2.3.8 Sandwich ELISA Detection of Serum CRP

A commercially available pentameric CRP detection kit (Duo Set ELISA kit, R&D Systems) was used to identify and determine levels of pCRP. Initially the protocol and antibodies were tested for recognition of mCRP and found to detect only the pentameric form (detailed within section 4.3.6), this allowed confident quantification of pCRP alone. A range of patient dilutions were assessed enabling optimisation of the seerum dilution that was best captured within the calibration range. Within the pCRP detection assay, patient samples were diluted 1:16000 in reagent diluent (R&D Systems) following optimisation of patient and control sample dilutions (Table 2.8). All antibodies used within the ELISA system are detailed within Table 2.9 with buffers and components used described within Table 2.10. The Duo Set protocol was designed to identify CRP within a range of 0.00 – 1000pg/ml, with a serially diluted calibration carried out on each plate using pentameric CRP (Scripps Labs).

The capture antibody used within the ELISA kit was a mouse anti-human IgG, reconstituted as per manufacturer's protocol in 1ml phosphate buffered saline (PBS) and further diluted 33µl within 12mls PBS (1µg/ml), this was a variation from the

recommended 66µl per 12mls (2µg/ml) protocol, final concentrations of this and all other antibodies used within ELISA are detailed within Table 2.9. A volume of 100µl of the diluted capture antibody was applied to a Corning CLS3590, 96 well plate, sealed and incubated overnight at room temperature.

Table 2.8 Table showing optimisation of serum samples within the pCRP detection ELISA. Samples were diluted between 1:1000 and 1:128,000 with dilutions found within the calibration scale shaded. Patient and control samples were diluted to 1:16,000 and 1:1000 respectively following optimisation.

Calibration pCRP		RA2		RA3		RA4		C03		Blank		Sample Dilutions
1000pg	1000pg											1:1000
500pg	500pg											1:2000
250pg	250pg											1:4000
125pg	125pg											1:8000
63pg	63pg											1:16000
32pg	32pg											1:32000
16pg	16pg											1:64000
0pg	0pg											1:128000

The plate was immersed in fresh wash buffer, phosphate buffered saline-tween (PBS-tween), prepared from PBS tablets (Sigma Aldrich), and carefully washed three times ensuring each well filled and liquid removed completely by inverting the plate and blotting against clean paper towels following each wash step. The plate was blocked by addition of 300µl of Reagent Diluent (R&D Systems) to each well and incubation at room temperature for 1 hour.

The wash step was repeated and patient samples (1:16000), or control samples (1:1000), diluted in reagent diluent (R&D Systems) and 100µl added per well, together with serially diluted calibration standards. Positive and negative controls were also added

to each plate, 0.5µg/ml each of pentameric CRP (Scripps Labs) and urea dissociated mCRP, produced as detailed within Chapter 3. The samples were incubated at room temperature in triplicate for two hours as per the sample layout in Table 2.11. Samples were flicked out and the wash cycle repeated before the addition of the biotinylated mouse anti-human CRP detection antibody (R&D Systems). This antibody was initially reconstituted in 1 ml PBS before further dilution of 66µl within 12mls PBS, and 100µl per well incubated for 30 minutes at room temperature.

Lastly Streptavidin-HRP (R&D Systems) was diluted 1:200 in Reagent Diluent and added at 100µl per well. Once again the plate was washed thoroughly three times in wash buffer before 100µl substrate solution was added. The substrate solution consisted of equal volumes Colour Reagent A (stabilised hydrogen peroxide) and B (stabilised tetramethylbenzidine), R&D Solutions. The substrate was left to develop for 30 mins in a dark place before 50µl of stop solution was added (R&D Systems). At this point the plate was read at both 450nm and 540nm with the latter removed prior to calculations in order to remove background plate absorbance.

Table 2.9 A summary table containing details of antibodies used within ELISA assays including final concentrations and suppliers.

Technique	Antibody/Protein	Supplier	Target	Concentration / Dilution	Species
ELISA mCRP detection	Capture Ab	Sigma C8284	Human CRP	4.6µg/ml	Goat
	Detection, 3H12	L. Potempa	Human mCRP	1:50	Mouse
	Secondary Ab	Sigma A9044	Mouse IgG	0.35µg/ml	Rabbit
ELISA pCRP detection	Capture Ab	R&D Systems 842676	Human pCRP	2µg/ml	Mouse
	Detection Ab (Biotinylated)	R&D Systems 842677	Human pCRP	90µg/ml	Mouse
	Streptavidin conjugated to HRP	R&D Systems 890803		1:200	
ELISA auto-antibody detection	PCh-KLH	Santa Cruz Biotechnology Sc-396490	pCRP	2µg/ml	Keyhole Lympet
	Conjugated anti-human IgG	Sigma A8667	Human IgG	0.35µg/ml	Goat
	Conjugated anti-human IgA	Invitrogen AH10	Human IgA	4.5ng/ml	Goat
	Anti-human IgM	Sigma I6385	Human IgM	40µg/ml	Mouse
	Secondary Ab	Sigma A5420	Goat IgG	186ng/ml	Rabbit
Antibody specificity Studies	Detection Ab Clone 8	Sigma C1688	pCRP / mCRP	9.1µg/ml	Mouse
	Detection Ab 8C10	L. Potempa	mCRP	1:50	Mouse
	Detection Ab 1D6	L. Potempa	pCRP	1:50	Mouse
	Keratin	Sigma		0-4µg/ml	Human
	Fc Specific anti-human IgG	Sigma A0170	Human IgG	1.72µg/ml	Goat
	PTX3	Sigma SRP6323		10µg/ml	Human
	SAP	Sigma 565190		10µg/ml	Human

Table 2.10 A summary table of all reagents used within the ELISA protocol, including monomeric CRP detection antibodies (shaded grey) and pentameric CRP detection antibodies unshaded.

Buffer Solution	Components	Concentration
PBS pH 7.4	1 PBS tablet (Sigma) / 200ml deionised water	One tablet dissolved in 200 ml of deionised water yields 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride.
PBS – Tween (Wash Buffer)	PBS	1.4 litre
	Tween	750 ml
Blocking Solution (R&D Systems) Reagent Diluent	R&D Reagent Diluent 10x	10x dilution in deionised H_2O provides 1% BSA
Bicarbonate Coating Buffer pH 9.6	Bicarbonate/carbonate	100mM
Capture Antibody 1:10,000 Dilution	Goat anti-human CRP (Sigma, C8284)	1µl
	Bicarbonate Buffer	9.999ml
Primary Antibody 1:50 Dilution	3H12 mouse anti-human mCRP	200µl
	Reagent Diluent	9.800ml
Secondary Antibody 1:30,000 Dilution	Rabbit anti-mouse IgG (Sigma, A9044)	0.5µl
	Reagent Diluent	14.9995ml
Capture Antibody R&D Systems	Mouse Anti-Human CRP Capture Antibody	33µl
	Reagent Diluent	12.0ml
Primary Detection Antibody	R&D Systems Biotinylated Mouse Anti-Human CRP	33µl

R&D Systems	Antibody	
	Reagent Diluent	12.0ml
Streptavidin-HRP A 1:200	Streptavidin-HRP A (R&D Systems)	60µl
	Reagent Diluent	11.940ml
Colour Substrate (R&D Systems)	Colour Substrate A (H^2O^2)	6.0ml
	Colour Substrate B (Tetramethylbenzidine)	6.0ml
Stop Solution (R&D Systems)	Stop Solution (2 N H_2SO_4)	5.0ml

Table 2.11 An example ELISA plate layout for pCRP detection assay with rows 1-3 containing various dilutions (ng/ml) of pentameric CRP (Scripps Lab) acting as calibration standards between 0-1000pg/ml. Samples were randomly allocated in triplicate with additional +ve and -ve controls of pCRP or mCRP 500 ng/ml also added in triplicate. Capture, detection and secondary antibody were used as directed within the DuoSet CRP kit (R&D Systems), patient samples diluted 1:16000 in reagent diluent and control samples 1:1000.

Pentameric CRP ELISA Plate Layout												
	1 pg/ml	2 pg/ml	3 pg/ml	4	5	6	7	8	9	10	11	12
A	1000	1000	1000	RA1	RA1	RA1	RA9	RA9	RA9	RA 17	RA 17	RA 17
B	500	500	500	RA2	RA2	RA2	RA10	RA10	RA10	RA18	RA18	RA18
C	250	250	250	RA3	RA3	RA3	RA11	RA11	RA11	RA19	RA19	RA19
D	1255	1255	1255	RA4	RA4	RA4	RA12	RA12	RA12	RA20	RA20	RA20
E	62.5	62.5	62.5	RA5	RA5	RA5	RA13	RA13	RA13	RA21	RA21	RA21
F	31.256	31.256	31.256	RA6	RA6	RA6	RA14	RA14	RA14	RA22	RA22	RA22
G	15.75	15.75	15.75	RA7	RA7	RA7	RA15	RA15	RA15	-ve Cont.	-ve Cont.	-ve Cont.
H	Blank	Blank	Blank	RA8	RA8	RA8	RA16	RA16	RA16	+ve Cont.	+ve Cont.	+ve Cont.

To enable detection and quantification of mCRP an individually designed ELISA was developed after assessment of antibody combinations for specificity and effectiveness. The protocol described previously for pCRP was used with specific amendments to capture and detection antibodies together with serum dilutions, and are detailed here. An mCRP calibration was included within each ELISA plate, ranging from 0 – 10ng/ml, using mCRP produced via dissociation and purification methods detailed within Chapters 2 and 3 (sections 2.3.4 & 3.2.2). All reagents and solutions used are detailed within Table 2.9 and Table 2.10 as described previously. Initially the ELISA plate (Corning) was coated overnight at 4^{o}C temperature with capture antibody polyclonal goat anti-human CRP IgG (Sigma C8284) at a dilution of 1:10000 in bicarbonate coating buffer pH 9.6 (100µl per well). Wash and block steps were carried out as previous, prior to the addition of 100µl of a 1:100 diluted serum in diluent solution (R&D Systems), calibration dilutions and positive control (0.5µg/ml pCRP).

The plate was incubated for one hour at room temperature. The ELISA plate layout was repeated as per Table 2.12 with samples arranged randomly in triplicate. The primary detection antibody used was antibody 3H12, a specific mouse anti-human mCRP monoclonal IgG (kindly donated by Dr L. Potempa, College of Pharmacy, Roosevelt University, Illinois), 100µl per well at a 1:50 dilution within reagent diluent solution. This was incubated at room temperature for one hour. The secondary antibody used was streptavidin conjugated rabbit anti-mouse IgG (Sigma) antibody, added at a dilution of 1:30,000 in reagent diluent solution and incubated for 30 minutes at room temperature. The plate was developed and read as described previously.

All ELISAs were carried out using 96 well flat bottom plates (Corning CLS3590), and sealed with pre-cut sealing tape (Thermo Scientific).

Table 2.12 A representative ELISA plate layout for mCRP detection rows 1-3 containing various dilutions (ng/ml) of urea dissociated monomeric CRP, acting as calibration standards between 0-10ng/ml. Samples were randomly allocated across the plate in triplicate with additional positive and negative controls of mCRP or pCRP 0.5µg/ml also added in triplicate.

Monomeric CRP ELISA Plate Layout												
	1 ng/ml	2 ng/ml	3 ng/ml	4	5	6	7	8	9	10	11	12
A	10	10	10	RA1	RA1	RA1	RA9	RA9	RA9	RA 17	RA 17	RA 17
B	5	5	5	RA2	RA2	RA2	RA10	RA10	RA10	RA18	RA18	RA18
C	2.5	2.5	2.5	RA3	RA3	RA3	RA11	RA11	RA11	RA19	RA19	RA19
D	1.25	1.25	1.25	RA4	RA4	RA4	RA12	RA12	RA12	RA20	RA20	RA20
E	0.625	0.625	0.625	RA5	RA5	RA5	RA13	RA13	RA13	RA21	RA21	RA21
F	0.3125	0.3125	0.3125	RA6	RA6	RA6	RA14	RA14	RA14	RA22	RA22	RA22
G	0.161	0.161	0.161	RA7	RA7	RA7	RA15	RA15	RA15	-ve Cont.	-ve Cont.	-ve Cont.
H	Blank	Blank	Blank	RA8	RA8	RA8	RA16	RA16	RA16	+ve Cont.	+ve Cont.	+ve Cont.

2.3.9 Competitive ELISA Detection of Serum Auto-antibodies

A competitive ELISA system was developed in order to allow detection of CRP auto-antibodies within human serum samples. This system allowed sensitive detection and avoided interaction between antibodies, blocking solutions and other components of the system. The principle is based upon competition between the auto-antibody and the CRP isoform of interest. The method varied between individual antibody/protein combinations but was based upon a consistent basic method.

2.3.9.1 ELISA Analysis of Serum Anti-p CRP IgG, IgA and IgM Auto-antibodies

A competitive ELISA assay was developed in order to identify auto-antibodies directed against pCRP as illustrated in Figure 2.4 which shows steps taken within the assay. Initially 100 µl of phosphocholine bound keyhole limpet haemocyanin (PCh-KLH), which allows pCRP binding, stabilisation and retention of conformation, was immobilised upon a Corning 96 well plate, diluted to 2µg/ml in bicarbonate buffer, sealed and incubated overnight at 4^{o}C temperature. Serum samples were prepared by diluting 1:200 in diluent alone (R&D Systems) and a second set in reagent diluent containing pCRP (Scripps Lab) at 5µg/ml and left to incubate at room temperature for an hour minimum. The plate was washed and blocked as described within section 2.3.8. pCRP (Scripps Lab) was diluted to 2µg/ml in diluent (R&D Systems) and 100µl per well applied to each plate. The plates were resealed and incubated for one hour at room temperature, before washing as previous. Samples were added to the plate in triplicate with half the plate containing diluted samples with and half without the addition of pCRP, as per Table 2.13, and left to incubate at room temperature for two hours. All buffers and reagents used within the assay are detailed in Table 2.14. Once again the plate was washed three times and 100µl detection antibody added, either goat anti-human IgG conjugated antibody (Sigma), diluted 1:30,000 in reagent diluent and incubated for 30 minutes or goat anti-human IgA conjugated antibody (Sigma), diluted 1:100,000 and incubated 1 ½ hours at room temperature.

The anti-CRP IgM assay was incubated first with mouse anti-human IgM antibody (Sigma), diluted 1:1000 in reagent diluent for 1 hour, washed 3 times and followed by a secondary streptavidin conjugated rabbit anti-mouse IgG antibody (Sigma) diluted 1:30,000 in reagent diluent. This was incubated for 30 minutes at room temperature.

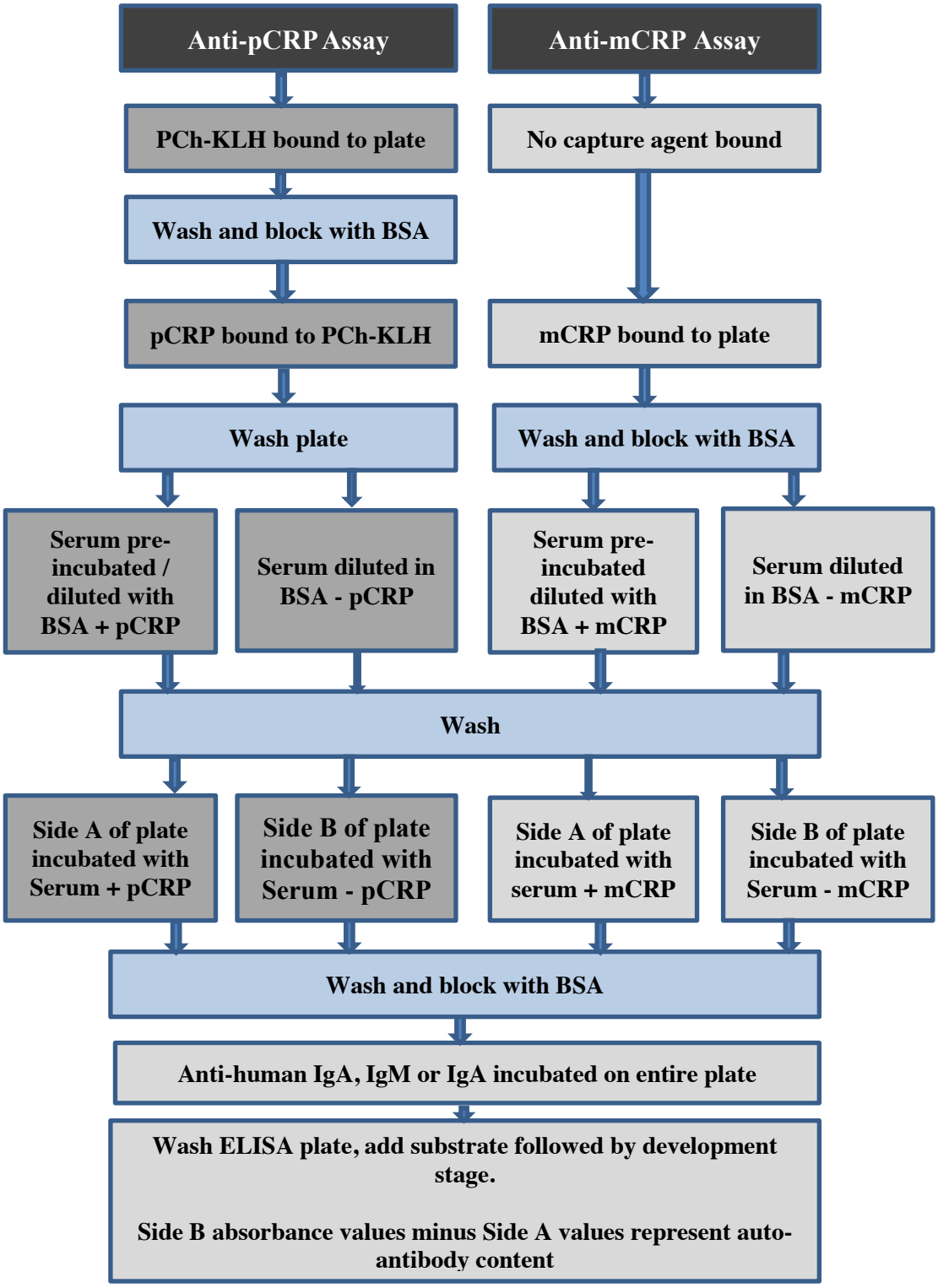


Figure 2-4 A flow diagram illustrating the steps carried out within the auto-antibody competitive detection ELISA for identification of anti-human pCRP or mCRP of IgG, IgA or IgM class.

Following a final three times wash step, each assay was developed as previously described in section 2.3.8, with the addition of 100µl substrate reagent (R&D Systems)

which was left to develop in a dark place for up to 30 minutes, before 50µl of stop solution was added (R&D Systems). Plates were then read at both 450nm and 540nm with the latter removed prior to calculations in order to remove background plate absorbance. A negative control was included on each side of the plate which omitted the serum. To avoid systematic errors, samples from patients and controls were randomly mixed on the ELISA plate. Results were calculated by removing the absorbance of wells with pre incubation of CRP from those without. Wells were triplicated with the mean of the closest two values taken, each plate was also processed in triplicate.

Table 2.13 A representative ELISA plate layout for the anti-pCRP auto-antibody detection assay. The plate is coated overnight with PCh-KLH 2µg/ml as a capture agent. Rows 1-6 contain contained 1:200 dilution of serum in reagent diluent only and rows 7-12 contained 1:200 dilution of serum preincubated in reagent diluent containing 2µg/ml pCRP. Samples were randomly allocated across the plate in triplicate.

Anti-Pentameric CRP Auto-antibody ELISA Plate Layout												
	1	2	3	4	5	6	7	8	9	10	11	12
A	RA01	RA01	RA01	RA09	RA09	RA09	RA01	RA01	RA01	RA09	RA09	RA09
B	RA02	RA02	RA02	RA10	RA10	RA10	RA02	RA02	RA02	RA10	RA10	RA10
C	RA03	RA03	RA03	RA11	RA11	RA11	RA03	RA03	RA03	RA11	RA11	RA11
D	RA04	RA04	RA04	RA12	RA12	RA12	RA04	RA04	RA04	RA12	RA12	RA12
E	RA05	RA05	RA05	RA13	RA13	RA13	RA05	RA05	RA05	RA13	RA13	RA13
F	RA06	RA06	RA06	RA14	RA14	RA14	RA06	RA06	RA06	RA14	RA14	RA14
G	RA07	RA07	RA07	+ve Cont.	+ve Cont.	+ve Cont.	RA07	RA07	RA07	+ve Cont.	+ve Cont.	+ve Cont.
H	RA08	RA08	RA08	-ve Cont.	-ve Cont.	-ve Cont.	RA08	RA08	RA08	-ve Cont.	-ve Cont.	-ve Cont.

Table 2.14 A list of reagents used within the ELISA protocol, both monomeric and pentameric CRP auto-antibody detection antibodies were the same, all buffers within the pCRP assay contained an additional 2mM $CaCl_2$ enabling pCRP binding to phosphocholine bound Keyhole limpet hemocyanin (PCh- KLH)

Buffer Solution	Components	Concentration
PBS pH 7.4	1 PBS tablet (Sigma) / 200ml deionised water	One tablet dissolved in 200 ml of deionised water yields 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride.
PBS – Tween	PBS	1.4 litre
	Tween	750 ml
Blocking Solution	R&D Reagent Diluent	10x dilution in deionised H_2O provides 1% BSA
Bicarbonate Coating Buffer pH 9.6	Bicarbonate/carbonate	100mM
Capture antigen 2µg/ml	PCh- KLH (Santa Cruz Biotechnology, sc-396490)	20µl
	Bicarbonate Buffer	9.980ml
Primary Antibody IgG 1:30,000 Dilution	Conjugated Goat Anti-Human IgG (Sigma,)	0.5µl
	Reagent Diluent	14.9995ml
Primary Antibody IgA 1:100,000 Dilution	Conjugated Goat Anti-Human IgA (Sigma)	0.5µl

	Reagent Diluent	49.9995ml
Primary Antibody IgM 1:1000 Dilution	Mouse Anti-Human IgM (Sigma)	15µl
	Reagent Diluent	14.985
Secondary Antibody IgM Assay 1:30,000 Dilution	Rabbit Anti-Mouse IgG (Sigma)	0.5µl
	Reagent Diluent	14.9995ml
Colour Substrate (R&D Systems)	Colour Substrate A (H_2O_2)	6.0ml
	Colour Substrate B (Tetramethylbenzidine)	6.0ml
Stop Solution (R&D Systems)	Stop Solution (2 N H_2SO_4)	5.0ml

2.3.9.2 ELISA Analysis of Serum Anti-mCRP IgG, IgA and IgM Auto-antibodies

Whilst many key aspects of the method highlighted within the anti-pCRP ELISA remain the same (section 2.3.9.1), key differences are detailed here with an overview illustrated previously within Figures 2.3 and 2.4. In order to identify auto-antibodies directed against mCRP, 100 µl of mCRP (previously created *in vitro* via 2M urea dissociation, detailed within section 3.2.2) was immobilised upon a Corning 96 well plate, diluted to 5µg/ml in bicarbonate buffer, sealed and incubated overnight at 4^{o}C temperature. Serum samples were prepared by dilution 1:200 in reagent diluent alone (R&D Systems) and a second set in reagent diluent containing mCRP at 2µg/ml and left to incubate at room temperature for an hour minimum. The plate was washed and blocked as previously described. Serum samples were added to the plate in triplicate with half the plate containing diluted samples pre-incubated with mCRP and the other half without the

addition of mCRP, as per table 2.15 and left to incubate at room temperature for two hours. Once again the plate was washed three times and 100µl detection and secondary conjugated antibodies were added as detailed section 2.3.9.1. The plates were also developed with the same previously mentioned method and once more read at both 450nm and 540nm with the latter removed prior to calculations in order to remove background plate absorbance. To avoid systematic errors, samples from patients and controls were randomly mixed on the ELISA plate. Negative controls were added to the plate on each side by the omission of sera. Results were calculated by removing the absorbance of serum wells pre-incubate with mCRP from those without.

Table 2.15 A representative ELISA plate layout for the anti-mCRP auto-antibody detection assay. The plate is coated with 2µg/ml urea dissociated mCRP as a capture agent. Rows 1-6 contain 1:200 dilution of serum preincubated in reagent diluent containing 2µg/ml mCRP and rows 7-12 contain 1:200 dilution of serum in reagent diluent only. Samples were randomly allocated across the plate in triplicate.

Anti-Monomeric CRP Auto-antibody ELISA Plate Layout												
	1	2	3	4	5	6	7	8	9	10	11	12
A	RA01	RA01	RA01	RA09	RA09	RA09	RA01	RA01	RA01	RA09	RA09	RA09
B	RA02	RA02	RA02	RA10	RA10	RA10	RA02	RA02	RA02	RA10	RA10	RA10
C	RA03	RA03	RA03	RA11	RA11	RA11	RA03	RA03	RA03	RA11	RA11	RA11
D	RA04	RA04	RA04	RA12	RA12	RA12	RA04	RA04	RA04	RA12	RA12	RA12
E	RA05	RA05	RA05	RA13	RA13	RA13	RA05	RA05	RA05	RA13	RA13	RA13
F	RA06	RA06	RA06	RA14	RA14	RA14	RA06	RA06	RA06	RA14	RA14	RA14
G	RA07	RA07	RA07	+ve Cont.	+ve Cont.	+ve Cont.	RA07	RA07	RA07	+ve Cont.	+ve Cont.	+ve Cont.
H	RA08	RA08	RA08	-ve Cont.	-ve Cont.	-ve Cont.	RA08	RA08	RA08	-ve Cont.	-ve Cont.	-ve Cont.

The ELISA techniques used within each detection assay are summarised within Table 2.16, which details which form of ELISA was used in the determination of mCRP, pCRP or auto-antibodies directed against each.

Table 2.16 A summary table of ELISA assays used within the detection of either mCRP , pCRP or autoantibodies directed against each of them.

ELISA	Detection Target	Capture Ab	Antigen	Detection Ab	Further Ab
Indirect Sandwich ELISA	mCRP	Goat anti-human CRP	mCRP	3H12	Anti-mouse IgG
Indirect Sandwich ELISA	pCRP	R&D mouse anti-human pCRP	pCRP	R&D mouse anti-human pCRP (Biotinylated)	Streptavidin conjugated to HRP
Competitive ELISA	Anti-human pCRP IgA/IgM/IgG	PCh-KLH	pCRP	Human Serum	Anti-human Iga/IgM/IgG
Competitive ELISA	Anti-human mCRP IgA/IgM/IgG		mCRP	Human Serum	Anti-human Iga/IgM/IgG

2.3.10 Statistical Analysis

All data analyses and statistical tests were carried out in Minitab® Statistical Software (Version 18). Power analysis was used to determine the validity of statistical analyses to detect significant differences in the pilot study data. A minimum sample size of 21 and 13, for mCRP and pCRP respectively, was needed to yield a power of 0.8. Hence the study sample size of 30 was valid and exceeded the minimum requirements. Quantitative data was graphically displayed to check distribution modality and Anderson-Darling normality test run to confirm normality ($p>0.05$) to ensure underlying statistical

test assumptions were met. The mCPR or pCRP auto-antibody group data did not have universally normal distributions and a Box-Cox transformation did not resolve the situation so raw values were used. Central tendencies are reported as mean ±SEM and median + interquartile range (IQR). A Mann-Whitney test was used to detect differences in mean CRP concentration and normalised auto-antibody data by gender (male, female) and health condition (healthy, rheumatoid arthritis). Spearman's rank was used to test for the correlation of mCRP concentration with pCRP concentration, CRP concentrations and normalised auto-antibody percentages and between auto-antibody groups.

During initial statistical analysis it became apparent that the male cohort auto-antibody data was affecting analysis and in many cases the combination of male and female data sets produced bimodal data making detailed analysis based on comparison of mean or median group data unreliable, in this case the groups were split by gender prior to statistical analysis. A Chi-Square test was used to test for an association in the number of samples with elevated levels of auto-antibodies between in the RA and control groups. All tests yielding P values of less than 0.05 were considered statistically significant, with significance levels of 0.05, 0.01 and 0.001 reported.

Chapter 3 - Production of Monomeric C-reactive Protein

3.1 Introduction

3.1.1 Overview

The structure of native human pCRP was determined via x-ray crystallographic studies in 1996 by Shrive *et al* who identified five protomers arranged around a central pore (Shrive *et al*., 1996). Interprotomer contacts result predominately from salt bridge interactions which stabilise the pentameric structure. Furthermore, the native conformation is found to be extremely stable under normal physiological conditions. Studies have reported an additional form of CRP is present within the body that of a discrete protomer referred to as monomeric CRP (mCRP). Growing evidence now supports the existence of this modified form although it is as yet unclear as to whether this isoform of CRP is membrane bound or soluble within the serum (Ying *et al*., 1989; Zhao and Shi, 2010; Ciubotaru *et al*., 2005; Sjowall and Wettero, 2007; Potempa *et al*., 1988; Zouki *et al*., 2002; Wang *et al*., 2007b; Singh *et al*., 2009).

Researchers report the monomeric form displays reduced solubility and a change in isoelectric point resulting in a reduction of pI from 6.4 to 5.4, pointing to possible disruption of the subunit structure (Potempa *et al*., 1983; Taylor *et al*., 2005). Studies suggesting the physiological existence of mCRP show the protein co-localised with various tissue including Aβ plaques, stroke neovessels, necrotic cells and upon damaged and activated cells and platelets (Slevin *et al*., 2010, 2015; Mihlan *et al*., 2011)(Ji *et al*., 2007). Emerging research supports the presence of mCRP as a serum protein *in vivo* (Wang et al., 2015). Auto-antibodies directed against the monomeric form have also been found within the serum of various disease state patients (Minatani 2001; Sjowall *et al*., 2004; Tan 2008).

This chapter will describe the production of mCRP from pCRP within an *in vitro* system, allowing its use within later work designed to identify mCRP and auto-antibodies directed against it within RA patient serum. Experimental procedures have been adapted from previously published research and seek to develop a system utilising methods as close to physiologically relevant as possible. This chapter will discuss the development of *in vitro* monomeric C-reactive protein, its stability and characterisation.

3.1.2 Dissociation of Pentameric CRP *in vitro*

The production of mCRP was first described by Potempa *et al*., in 1983 and detailed the methods employed to dissociate the pentamer into individual subunits. They were able to show *in vitro* that native pentameric CRP will dissociate following treatment with various and often harsh, non-physiological denaturing conditions treatments, including heat, pH4.0 and 6M urea (Potempa et al., 1983, Kresl et al., 1998). Other groups reported pentamer dissociation following calcium dependent binding to membranes, including cell membranes and liposomes, forming an intermediate membrane bound form of monomeric CRP (Ji *et al*., 2007), and also during interaction with activated platelets (Eisenhardt *et al*., 2009). Following on from Potempa *et al*., (1983), other groups explored methods of dissociation, including Kresl *et al*., (1998) who studied the effects of 8M urea and 10mM EDTA which caused a rapid and irreversible dissociation of pentameric CRP into monomers within two minutes. They were able to show at 30 minutes only partial unfolding using 6M urea which reached 88% dissociation after 60minutes. The earlier study by Potempa *et al*., (1983), reported a change in electrophoretic mobility and new antigenic properties following 6M urea or heat to 63^{o}C for five minutes. These changes were only apparent when calcium concentrations fell below 0.7mM (Potempa *et al*., 1983). However, these methods of dissociation are extremely harsh and would not be replicated

within the body. It is therefore important that *in vitro* treatments developed reflect physiologically relevant conditions where possible, and that these must also be replicable and reliable. The first conditions trialed took place within a Tris, sodium chloride, calcium (5mM) buffer at pH4, which is outside the range of acidosis found even within some infection patients (Waugh & Grant, 2014) and therefore not physiologically relevant.

3.1.3 Experimental Aims

The aim of experimentation within this chapter was to develop a technique which was both accurate and reproducible, and not too harsh, allowing production of a constant supply of mCRP for use within further experimentation. This would be used as a capture protein within ELISA studies, and western blot analysis of serum samples. It was also required to determine the specificity and affinity of antibodies used throughout the research. Patient serum analysis requires a high concentration of pure, stable monomeric CRP stock solution, therefore a reliable method of preparation and storage is essential. Retention of the structural and functional integrity of the protein was required and as such production methods were developed to produce a more physiologically relevant form of the protein. It became essential that methods replicating the dissociation found within studies at 6M urea be sought, however, using less harsh denaturing effects and therefore allowing a more meaningful comparison of future findings with those physiologically possible *in vivo*.

3.2 Materials and methods

3.2.1 C-reactive Protein Storage

Native pentameric C-reactive protein (pCRP) was purchased from SCRIPPS laboratories (C0129), at a concentration of 2.43mg/ml (confirmed to be >99% pure by SDS PAGE analysis). The protein was supplied within a standard storage buffer composed of 20mM Tris, 280mM NaCl, 5mM $CaCl_2$, 0.01% sodium azide pH 8.0 and was stored at 4^{o}C until needed. These storage conditions were adopted for dissociated samples following purification and concentration.

3.2.2 Dissociation of Pentameric CRP

Dissociation methods were tested to assess the least harsh and non-denaturing dissociation treatments, presumed closest to physiological relevant conditions, allowing production and collection of pure mCRP. The first trials assessed the ability to dissociate pentameric CRP (SCRIPPS) in association with calcium (5mM). Results from dissociation treatments were analysed by both native PAGE and FPLC, allowing identification and separation/purification of the monomer from part dissociated samples.

Acidic conditions were assessed at pH 4.0 and pH 6.0, in both calcium free and 5mM calcium buffer. Calcium free buffer (20mM Tris, 280mM NaCl and 1mM EDTA) was adjusted to pH4 or pH6 using 2M HCL. 50µl of CRP (121.5µg) was added to 24.95ml of either pH4 or pH6 buffer which was further incubated at room temperature for 5 minutes before readjustment to pH8 using 2M NaOH. This dilution was concentrated as described within section 2.3.5 to 500µl and a concentration of approximately 200µg/ml CRP determined via Nano drop spectrophotometer as described within section 3.2.5. In a further experiment testing the combined effects of temperature and acidity the CRP/pH4 calcium

free, 1mM EDTA sample was heated to 63^{o}C for 5 minutes before rapidly cooling on ice. This condition was repeated in 5mM $CaCl_2$.

Standard Tris buffer (20mM Tris, 280mM NaCl) with the addition of urea to a final concentration of either 2M or 6M including added CRP, was tested for the ability to dissociate CRP. Once again in both calcium free (1mM EDTA), and calcium (5mM $CaCl_2$) conditions. Initially 50µl (121.5µg) of CRP was added to 450µl buffer and incubated at 4^{o}C; this was optimised in later trials and increased to 100µl (243µg) CRP with 400µl buffer, providing greater levels of resulting mCRP due to a higher ratio of CRP to buffer. These treatments were incubated at 4^{o}C for various periods between two days and 12 weeks to assess optimum dissociation times. Dissociation was determined by both native PAGE and FPLC size exclusion chromatography.

The possible re-association of successfully dissociated, monomeric CRP was tested by native gel and FLPC size exclusion chromatography, following up to 12 weeks storage at 4^{o}C in standard pH8, 20mM Tris, 280mM NaCl buffer.

3.2.3 Calibration of Gel Filtration Size Exclusion Chromatography Column

The Fast Protein Liquid Chromatography (FLPC) system with a HiLoad 16/60 Superdex 200pg Column was used to identify and purify mCRP and pCRP. Prior to dissociation experimentation the FPLC was fully calibrated using a Gel Filtration Calibration Kit (GE Healthcare). This process is described within section 2.3.3.

3.2.4 Purification of Pentameric and Monomeric C-reactive Protein

Determination of the molecular weight of the pentameric form of CRP following dissociation or reassociation trials was achieved by the use of an AKTA explorer 100, Fast Protein Liquid Chromatography (FPLC) system (GE Healthcare). The system, software and column are the same as described within section 2.2.1 and all buffers and components

detailed within Table 2.3. The column was pre-equilibrated using one column volume ~120ml, of filtered deionised water followed by two column volumes of calcium free elution buffer (20mM Tris, 280mM NaCl, pH 8.0) as described within section 2.3.4. Initial trials involved 500µl of treated CRP sample ~ 200µg/ml, injected onto the 2ml sample loop and loaded onto the HiLoad 16/60 Superdex 200 pg column (GE Healthcare). A flow rate of 1ml per minute using calcium free elution buffer was used in all assays. Fractions were collected in separate 5ml elution volumes within 20ml universal tubes. This method was used to determine molecular weight and purity for the native pentamer, pH6, pH4, heat treated and urea treated samples. It was further used to determine possible reassociation of monomeric CRP samples. Optimum dissociation conditions were assessed and further experiments continued with 100µl CRP and 400µl buffer to a final concentration of 2M urea.

3.2.5 Quantification of C-reactive Protein

Following sample concentration, the amount of protein within each sample was measured by NanoDrop spectrophotometer, which allowed 2µl to be tested at $A_{280.}$ The NanoDrop was first blanked with deionised water followed by sample buffer which should result in a reading no greater than 0.04 absorbance units from the baseline 280nm reading. The buffer is then reloaded and system blanked before adding the protein sample. A molar extinction coefficient must be used to quantify the protein, which is directly related to the tryptophan, tyrosine and cysteine amino acid content of the protein. The coefficient for human CRP was calculated using the online programme *ProtParam* from Expasy (http://web.expasy.org/protparam/). This program uses the protein sequence to calculate the coefficient which for human CRP is 1.7. The absorbance at 280nm divided by the coefficient results in the concentration in mg/ml.

3.2.6 Native Polyacrylamide Gel Electrophoresis

After isolation of pentameric and monomeric CRP fractions following FPLC, and subsequent concentration, samples were analysed using native PAGE to determine the purity and subunit size of CRP within the eluted fractions. Native pentameric and dissociated samples of CRP at a minimum of 1µg (typically 10µl) were combined 1:1 with a non-reducing, non-denaturing sample buffer and loaded onto a native PAGE non-gradient gel (12.5% resolving, 4% stacking) with or without the addition of 1/20th SDS as detailed within section 2.3.6, to both sample and running buffers. Standard low or high molecular weight markers were used (Amersham) in order to estimate the band molecular weight. CRP does not reliably correspond to these markers in native gels due to differences in pI, resulting in variations within charge to mass ratios between proteins; therefore the position of resulting bands was correlated with the elution peaks determined within the FPLC assay. The protein molecular weight could be calculated from the calibration previously performed on the FLPC system as described within section 3.2.3, allowing enhanced determination of mCRP and native CRP (SCRIPPs) molecular weights.

All gels were electrophoresed between 55 – 90 minutes, at either 100V or 200V utilising a BioRad Mini-PROTEAN II electrophoresis system, within 500ml running buffer. Bands present were visualised using Instant Blue (Expedeon), or Bright Blue (BioRad) Coomassie staining between 30 minutes and one hour. Gels were gently washed in deionised water and scanned on a Syngene GBOX image detector. Alternatively, resulting gels were analysed by western blot rather than Coomassie stain.

3.2.7 Western Blotting of C-reactive Protein Samples

Protein samples of interest were further analysed following FPLC purification and native PAGE by western blotting in order to ensure bands present were in fact recognised

by specific antibodies to CRP. Samples were quantified as described within section 2.3.6 and calculated to ensure 1μg protein was loaded to each well before separation by native PAGE (see section 3.2.7). The polyacrylamide gel was run until the dye front left the gel bottom was then carefully removed and resolving/stacking gels separated.

The resulting resolving gel was then placed within a Tris/Glycine transfer buffer (Table 2.4) for up to 30 minutes in order to reach equilibrium together with the 0.2μm nitrocellulose membrane, filter paper and fibre pads. Once equilibrated a 'sandwich' was made of the fibre pads, filter paper, nitrocellulose membrane and gel, clamped within the cassette, and loaded together with a pre frozen ice pack within a Mini Trans-Blot Electrophoretic Transfer Cell (BioRad) topped up with 500ml transfer buffer. The transfer unit was kept at 4^{o}C and run at either 80V for two hours of 30mA overnight.

Once transfer was complete the membrane was carefully removed from the cassette and rinsed in deionised water before being placed within a tray containing 20ml Blocking Solution (see Table 2.6) and incubated overnight whilst agitating on a mechanical plate rocker. The membrane was then washed five times with BLOTTO solution (see Table 2.6), rocking for five minutes each wash. The primary antibody used was Clone-8 (Sigma Aldrich) a monoclonal mouse anti-human CRP antibody supplied in ascites fluid. This antibody was diluted 1:1000 with BLOTTO and incubated with the membrane overnight at 4^{o}C, again whilst rocking. Following a second series of five times five minute washes in fresh BLOTTO, the membrane was then incubated at room temperature for one hour with a 1:40000 dilution (in BLOTTO) of the secondary antibody, a polyclonal rabbit, anti-mouse IgG antibody, HRP conjugate (Sigma Aldrich, A9044). The membrane was then washed six further times for five minutes in BLOTTO before visualisation via chemiluminescence was achieved as described within section 2.3.7.

3.3 Results

3.3.1 Calibration of the Size Exclusion Gel Filtration System

Size exclusion chromatography was used to purify and separate dissociated monomeric CRP from remaining pentameric native CRP and therefore assess the effectiveness of dissociation methods. Initially a calibration of the FPLC system was performed using proteins of known molecular weight, Ferritin 440kDa, Aldolase 158kDa, Conalbumin 75kDa, Ovalbumin 44kDa and Ribonuclease A 13.7kDa, using the methods previously described within section 2.3.3. Figure 3.1 shows the chromatography trace for the molecular weight standards. Blue Dextran a high molecular weight glucose polymer, was also used in order to calculate the void volume of the column which was 46.79ml.

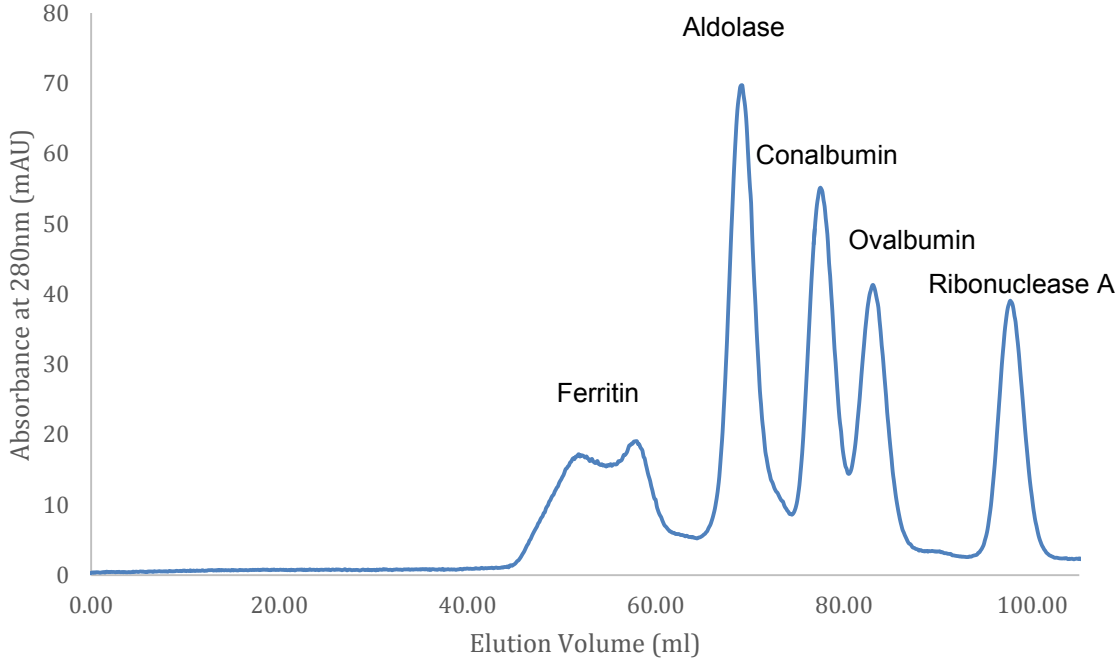


Figure 3-1 A size exclusion chromatography trace for the molecular weight standards calibration - Ferritin 440kDa (59.67ml), Aldolase 158kDa (70.62ml), Conalbumin 75kDa (79.04ml), Ovalbumin 44kDa (84.75ml) and Ribonuclease A 13.7kDa (99.38ml).

The resulting elution volumes relative to molecular weight of each protein were used to construct a calibration graph after determining the Kav value for each which was calculated using the following equation:

Equation 3.1

Kav = (Elution Volume – Void Volume) / (Total Column Volume – Void Volume)

The void volume was calculated to be 46.79ml, taken from the Blue Dextran chromatography run and the column volume calculated to be 124.5ml.

Table 3.1 A table illustrating protein standards their elution values, calculated Kav and Log of molecular weights.

Protein Standard	Molecular Weight (kDa)	Elution Volume (Ve) (ml)	Kav	Log of Molecular Weight
Ferritin	440	59.67	0.150	5.64
Aldolase	158	70.62	0.289	5.20
Conalbumin	75	79.04	0.397	4.88
Ovoalbumin	44	84.75	0.470	4.64
Ribonuclease A	13.7	99.38	0.656	4.14

Table 3.1 contains calculated values for each protein standard's Kav and the Log of each molecular weight. The two values were plotted against each other in order to construct the calibration graph shown in Figure 3.2.

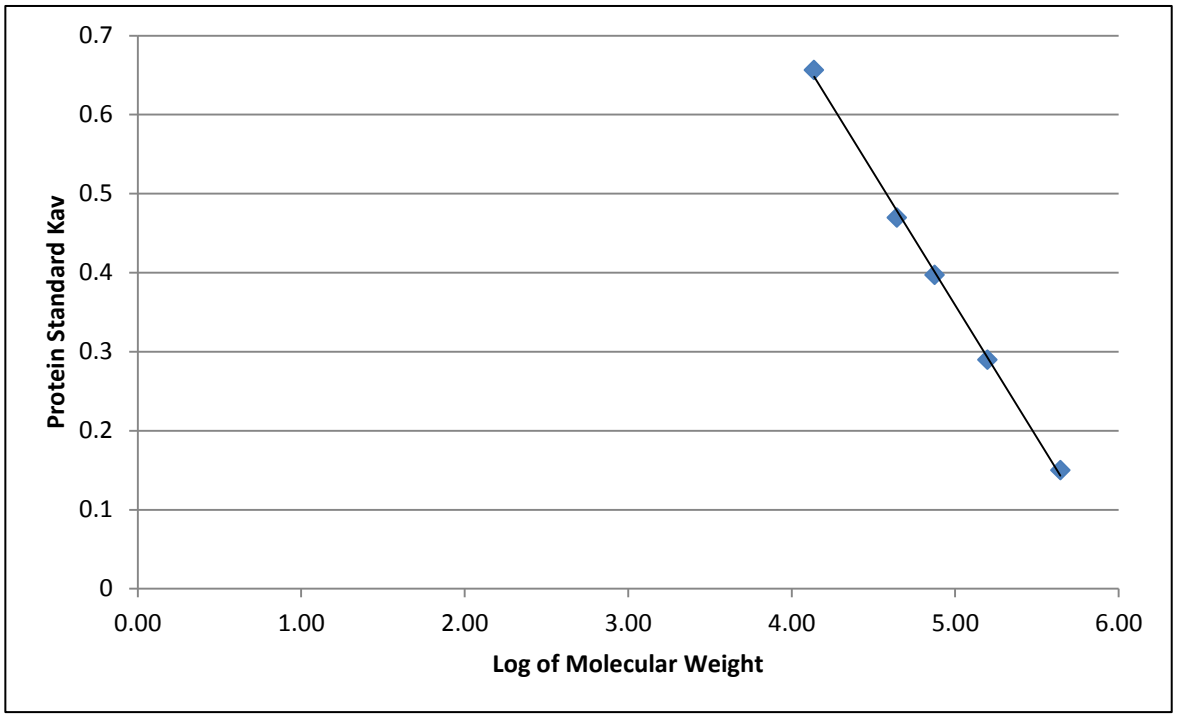


Figure 3-2 A calibration graph showing both Kav and Log of molecular weight values for each protein standard. The R^2 value is 0.9986 and the line equation: $y = -0.3348x + 2.0329$.

The calibration graph shows the relationship between molecular weight (MW) and elution volume, as Kav (representative of elution volume) rises Log molecular weight reduces, producing an R^2 value of 0.9986 indicating a strong correlation between the two data sets. The line equation: $y = -0.3348x + 2.0329$ describes an inverse relationship between molecular weight and elution volume where the latter rises as the former increases. The equation allowed accurate determination of a protein's molecular weight from its size exclusion chromatography elution volume, and enabled monomeric or pentameric CRP conformation characterisation to be established within further experiments.

3.3.2 Dissociation of Native Pentameric C-Reactive Protein

Following FPLC calibration, native pentameric CRP (Scripps Labs) was tested to ensure correct analysis of the protein size. The resulting ~17 mAU peak at 73ml elution volume illustrated in Figure 3.3, was used to calculate native CRP molecular weight as 115kDa using the equation constructed from calibration graph data illustrated within section 3.3.1. This allowed confidence within the determination of CRP conformation by FPLC and provided a comparison for pentameric CRP in future samples.

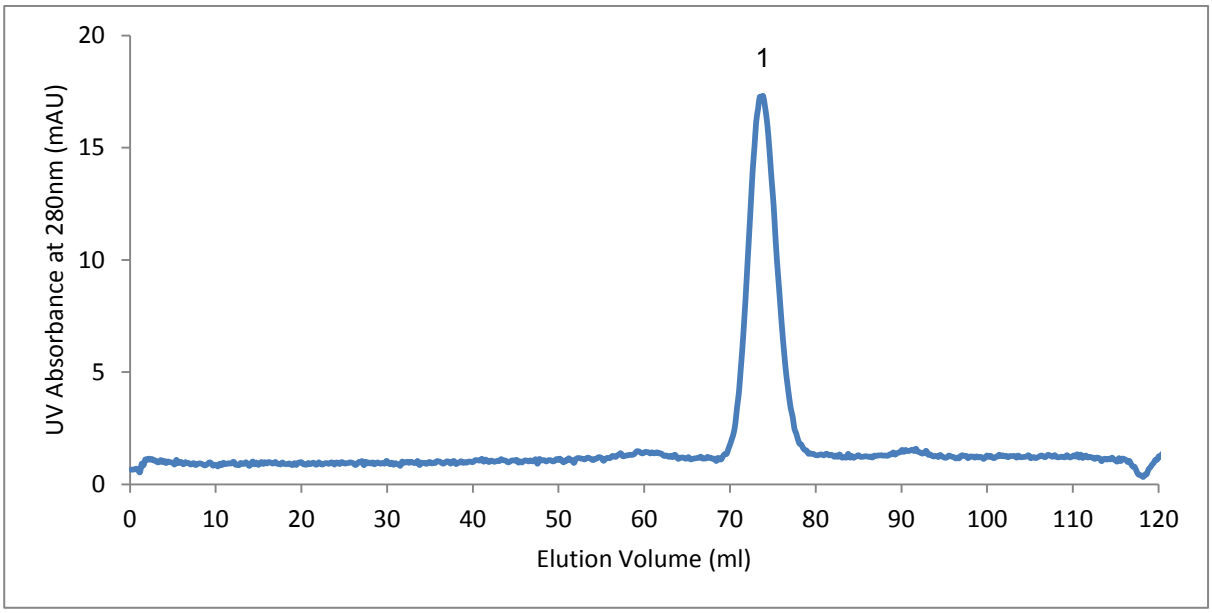


Figure 3-3 A size exclusion chromatography trace for the pCRP control assay. 200µg of the protein was run through the column. The main peak on the chromatogram, labelled 1, has an elution volume of 73ml, (17mAU).

The native pentameric (SCRIPPS) sample applied to the FPLC column in Figure 3.3, resulted in an elution volume of 73ml, which calibrated correctly to 115 kDa, known to be the native conformation molecular weight.

3.3.2.1 The Effects of pH4 and Calcium on CRP

Initial dissociation conditions tested explored the effects of pH on the stability of pentameric C-reactive protein. Using the equation determined from calibration data in

section 3.3.1 it was calculated that an elution peak resulting from the monomeric form of CRP would be found in the region of 91ml, which in turn calibrated to 23,508 Daltons, consistent with that of the monomer, allowing confirmation of its position in future studies.

This treatment provided little or no dissociation as can be seen in Figure 3.4 (a.), in which the chromatograph shows one elution peak at 73ml consistent with that of the pentamer and an absence of a secondary monomeric peak – expected to be in the region of 91ml elution. This condition was unable to initiate dissociation, therefore calcium was omitted and 1mM EDTA, a calcium chelator, included in the next condition trialed. An omission of calcium within the dissociation buffer resulted in a small amount of dissociation, as can be seen in Figure 3.4 (b.) which shows both a 73ml pentameric elution peak (8 mAu), together with an additional 91ml elution peak (0.3 mAu), indicating the presence of the monomer. The third peak at 110-117ml, visible on many chromatographs is presumed to be degraded protein of low molecular weight.

Under the calcium free conditions less than 4% of pentamer was dissociated, indicating more extreme dissociation methods were required (calculated by comparison of the UV absorbance of treatment peaks against control peaks). Heat was therefore applied to the pH4 EDTA sample (63^{o}C), which caused slight precipitation, removed by centrifugation before the FPLC assay. Figure 3.4 (c.) shows the chromatograph of the heat/acid conditions and it is apparent that a greater amount, approximately half that of pentameric CRP, was discovered at 110ml (~2400Da) indicating a breakdown of protein. No monomeric fraction was observed at 91ml elution volume and the recovery of pentameric protein was greatly reduced from that seen in either 3.4 (a.) or (b.).

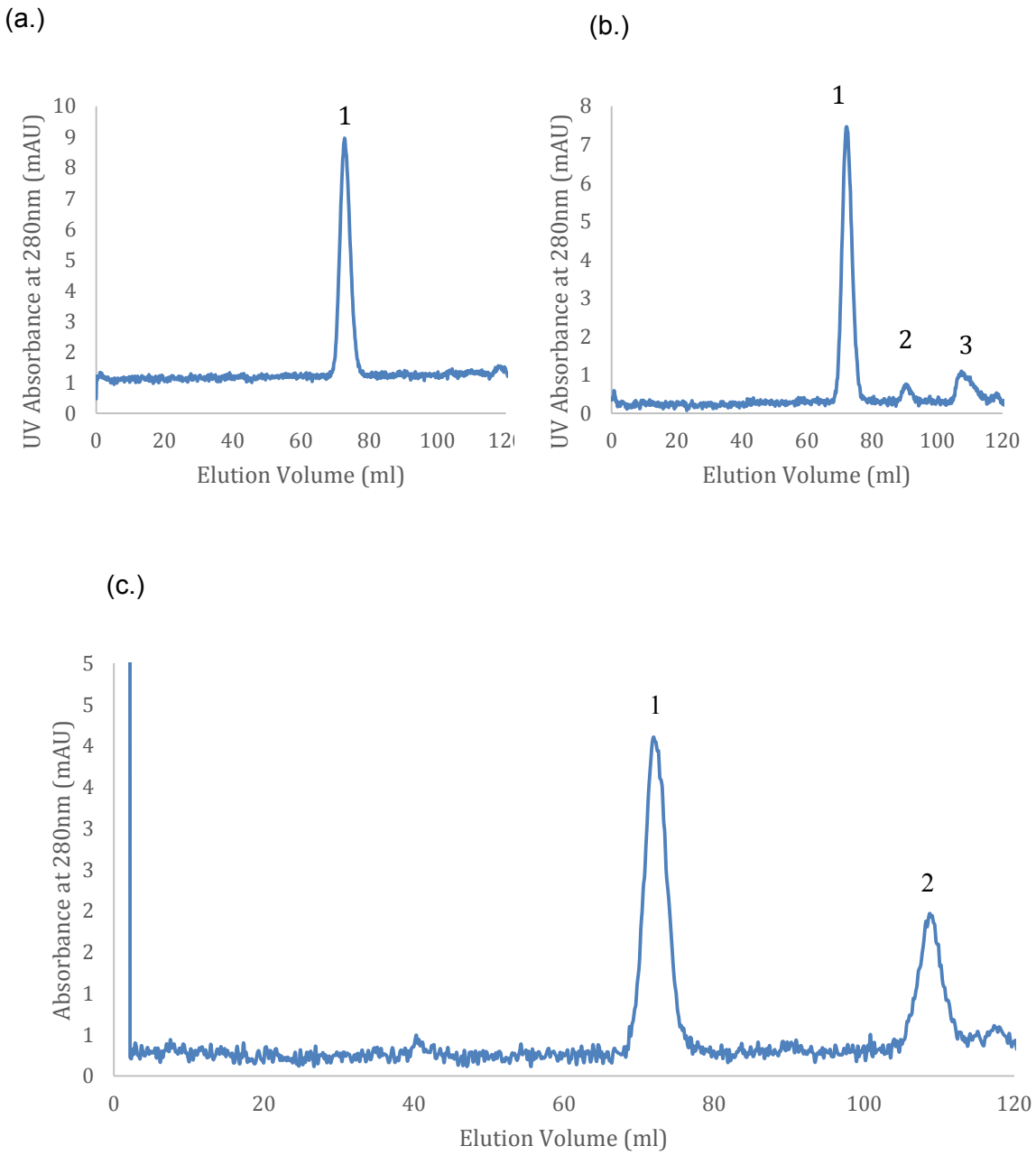


Figure 3-4 Size exclusion chromatograph trace showing the effects of pH4, with and without calcium and heat (a.) Chromatograph of pH4, 5mM $CaCl_2$ unheated CRP following 6 days incubation, showing a pentameric peak labelled 1, (b.) Chromatograph of pH4, EDTA unheated CRP following 14 days incubation, showing a pentameric peak (labelled 1) and small monomeric peak (labelled 2) at 91ml, together with small peptide peaks (labelled 3, 110-117ml), (c.) Chromatograph of pH4, EDTA, 63^{o}C heat treated CRP, precipitate spun out pre FPLC following 4 days incubation, showing pentameric (labelled 1) and peptide peaks (labelled 2).

The monomer was present in calcium depleted treatments only, indicating calcium's known importance in stabilising the pentameric conformation. Physiologically normal conditions are pH 7.4 and 2.2 to 2.6mM calcium (Zhang *et al*., 2009), slight acidosis and hypocalcaemia within inflammation-infection patients can result in local pH6.5 and 2 to 2.12mM calcium levels (Zhang *et al*., 2009, Aderka *et al*., 1987). These transient inflammatory states may not be of low enough pH or calcium level to dissociate the native CRP protein and are unlikely to be the sole cause of physiological monomers. It is suggested that the monomer has reduced solubility (Singh *et al*., 2009, Ji *et al*., 2009b) and this may explain the absence of a monomer, Figure 3.4(c.), following removal of the precipitate, it is possible that the dissociated CRP fraction was within this precipitate. The resulting chromatograph seen in Figure 3.4 (c.), shows a reduced amount of pentameric fraction (3.5 mAu) and increased degraded protein (1.6 mAu), but absent 91ml elution volume peak of the monomer.

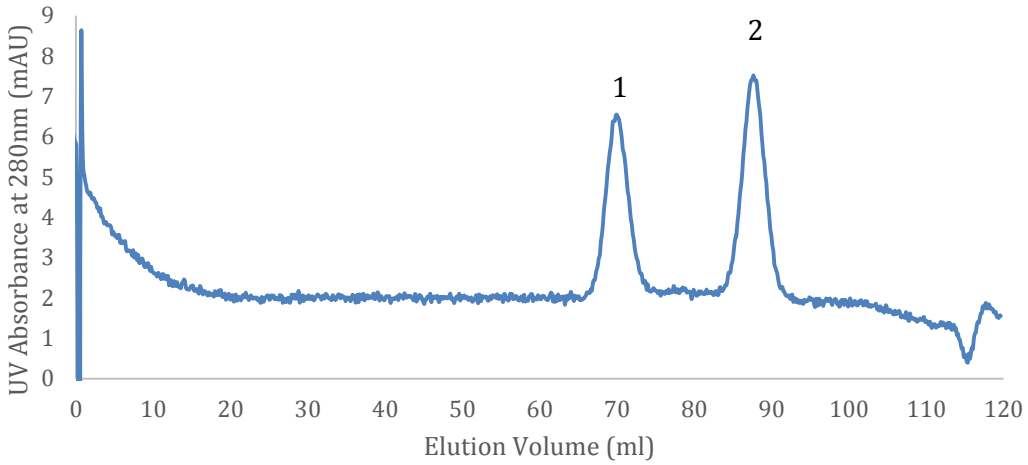
3.3.2.2 The Effects of Urea and Calcium on CRP

The amount of mCRP generated within pH4 conditions was not great enough to accumulate effective volumes of mCRP; therefore urea dissociation treatments were tested. Initial dissociation trials included both 6M and 2M urea treatments both of which showed a second peak at ~ 91ml elution volume, confirmed as the monomer position. The levels of dissociation in 6M urea conditions (data not shown) were not greatly increased to those of 2M conditions and it was therefore decided to proceed with gentler 2M conditions for all following urea trials.

Having assessed the effects of calcium inclusion within pH dissociation conditions it was necessary to determine how the addition or removal of calcium would affect dissociation rates within urea treatments. Pentameric CRP was incubated at 4^{o}C in 2M urea buffer for approximately 3 weeks with and without calcium. Figure 3.5 shows the sample

without calcium beginning to dissociate into its monomeric subunits (a.), whereas the sample with calcium (b.), has remained in a pentameric conformation.

(a.)



(b.)

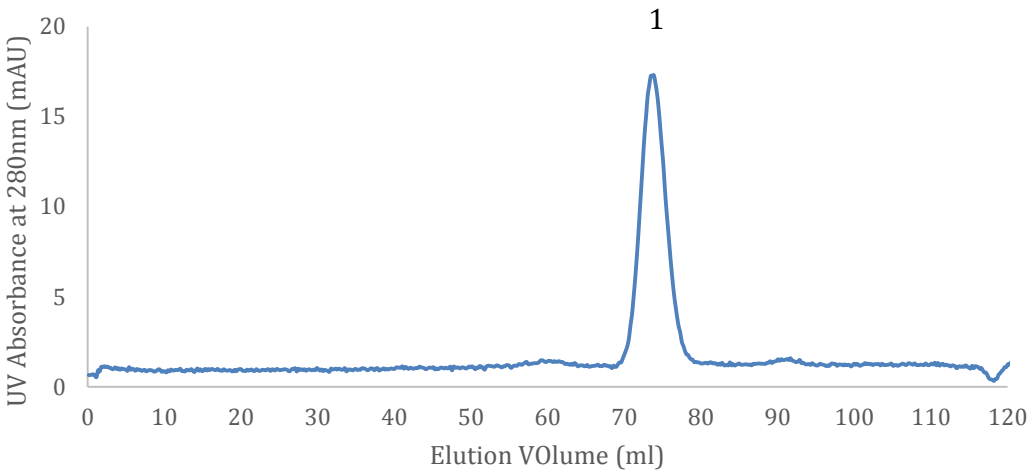


Figure 3-5 Size exclusion chromatograph trace showing the effects of 2M urea on CRP with and without calcium (a.) 2M urea and 1mM EDTA (no calcium) following 24 days incubation showing both pentamer (peak 1) and monomer (peak 2) conformations. (b.) CRP with 2M urea and $CaCl_2$ following 21 days incubation showing only the pentameric peak (labelled 1).

The dissociation effects of 2M urea are greater than those of pH4, and this condition was therefore used for the accumulation of mCRP for future studies.

Initially native PAGE was also used to detect monomer formation; however the monomeric band was never apparent, even in samples of pure monomer post FPLC separation. This problem was also encountered by Taylor *et al*., *(*2005), who suggested an insolubility of the monomer may prevent it running correctly within the gel. They advised the addition of 1/20th SDS to the running buffer and sample buffer, but not the gel itself, would allow detection of the monomer (Taylor *et al*., 2005). This method was tried, found to be successful, and used in later native PAGE gels to detect levels of dissociation in conjunction with the FPLC chromatograms. The dissociation of CRP using 2M urea and EDTA can be seen in a 1/20th SDS native PAGE, Figure 3.6 (a), lanes 5 and 6 illustrating two replicated samples analysed before FPLC separation, each with two bands (pentameric and monomeric). Lanes 2 and 3 show the separated pentameric and monomeric fragments produced following FPLC separation, and comparative native pentameric (lane 1) and monomer formed by pH4, EDTA and heat treatment (lane 4).

The stabilising effects of calcium can once again be seen in Figure 3.6 (b.), again using a 1/20th SDS native PAGE system which shows a partially dissociated sample treated with 6M urea and $CaCl_2$ in lane 4, clearly showing both monomer and pentamer bands, compared to the single monomeric band produced following 2M urea and EDTA lane 3.

(a.) (b.)

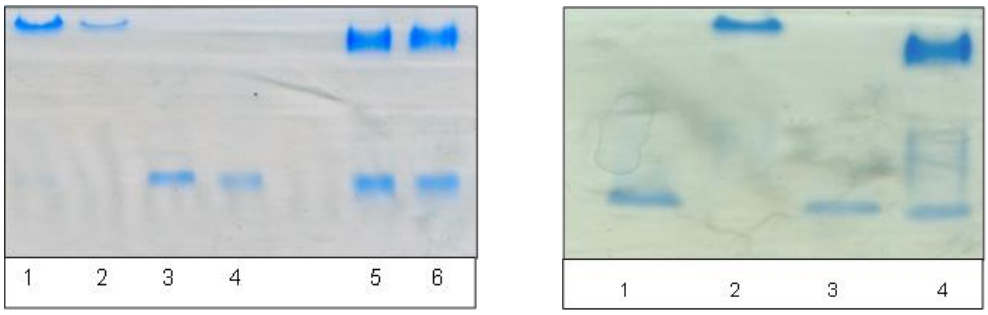


Figure 3-6 Native PAGE showing dissociation of native CRP Gels prepared with 1/20th SDS (a.) 2M urea, EDTA treated CRP, Lane 1 – pentameric CRP; Lane 2 – pCRP, 73ml FPLC elution peak; Lane 3 – mCRP, 92ml FPLC elution peak; Lane 4 - CRP pH4, EDTA, heat treated; Lanes 5 & 6 – replicate samples of 2M urea/EDTA dissociated samples pre FPLC separation showing both pCRP and mCRP. (b) Lane 1 – CRP pH4, EDTA, 63°C, showing monomer; Lane 2 – native CRP, showing pentamer, lane 3 – 2M urea, EDTA treated showing one band equivalent to the monomer, lane 4 – 6M urea, 10mM $CaCl_2$ treated, showing two bands, predominantly pentamer and also monomer. Bands are visualised following incubation for 10 minutes in Instant Blue (Expedeon).

Table 3.2 A table summarising the CRP dissociation methods trialled with representative yields gained following FPLC analysis. Urea dissociation incubations took place over between 21-24 days prior to FPLC, [a] precipitation was removed by centrifugation prior to FPLC, but assessed by native PAGE. Urea dissociation provided the greatest yield when calcium omitted, with little yield difference between 6M and 2M, therefore the least harsh dissociation condition of 2M urea 1mM EDTA (- $CaCl_2$) was taken forward.

Dissociation Method	Dissociation Conditions	FPLC Yield	Native PAGE mCRP +/-
pH4	+ $CaCl_2$	0%	-
pH4	EDTA (- $CaCl_2$)	9%	+
pH4 + heat	EDTA (- $CaCl_2$)	0% [a]	+
2M urea	+ $CaCl_2$	0%	+
2M urea	EDTA (- $CaCl_2$)	50.88%	+
6M urea	EDTA (- $CaCl_2$)	55%	+

3.3.2.3 The Effects of Incubation Time on 2M Urea Dissociation of CRP

It was then necessary to optimise the minimum dissociation time for monomer preparation, as illustrated by Figure 3.7 (a-e). Following two weeks incubation (2M urea, EDTA), a third of the protein has dissociated (a.), this dissociation increases over time until

~ 90% of the sample dissociates at 8 weeks (d.), this level of dissociation does not increase substantially even after 12 weeks (e).

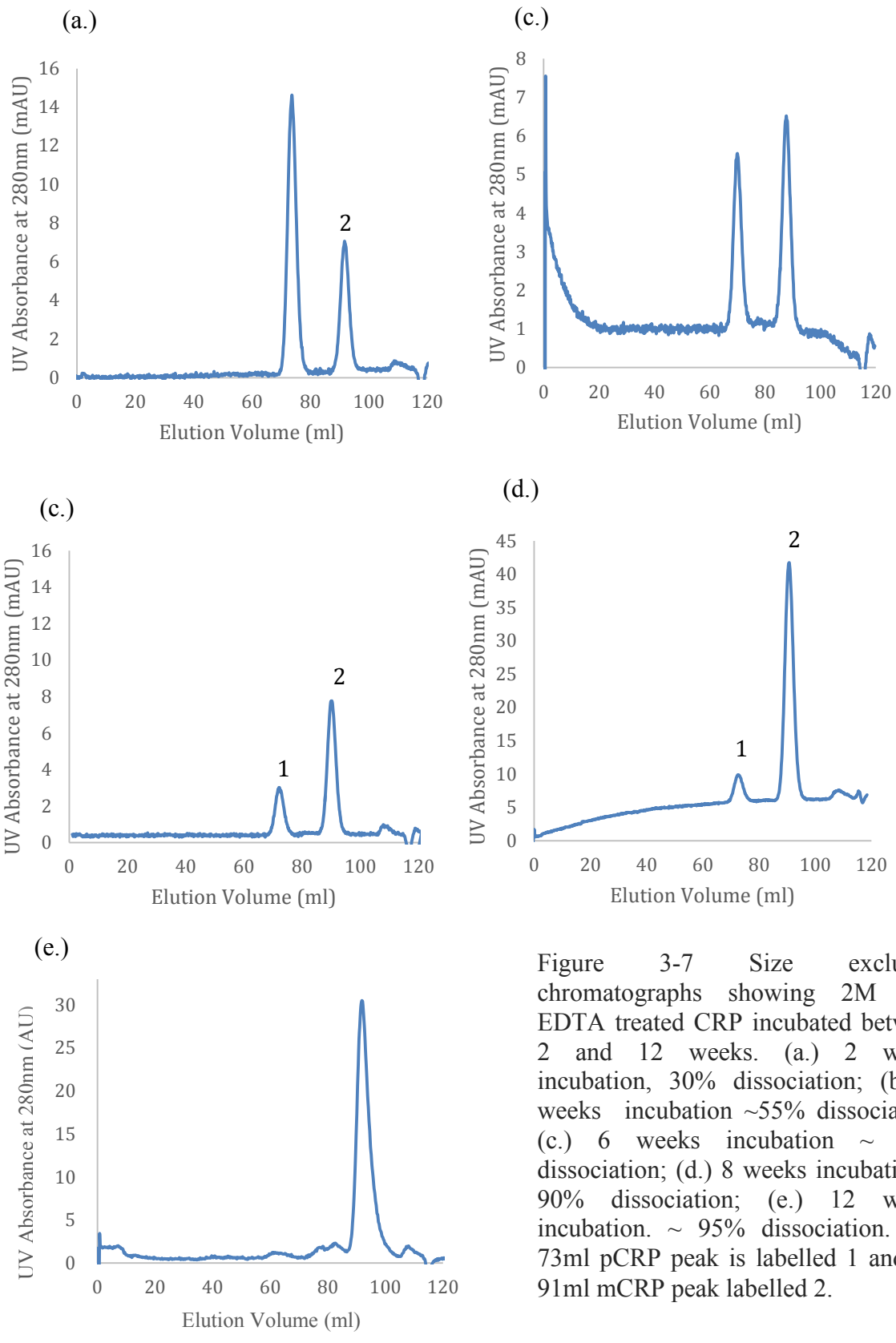


Figure 3-7 Size exclusion chromatographs showing 2M urea EDTA treated CRP incubated between 2 and 12 weeks. (a.) 2 weeks incubation, 30% dissociation; (b.) 3 weeks incubation ~55% dissociation; (c.) 6 weeks incubation ~ 75% dissociation; (d.) 8 weeks incubation ~ 90% dissociation; (e.) 12 weeks incubation. ~ 95% dissociation. The 73ml pCRP peak is labelled 1 and the 91ml mCRP peak labelled 2.

3.3.2.4 Re-association of Monomeric CRP

Whilst it was possible to produce dissociated CRP using 2M urea, it was important to determine the stability of samples during long term storage. A sample of monomer was left at 4°C for 12 weeks within both calcium containing and non-calcium EDTA buffers, and then re-assessed by FPLC in the same manner as previously described in section 3.2.4. Figure 3.8 shows a peak at the ~91ml elution volume position which correlates to the correct monomer molecular weight with an absence of pentameric peak at position 73ml. This indicates no re-association of monomer has taken place and the effects of urea during dissociation prevent future pentamer formation from the subunits created.

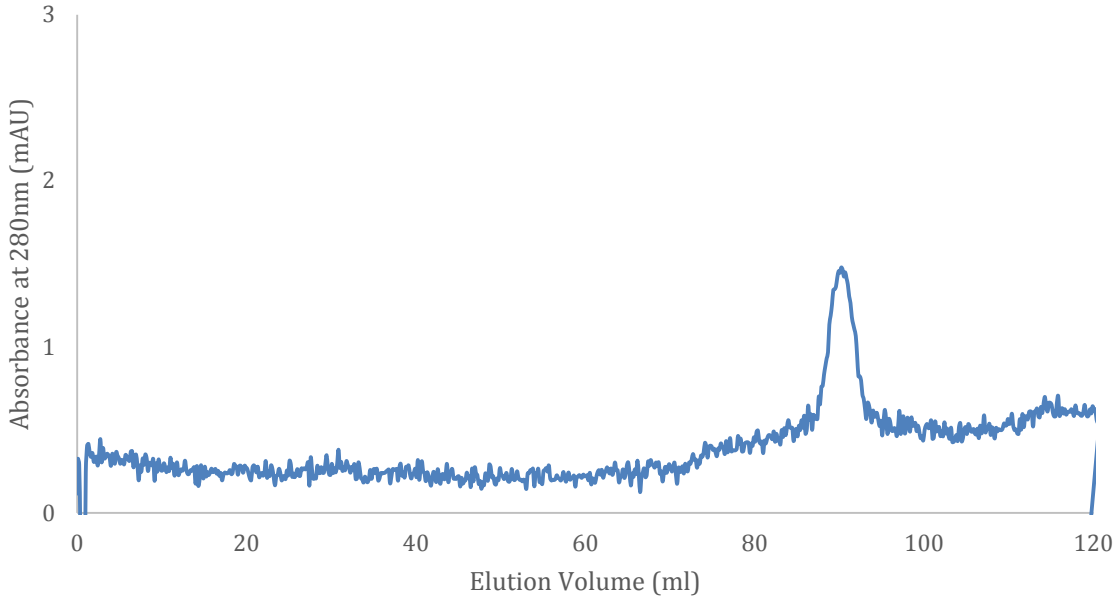


Figure 3-8 Size exclusion chromatograph showing re-association trial following 12 weeks storage of monomeric CRP previously dissociated using 2M urea. There is no apparent re-association visible with the only peak at ~91ml as indicated for the monomeric protein (23kDa).

3.4 Discussion

3.4.1 Production and Purification of *in vitro* Monomeric C-reactive Protein

The purification and isolation of different conformations of CRP were carried out by the use of size exclusion chromatography. Based on the results of the calibration of the column the elution volumes theoretically calculated for pCRP and mCRP were 73ml and 91ml respectively. The standard commercially available pCRP when applied to the column also ran at an elution volume of ~73ml (Figure 3.3) and therefore corresponded to the molecular weight expected of 115kDa. The calibration of the system allowed confident analysis of samples subjected to various dissociation techniques.

The native pentameric form of CRP has been studied and characterised extensively, however the physiological conformation of the discreet subunit mCRP, its function and creation mechanism are still under consideration. What is less in doubt is the ability to create a form of monomeric CRP *in vitro* which can in turn be studied, enabling clarification of its structure and function. It is known that over time natural low levels of dissociation are found within stored native CRP, even within physiologically relevant buffer concentrations (Slevin *et al*., 2010). It is also documented that pentameric CRP added to polystyrene flasks results in a partial dissociation of the pentamer into subunits which express novel antigenic sites as detected by specific antibody tests (Eisenhardt *et al*., 2011). It therefore suggests that the pentameric protein whilst generally stable at correct calcium and pH levels becomes less stable when in contact with and able to bind a surface, indicating a physical method of separation may be more relevant *in vivo* than environmental. Recombinant CRP monomers have been produced using modifications to selected amino acids allowing the protein to remain soluble at higher concentrations, preventing aggregation (Potempa *et al*., 2015). Whilst useful within functional studies, they

lack complete homology with the sequence of naturally occurring mCRP and as such may give rise to functions *in vitro* not naturally occurring *in vivo*.

Initial investigations using a combination of acidic conditions, heat and the removal of calcium were ascertained for effectiveness in an effort to balance the requirement to dissociate CRP with that of minimising the use of harsh dissociation techniques. Using acidic conditions of pH 4.0 within a calcium buffer resulted in no discernible dissociation, once calcium was removed however, dissociation can be seen, although at very low levels, as shown in figures 3.4, it is clear that additional calcium provided a protective effect even within these relatively mild conditions. Upon heating at pH 4.0, in the absence of calcium, the protein was denatured and appeared as a precipitate, no longer soluble at the concentration of 200ug/ml, with reduced recovery of pentameric CRP and no apparent peak indicating monomeric CRP within the sample (Figure 3.4). Whilst these conditions were not continued due to a lack of effective dissociation, they were also not conditions occurring naturally within the body. Although some diseased states result in localised drops to pH, they are not found to fall as low as pH4.0. It is reported that lactic acidosis associated with septic shock is defined upon detection of pH ≤ to 7.35, metabolic acidosis ≤ pH 7.2 (occurring with approximately 50% mortality rate) and severe lactic acidosis during clinical shock found at levels below pH 7.0, with no survival reported at this pH level or below in a study by Kimmoun *et al*., *(*2015). Within cancer tumours, localised reductions in pH have also been reported, however again these are contained within a narrow range of between pH 6.9-7.1 (Helmlinger *et al*., 1953). It is therefore unlikely pH levels within the body would be within a range that would support the minimal amount of dissociation found within this *in vitro* testing system.

It was not appropriate to reduce the pH further and therefore another method of dissociation was sought. The ability of urea to interrupt subunit bonding is not unique to

CRP as many proteins are sensitive to urea and dissociate fully at high concentrations (Caballero-Herrera et al., 2005, Wang et al., 2014). The effects of urea within this study can be seen in figures 3.5, 3.6, and 3.7, which reflect up to 95% dissociation to monomeric units within the protein solution. The lower concentration levels of 2M have taken many weeks of incubation with an optimum period of 8 weeks compared to 6M urea which also produced monomer within the 8 week period (data not shown). Although dissociation levels were slightly higher using 6M urea at the same time point it, was judged to be too harsh, and therefore the lower urea concentration of 2M was taken forward.

The exact way in which urea causes dissociation is not fully understood and it remains unclear as to what if any structural or functional modifications may have occurred to the protein. Future X-ray crystallography studies may enable full determination of the monomeric subunit following different dissociation techniques. Urea is able to interact with polar and non-polar components making it a powerful denaturant, able to disrupt non-covalent bonds within proteins, it is one the commonly used denaturants within proteomic studies (Zou *et al*., 1998; Rossky, 2008). Hydrophilic group interactions are suggested to occur by formation of hydrogen bonds or other polar interactions whereas hydrophobic interactions are weakened (Rossky, 2008; Zaingi *et al*., 2009). The effects of urea on protein denaturation and unfolding are suggested to be either of two possibilities, the first of which is a proposed indirect mechanism in which urea alters the solvent structure weakening the hydrophobic effect between subunits. This occurs via a change in the structure of the hydrogen bond network of water surrounding hydrophobic regions of the protein, which in turn increases solubility of the protein and weakens the hydrophobic effect, leading to protein unfolding (Caballero-Herrera *et al*., 2005; Hua *et al*., 2008). The second is a direct mechanism; this suggests the protein is solvated by water and urea, with urea directly interacting with the proteins backbone via hydrogen bonds or electrostatic

interactions compared to water. The greater favourability in hydrogen bond formation between the peptide amide units and urea over those with water, allow urea to intrude within the protein structure, leading to alterations within protein structure and stability. These changes are postulated to be the driving force in protein unfolding and denaturation (Caballero-Herrera *et al*., 2005; Hua *et al*., 2008; Stumpe & Grubmuller, 2007). Although CRP protomers are held together by salt bridges, hydrophobic interactions are also involved, and these may be targeted by urea causing dissociation (Caballero-Herrera *et al*., 2005).

It is also likely that the salt bridges are involved within the subunit dissociation by urea. Urea when within an aqueous solution can spontaneously dissociate forming cyanate and ammonia which then form isocyanic acid. When in contact isocyanic acid can result in carbamlylation of N terminal proteins, peptides or lysine and arginine residue side chains groups (Sun *et al*., 2014). The rate of urea dissociation is dependent on temperature, pH and concentration together with the time in contact with the protein, therefore these factors impact on the degree of carbamylation. Residues 40-42 and 197-202 on one protomer interact with the loop of the next protomer, with salt bridges formed between Glu101-Lys201, Lys123-Glu197 and Arg118 to the carbonyl region of Pro202 (Shrive *et al*., 1996). The urea induced carbamlyation of salt bridges may explain the dissociation effect of urea on CRP.

Once again the protective effects of calcium were shown within the 2M urea trials. Figure 3.5 shows calcium prevention of dissociation even within the 2M urea conditions, which alone is not able to overcome the inclusion of calcium. The possible protective effects of calcium may lie in its ability to prevent the proteolytic cleavage of subunits and possible instability resulting from this. Calcium when bound to the protomer enables a loop formed by residues 140-150 to fold in protecting proteolytic sites (Ramadan *et al*., 2002).

When calcium is depleted, the loop moves away from the molecule exposing these hidden sites (Shrive et al., 1996) allowing cleavage. Nagase protease and pronase cleavage of calcium depleted CRP between Phe 146, Asn 145 and 146, 147 respectively was found to be inhibited by the addition of 1mM $CaCl_2$ ((Kinoshita *et al*., 1989; Ramadan *et al*., 2002). Although these residues are not part of the intersubunit associations found between protomers, it is possible that slight conformational change and proteolytic susceptibility in calcium depleted CRP is responsible for the overall instability of the pentamer in its absence. The absence of calcium ions may weaken the interprotein contacts making them more susceptible to urea treatment.

It could be expected that the removal of urea and EDTA and replacement of calcium may create conditions enabling a monomeric CRP solution to undergo spontaneous re-association to pentameric CRP. As seen in figure 3.8 this was not the case, it is not possible to instigate a return to pentameric CRP after urea dissociation as described in a study by Kresl *et al*. (1998) It is possible that during the final stages of protein assembly a chaperone protein is involved in bringing the subunits together or that the 19 residue N-terminal signal peptide is instrumental in association of subunits pre cleavage. Alternatively, the salt bridges between CRP protomers may become carbamylated by urea, preventing reassociation of subunits. Although it is not fully understood why the protomers are not able to re-associate, it is confirmed that once dissociated the solution is stable and results seen are not affected by pentameric contamination

The creation of monomeric CRP via urea or acidic treatments and that observed in studies following membrane or polystyrene binding; enable several theories to form explaining how it may arise *in vivo* and what mechanisms may be involved. It is unknown whether all physiological monomeric CRP arises locally via mechanical dissociation initiated following membrane binding, in response to changes within the extracellular

environment such as inflammatory driven changes in calcium concentrations or reduced blood pH, or is actually expressed as a monomer rather than a pentamer. It may even be a combination of these actions dependent on disease or inflammatory states within the body. The use of urea as a method of dissociation within this research allowed for a continual source of manufactured monomer, but does not in itself add understanding to the mechanism of monomer creation within an intra or extracellular environment.

3.4.2 Conclusion and Future Work

The techniques used within this study provided a reliable method of producing stable monomeric CRP for further investigation within patient serum and antibody studies. The results confirmed those previously reported; however these methods have been adapted to reduce the harsh techniques used within much existing research and attempt to reflect functions and affects found physiologically, with a protein structurally resembling that encountered within the body. The monomer created has been confirmed as 23KDa via a calibrated FPLC and travels on native PAGE to a position estimated to be that of a monomeric subunit, in a distinctly different way to the pentamer. Western blotting discussed within chapters two and five further confirms the identity of the created subunit through the use of specific antibodies designed to recognise the intersubunit epitopes. It has not been possible to re-associate the subunits due to suggested *in vivo* mechanisms present within the cell prior to secretion and absent *in vitro*. It is also possible to see small peaks between 110-115ml; these calibrated to between ~ 2400-4400 Da, and may represent the breakup of tertiary protein structure and gradual degradation of the protein.

Whilst the methodology of monomer generation within our study does not mirror physiological conditions, it is less harsh than several methods used within previous studies, including those of 8M urea and both heat and extremes of pH. Despite this it is unknown

what effect the conditions have had on the protein and future work involving either mass spectrometry or X-ray crystallography would aid in the determination of urea dissociation changes to structure.

Chapter 4 - Antibody Specificity Studies

4.1 Introduction

4.1.1 Overview

Consistency and regulation of all results within the study are essential, with the interaction between CRP, its structural conformations and detection antibodies key to a full understanding. Antibodies directed towards both pCRP and mCRP may recognise other proteins within the serum and as such must be tested for specificity. ELISA relies upon the binding affinity of proteins to solid surfaces such as micro plates, with indirect ELISA determining serum auto-antibody content subject to the inherent binding properties of serum immunoglobulins to the solid surface, creating possible false positives. There are several possible interactions likely to impact upon results of the ELSIA assays and as such it was important within this study to investigate and eradicate any unwanted interactions. It is known that CRP is capable of spontaneous dissociation when bound to solid phase such as polystyrene ELISA plate wells (Potempa *et al*., 1983; Sjowall *et al*., 2004; Singh *et al*., 2009), which could cause conformational changes to pentameric CRP and exposure of non-native sites when bound directly as a capture protein. This spontaneous dissociation would confuse results as conformation of the target protein would be changeable, preventing correct identification of the auto-antibodies present within a sample tested. Stability of the protein is therefore essential within the design of experiments seeking to identify and quantify either CRP conformation.

Human serum may also contain anti-BSA antibodies, or interact with other blocking agents or capture antibodies (Hilger *et al*., 2001; Waritani *et al*., 2017). There is also potential for interactions between detection antibodies and other pentraxins such as

long pentraxin 3 (PTX3) and serum amyloid P (SAP), both of which share sequence homology with CRP (Agrawal *et al*., 2009).

The correct interpretation of results is therefore dependent upon an experimental design which eliminates or mitigates fully for any unrequired interactions caused by these or any other factors. With this in mind a range of experiments were performed to characterise CRP and antibody interactions within the chosen immune assays to ensure consistency between results and eliminate variables likely to be encountered.

4.1.2 Serum Anti-BSA Antibodies

Human serum antibodies may interact with the blocking agents or capture antibodies used within ELISA and western blotting procedures (Hilger *et al*., 2001; Waritani *et al*., 2017). Bovine serum albumin is a common blocker used within ELISA to ensure prevention of non-specific binding. In some studies however it has been reported that serum within both healthy and diseased individuals contains antibodies directed against BSA, for instance the serum of up to 55% of healthy individuals and diseased patients were found to contain anti-BSA IgG in a study by Mogues *et al*. (2005) in an investigation looking at patients with lung cancer (Mogues *et al*., 2005). An earlier study in 1982 found anti-BSA antibodies in the serum of patients with upper but not lower gastro-intestinal diseases such as oesophageal cancer, gastric ulcer and duodenal ulcer, rather than Crohn's disease or colon cancer, with a greater prevalence in women than men (Tamura *et al*.,1982).

In 2011 Sjowall *et al* published an article detailing the effect of BSA on their own findings due to its use as a stabiliser of some cytokine preparations used within their group. They then went on to test a cohort of RA patients of whom 63% were RF-positive, with 50% of RA and 62% of controls anti-BSA positive. No significant difference was found

between the groups, nor between male and females (Sjöwall *et al*., 2011). It is unknown whether the interaction between BSA and autoantibodies is due to natural (innate) antibodies or due to MHC antigen presentation, however it is suggested the latter as BSA is a dietary antigen (Sjowall *et al*., 2011). Anti-BSA antibodies are also increased in insulin dependent diabetes mellitus (IDDM) patients, where a cross reactivity found between a BSA peptide sequence and a pancreatic β-cell surface protein has been suggested as an important area of study (Karjalainen *et al*., 1994). Cow's milk has also been discussed as a possible trigger of IDDM (Saukkonen *et al*., 1998). In addition to anti-BSA IgG, IgA antibodies have also been reported. In a study comparing IDDM patients and a healthy group, highest titeres were found in 0-10 year olds, with levels declining as age progressed. IDDM patents had significantly higher levels of both IgA and IgG anti-BSA in their serum compared to non-IDDM controls (Hilger *et al*., 2001).

Therefore the specificity of blocking components must be considered within the ELISA system ensuring that suitable steps are taken to insure false positive results do not occur via binding of patient auto-antibodies within serum.

4.1.3 C-reactive Protein Conformational Changes upon Binding of Microwells

Studies have provided evidence showing that not only may CRP dissociate on the surface of cell membranes, but that contact with the polylysine of an ELISA plate or other solid surface may provide an environment allowing relaxation of the pentameric form and exposure of neo-epitopes (Ji *et al*., 2009; Potempa *et al*., 1987; Thiele *et al*., 2015). This provides challenge when using ELISA as an assay in order to determine the conformation of CRP within serum, as the CRP present within the serum can dissociate upon binding the ELISA plate and therefore create difficulties in accurate determination of monomeric or pentameric CRP content. Within auto-antibody studies this also becomes a challenge due

to the nature of the protein bound to the plate, without knowledge of the conformation bound, it is not possible to determine which auto-antibodies are present within a sample.

4.1.4 Capture and Detection Antibodies

For many years, research studies have used a commercially available mouse anti-human CRP antibody - Clone 8 (Sigma), (Torzewski *et al*., 2000; Burke *et al*., 2002; Eisenhardt, Habersberger, Murphy, *et al*., 2009; Eisenhardt, Thiele, *et al*., 2009) as a detection antibody, which was at one time assumed to detect pentameric CRP alone. It is now known that the antibody interacts with both forms of CRP, and appears to have higher affinity for the monomeric rather than pentameric form of the protein (Ciubotaru *et al*., 2003; Jabs *et al*., 2003; Khreiss *et al*., 2005; Schwedler *et al*., 2003) and therefore is useful to identify the presence of CRP but not the conformation. It is suggested that early studies using this antibody to determine CRP may have recognised mCRP instead of or in addition to pCRP adding to the opposing results regarding both pro and anti-inflammatory actions of CRP conformations.

To enable identification of not only the size of the CRP protein within serum enabled by western blotting, but also the concentration within an ELISA, a range of antibodies which were capable of determining monomer from pentamer were required. Several research groups have used antibodies produced and supplied by Dr Lawrence. Potempa (Thiele, 2015; Trial *et al*., 2016; Slevin, 2015; Kreiss 2004; Wang, 2001), and he is gratefully acknowledged for his generosity in providing 3 antibodies for this research – Mouse anti-human mCRP monoclonal antibodies specific against the mCRP - 3H12 and 1D6 together with 8C10 specific for the pCRP. These were initially examined for their suitability before use within this research.

4.1.5 Exploration of Potential Antibody Interactions with other Immune Proteins

Human serum in diseased and non-diseased states will contain a range of proteins and immune complexes, potentially also other pentraxins. There is therefore potential for the selected detection antibodies to interact with other serum immune proteins such as the acute phase proteins PTX3 and SAP. The pentraxins are a family of evolutionarily highly conserved pattern recognition proteins characterised by their multimeric cyclic structures and fall into two categories – the long and short pentraxins. Both CRP and SAP fall into the short pentraxin category, whilst pentraxin 3 (PTX3) is characterised as a long pentraxin (Mantovani *et al*., 2006). The shared structural components are an indication of their common properties, including the activation of complement, and opsonisation of apoptotic cells and microorganisms.

Pentraxins share an approximate ~200 amino acid long domain with a distinctive primary motif, the so called "pentraxin signature". This signature is composed of the sequence His-x-Cys-x-Ser/Thr-Trp-x-Ser, where x is any amino acid (Manovani *et al*., 2008). SAP and CRP share 51% identical protein sequence (Agrawal *et al*., 2009), additionally PTX3 shares a C-terminal pentraxin like domain with CRP and SAP (Woof & Mestecky, 2015). Therefore there is some structural homology between these proteins and CRP and therefore it is important that cross reactivity be eliminated.

Where CRP and SAP are mainly expressed via hepatocytes in response to IL-6, PTX3 is expressed in cells across the body such as endothelial, fibroblast and hepatic cells via IL-1β and TNF-α, but unlike CRP not IL-6 signalling. The PTX3 transcript consists of 1861 base pair and is predicted to 381 amino acids. In the COOH-terminal half, PTX3 contains a "typical 8 amino-acid pentraxin sequence" and is composed of 3 exons, the third of which bears sequence homology to CRP (Breviario *et al*., 1992). In contrast to human CRP pentameric conformation, PTX3 structure is composed of covalently linked octamers.

It is also involved within host defense against infections caused by pathogens and helps regulate scavenging activities of macrophages and dendritic cells. PTX3 also modulated complement activity by binding C1q (Inforzato *et al*., 2006)

4.1.7 Experimental aims

The aim of this chapter was to determine the affinity and specificity of the chosen capture and detection antibodies towards different isoforms of CRP. Once determined the most appropriate antibody combination would be taken forward for use within all future experiments. It was also necessary to determine the effects of CRP interactions with the ELISA plate and assay components to ensure eradication of non-specific binding.

4.2 Materials and methods

4.2.1 Analysis of Detection and Capture Antibody Specificity

4.2.1.1 Western Blotting

Monomeric CRP was produced *in vitro* using the 2M urea dissociation method as described in section 3.2.2. Both mCRP and pCRP were separated by native PAGE as described within section 2.3.6, each lane was loaded with 1µg of both monomeric and pentameric CRP and run at 100V for 1 ½ hours. The selected antibody was then used to probe mCRP or pCRP within a western blot to gauge antibody specificity (methods described within section 2.3.7). The primary detection antibodies used were antibody 3H12, a specific mouse anti-human mCRP monoclonal IgG; 1D6, a specific mouse anti-human pCRP monoclonal IgG and 8C10, a specific mouse anti-human mCRP monoclonal IgG (Dr Lawrence. Potempa, College of Pharmacy, Roosevelt University, Illinois), at a 1:50 dilution within Blocking solution. In addition to these Clone 8 (Sigma) was also tested at a dilution of 1:2000. The primary antibody was incubated for two hours at 4^{o}C. The secondary antibody used was streptavidin conjugated rabbit anti-mouse IgG (Sigma) antibody, added at a dilution of 1:30,000 in Blotto and incubated for 30 minutes at 4^{o}C.

4.2.1.2 Enzyme Linked Immunosorbent Assay

Initial determination of antibody specificity utilised a direct ELISA method as described in section 2.2.8, in which the protein in question was bound directly to the plate within a coating buffer at concentrations between 0.25µg/ml to 4µg/ml (all reagents detailed within table 2.7). The method was as described in section 2.3.8, with additional primary and detection antibodies used whilst developing the final assay. The primary detection antibodies used were antibody 3H12, 1D6, and 8C10, at a 1:50 dilution within reagent diluent and Clone 8 (Sigma) mouse anti-human CRP was also tested at a dilution

of 1:30000 with the secondary rabbit anti-mouse IgG conjugated antibody (Sigma) also at a dilution of 1:30000.

Within investigations considering the interaction between CRP and goat anti-human IgG, the detection antibody was omitted and the direct interaction between protein and conjugated secondary antibody detected. In addition a rabbit anti-mouse IgG (Sigma) was also tested directly against human CRP in varying dilutions and lastly a conjugated rabbit Fc specific anti-human IgG (Thermo Scientific) was also used at varying dilutions. This assay was extended to further test human proteins PTX3 (Sigma), SAP (Sigma) and keratin (Sigma).

4.2.2 Analysis of Sodium Azide and Peroxidase Interactions

The indirect ELISA method was once again used (section 2.3.8) to investigate possible sodium azide inhibition of peroxidase within CRP/anti-IgG studies. The ELISA preceded as previously described (section 2.3.8) however mCRP was pre-incubated with sodium azide at 0.1% to match the storage conditions of pCRP, the secondary conjugated goat anti-human IgG antibody was used at a dilution of 1:30000.

4.2.3 Human C-Reactive Protein DuoSet ELISA Specificity

A commercially available CRP detection ELISA assay (R&D systems Human CRP DuoSet ELISA) was used to detect pCRP. It was first tested for specificity against both mCRP and pCRP in order to determine its specificity for the pentameric form only. The protocol was followed as described in section 2.3.8, with all samples diluted in reagent diluent, however the manufacturer's suggested protocol was initially applied with no reduction in capture antibody dilutions. To optimise the protocol for patient pCRP detection a range of patient, control and antibody dilutions were tested.

4.2.4 Optimisation of Auto-antibody Detection ELISA

The competitive ELISA method detailed within sections 2.3.9.1 and 2.3.9.2 was first optimised to ensure avoidance of interactions between assay components. Initially an ELISA was developed directly coating each well with patient samples diluted 1:200 in bicarbonate coating buffer (Table 2.13). Half of the plate (side A) was coated with either mCRP or pCRP at 2μg/ml in bicarbonate and the other half (side B) in bicarbonate buffer alone and incubated overnight at 4^{o}C. The plate was then washed as described in section 2.3.9.1 and patient samples diluted 1:200 in reagent diluent (R&D Systems), added to both sides of the plate and incubated at room temperature for two hours. The plate was once again washed and the anti-human IgG, IgM or IgA antibody added as per Table 2.9. The remaining protocol was as described in section 2.3.9. The resulting absorbance for side B were removed from side A. Following optimisation the protocol described within section 2.3.9 was adopted.

4.3 Results

4.3.1 Analysis of Antibody Specificity

The interaction between a commercially available anti-human CRP antibody, Clone 8 (Sigma) was investigated to confirm specificity for pentameric, monomeric or both forms of CRP. Analysis by western blotting using native human pentameric CRP (Scripps) and dissociated CRP (mCRP) were first performed using Clone 8 as a detection antibody, the results of which are shown in figure 4.1.

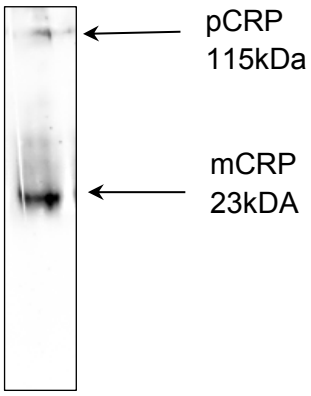


Figure 4-1 Western blot image of a native PAGE of mCRP and pCRP including 1/20th SDS within both sample and running buffer. Containing 1µg of both mCRP and pCRP. The primary detection antibody used was Clone 8 1:1000 dilution. Although both proteins were loaded in equal amount the intensity of the monomeric band is greater

It is apparent that not only does Clone 8 recognise pCRP, but it is also very effective in determining the presence of mCRP. Whilst both forms of CRP have been loaded in equal concentration an mCRP band of greater intensity is visible in the position expected for a band of 23kDa. Whilst western blotting allows confirmation of the size (monomer or pentamer) of CRP, it is also essential that this can be determined within an ELISA to enable confident determination and concentration of CRP conformation within human serum samples. For this reason it became necessary to have an antibody specific to one form of CRP and not the other as opposed to Clone 8 which recognised both. Antibodies supplied by L. Potempa (3H12 and 8CID - specific to monomeric CRP, and 1D6 specific to pentameric CRP), were tested for specificity via western blotting. Figure 4.2 shows a representative western blot (n=3) which reveals the specificity of each, 1µg of

both monomer and pentamer per well were run on 1/20th SDS native PAGE and blotted with each antibody. Lane 2 used Clone 8 as a detection antibody resulting in a monomeric band only, lanes 3-5 show detection of monomer not pentamer by 3H12 and lanes 6-8 have a very faint detection of monomer by 8C10 and strong detection of the pentamer in lanes 9-10 by 1D6.

It was also apparent following retention of the stacking gel before transfer that the pentameric CRP sample was unable to move fully into the resolving gel, with the majority remaining within the stacking layer. The western blot analysis during antibody specificity testing at various exposure times allowed detection of several bands present when probed with the anti-monomeric antibody 3H12. In addition to the expected monomeric band position two others are visible – indicating possible dimer or trimer conformations. The monomer tested was prepared as described and isolated by FPLC (Section 2.3.4), however there may have been some aggregation or stacking within the gel or within storage following FPLC separation.

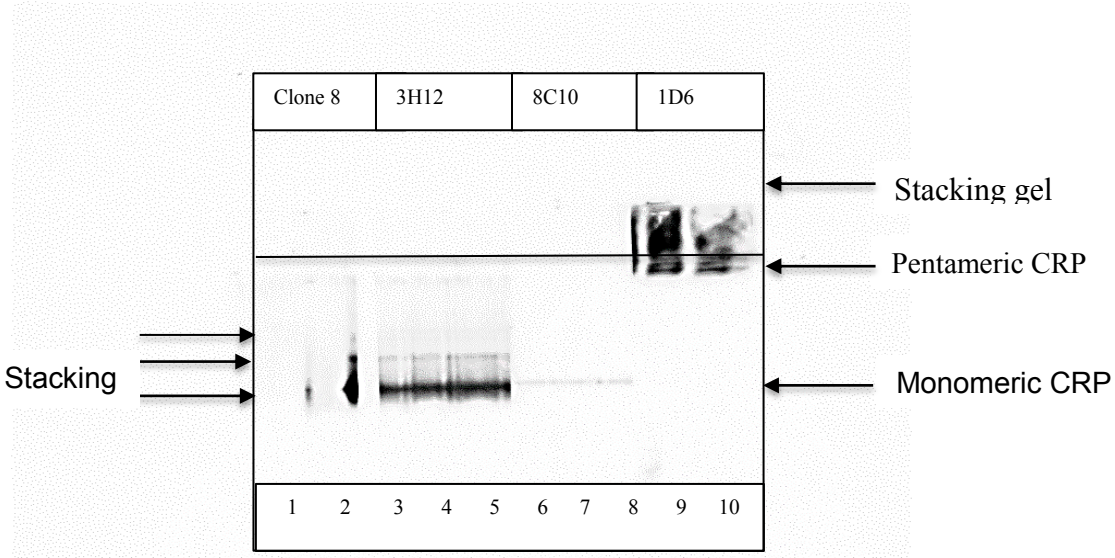


Figure 4-2 Western blot image of antibody specificity, Clone 8 (1:1000), 3H12, 8C10 and 1D6 (1:50). Native PAGE gel with 1/20th SDS within both the sample and running buffers, the stacking gel was not removed prior to blotting. 1ug of both mCRP and pCRP were loaded into each lane. Lane 1 is a negative control without primary detection antibody; Lane 2 – the positive control (Clone 8); Lanes 3-5 (3H12) showing an intense band at the monomer position; Lanes 6-8 (8C10) with a single less intense band at the expected monomer position and lastly Lanes 9-10 (1D6), which show a band below the stacking gel where expected for pentameric CRP, however the majority of the pentamer has remained within the stacking gel and not entered into the resolving section, *n*=3.

4.3.2 Antibody Interactions with Immune Proteins PTX3 and SAP

Further ELISA and western blots were performed to test for interactions between PTX3, SAP and antibodies. It is known that PTX3 may be found on the surface of cells and within serum as is SAP, therefore this test ensured that any positive interaction within serum were detection of CRP and not either of these proteins. Figure 4.3 shows the comparison between detection of 1µg of mCRP and PTX3 within an ELISA with Clone 8, 3H12, 8C10 and 1DC used as primary detection antibodies. PTX3 was not detected in any of the assays by anti-CRP antibodies. Human SAP was separately tested for anti-human IgG binding, and with anti-CRP antibodies 3H12 and Clone 8, and was also not found to have any interaction (Figure 4.3).

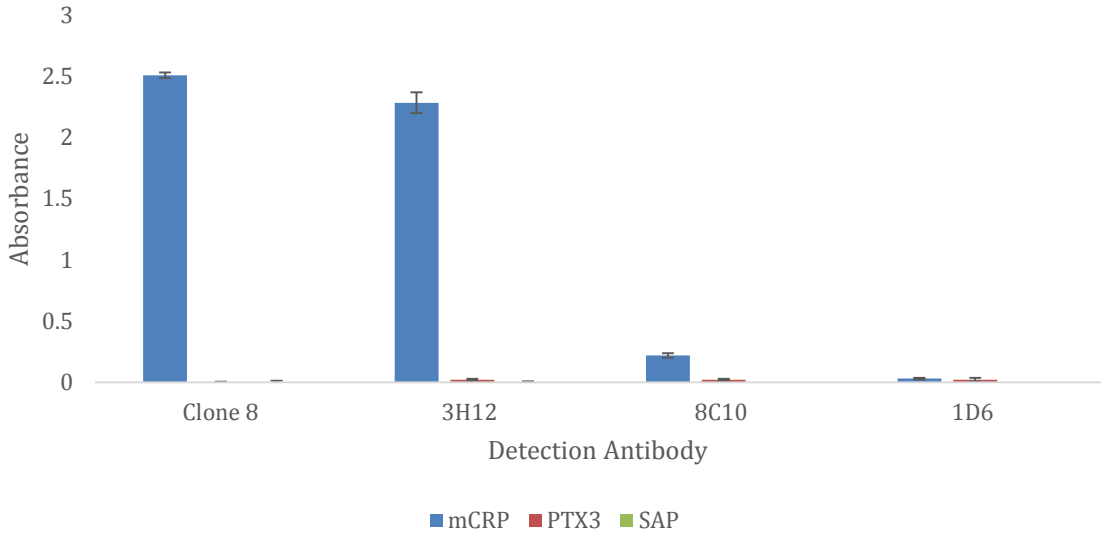


Figure 4-3 Bar graph showing the interaction between detection antibodies mCRP, PTX3 and serum amyloid protein (SAP), (SAP tested with Clone 8 and 3H12 alone), showing a strong interaction between mCRP, 3H12 and Clone 8 and weaker with 8C10 n = 3. No interaction is shown between mCRP and pentamer specific 1D6, or between PTX3 and any of the four antibodies. SAP was also not detected by either Clone 8 or 3H12), n = 2.

4.3.3 Interactions between Anti-Human IgG and CRP

During ELISA optimization an interaction was seen within control wells between CRP and goat anti-human IgG. The effect was further investigated and found to be greater within lower antibody dilution and at higher CRP concentrations; more interesting was the difference between anti-human IgG and mCRP or pCRP. The interaction between mCRP and anti-human IgG was more than five times higher than pCRP and anti-human IgG samples at 1:1000 antibody dilution, as illustrated in Figure 4.4, absorbances 1.35 and 0.253 respectively at 4 µg/ml. A less pronounced interaction can be seen between 4µg/ml pCRP and higher antibody dilutions, and also with lower CRP concentrations, with virtually no interaction at 0.5 µg/ml pCRP.

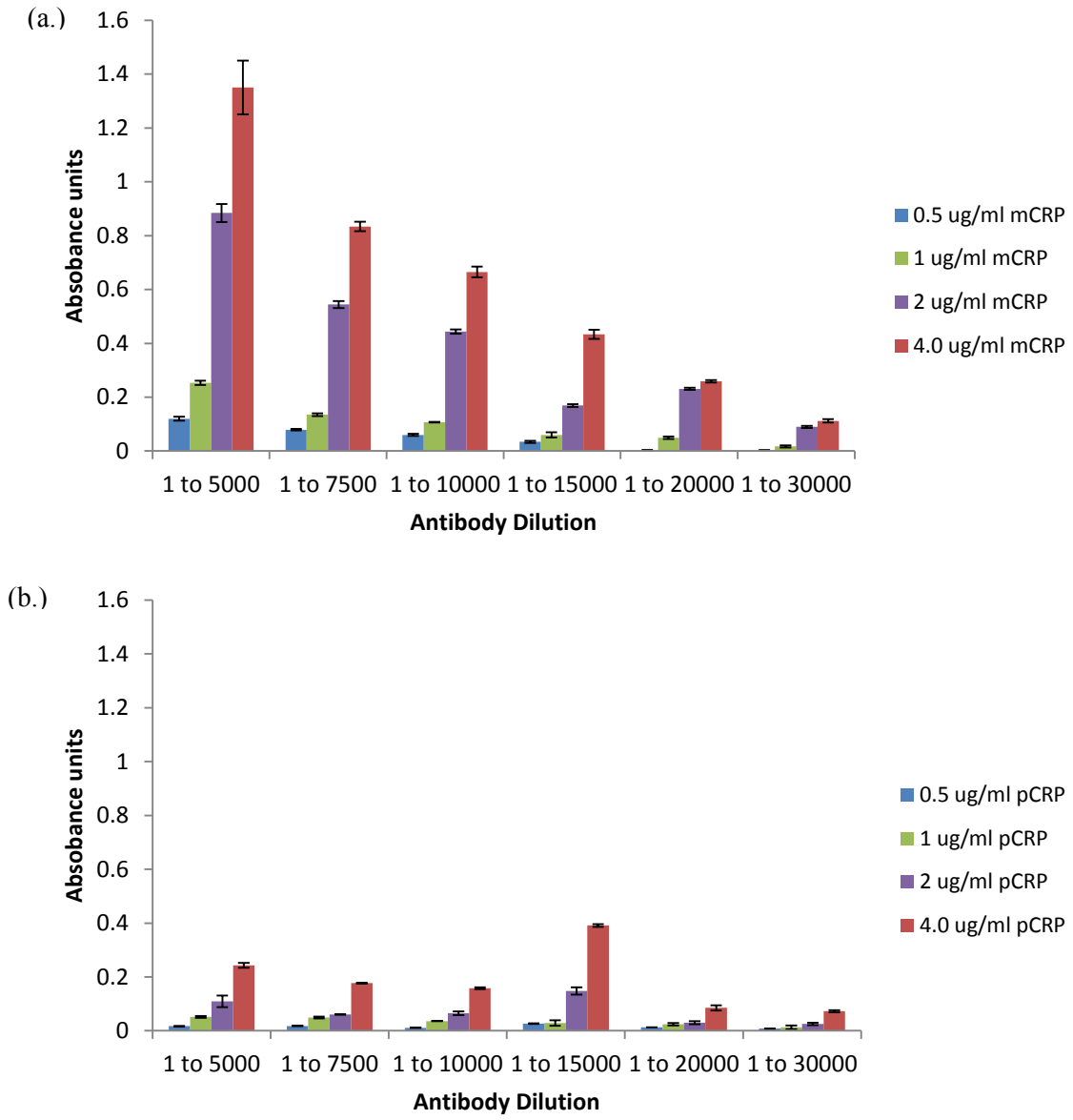


Figure 4-4 Bar graph showing the interaction between CRP and goat anti-human IgG at different amounts of bound CRP protein and antibody concentrations. (a.) mCRP dilutions ranging from 0.5µg/ml to 4µg/ml (b.) pCRP dilutions ranging from 0.5µg/ml to 4µg/ml.

The nature of the interaction between CRP and anti-human IgG was further investigated by pre-incubating anti-human IgG with either mCRP or pCRP to see if this abrogated the effect. As can be seen in Figure 4.5, not only does anti-human IgG interact more strongly with monomeric CRP but the IgG binding is reduced by half during pre-incubation with the mCRP when compared to pre incubation with pCRP.

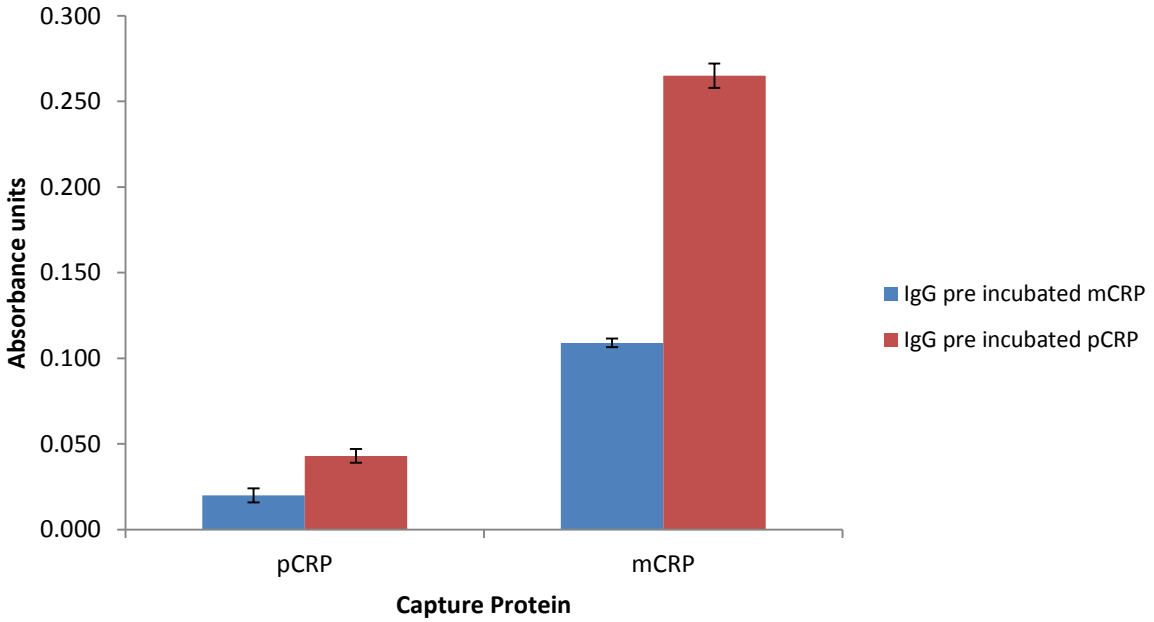


Figure 4-5 Bar graph showing binding inhibition of anti-human IgG pre incubated with either mCRP or pCRP. Anti-human IgG (1:5000) dilution, pre-incubated with either mCRP or pCRP (1µg/ml) overnight. The capture protein is either mCRP or pCRP at 2ug/ml coated overnight. Monomeric CRP abrogates the binding of anti-human IgG to both the bound monomer and pentamer to a greater degree than pre-incubation with pCRP.

The interaction between CRP and anti-IgG was further investigated with the secondary antibody used within many of the assays, rabbit anti-mouse IgG. There was once again an interaction found, however there was less difference between pCRP and mCRP. Figure 4.6 shows the binding of 2µg/ml mCRP and pCRP by rabbit anti-mouse IgG across and antibody dilution range between 1:250 and 1:16000.

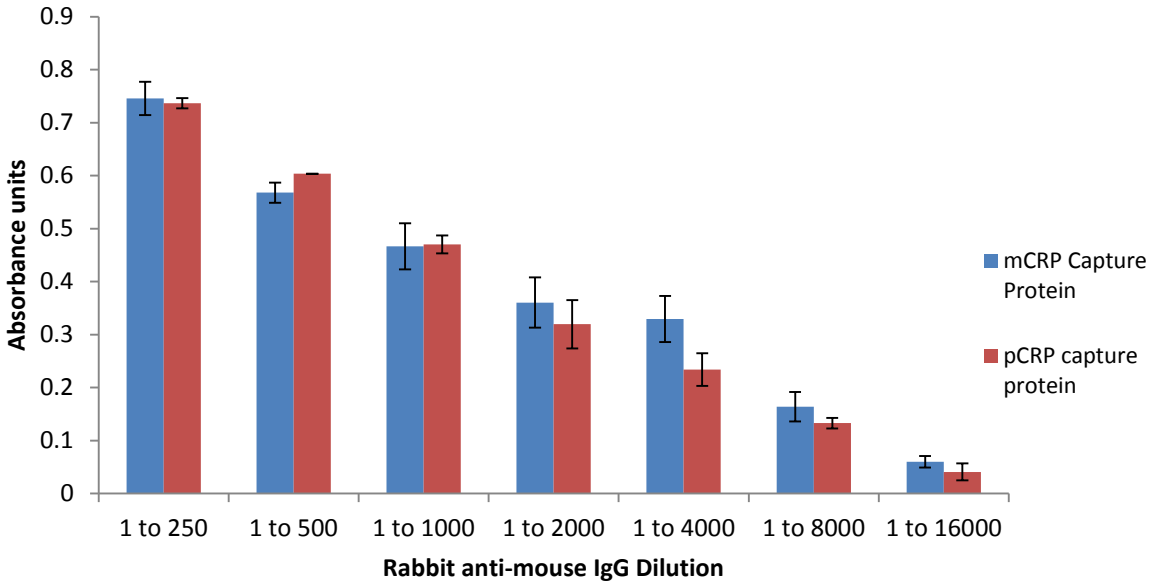


Figure 4-6 Bar graph showing interactions between human CRP and anti-mouse IgG. An ELISA plate was coated overnight with 2µg/ml mCRP or pCRP and probed with a rabbit anti-mouse IgG at various dilutions. The absorbance value for an antibody dilution of 1:8000 is 0.164 and 1:16000 dilution is 0.06 when captured by the monomer and is a little lower when captured by the pentamer.

Although an interaction can be seen between anti-mouse IgG and CRP in Figure 4.6, there is no significant difference between that captured by mCRP or pCRP. Figure 4.6 also shows an absorbance of 0.164 units for mCRP at an antibody dilution of 1:8000; the comparison figure of 0.544 absorbance units at an antibody dilution of 1:7500, over three times larger can be seen in Figure 4.4, showing greater interaction/binding of anti-human IgG to mCRP as opposed to anti-mouse IgG.

4.3.4 Interactions between CRP and Fc specific anti-human IgG

The interaction between the secondary antibody (anti-human IgG) and CRP was further investigated using an Fc specific anti-IgG, this was performed in order to determine whether the antibody recognition sites resembled Fc regions. Figure 4.7 shows that whilst

anti-human IgG recognises monomeric CRP and to a lesser extent pentameric CRP, an Fc specific anti-human IgG shows no recognition of either conformation.

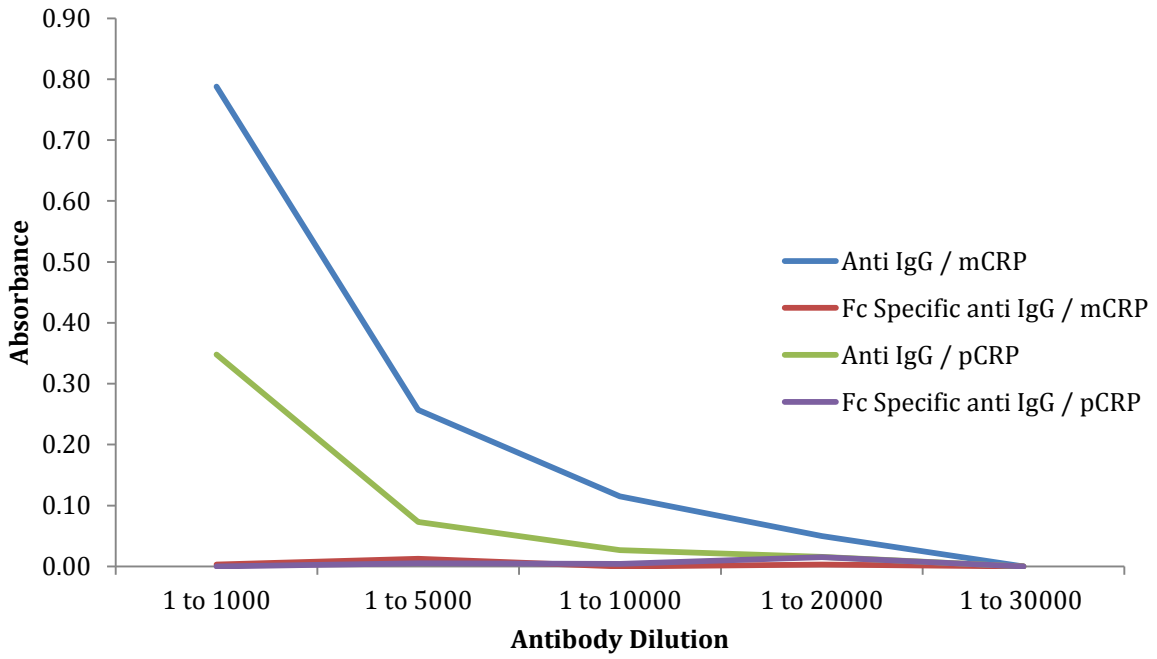


Figure 4-7 Line graph showing the interaction between mCRP, pCRP and both anti-human IgG and Fc region specific anti-human IgG. There is little interaction between the Fc specific antibody, although a large interaction between anti IgG and mCRP.

The specificity of the anti-human IgG was further tested using additional human proteins. Keratin was easily available and initially used; this also resulted in an interaction as can be seen in Figure 4.8. The interaction is comparable to mCRP but lower, with the absorbance at an antibody dilution of 1:5000 for 4µg and 2µg keratin 1.106 and 0.49 respectively. The equivalent values for mCRP were 4µg - 1.35 and 2µg - 0.884 and pCRP 4µg - 0.243 and 2µg - 0.109. Once again the interaction was assessed for Fc site specificity and as with both mCRP and pCRP no binding was present when anti-human IgG Fc specific antibodies were used, as seen in Figure 4.9.

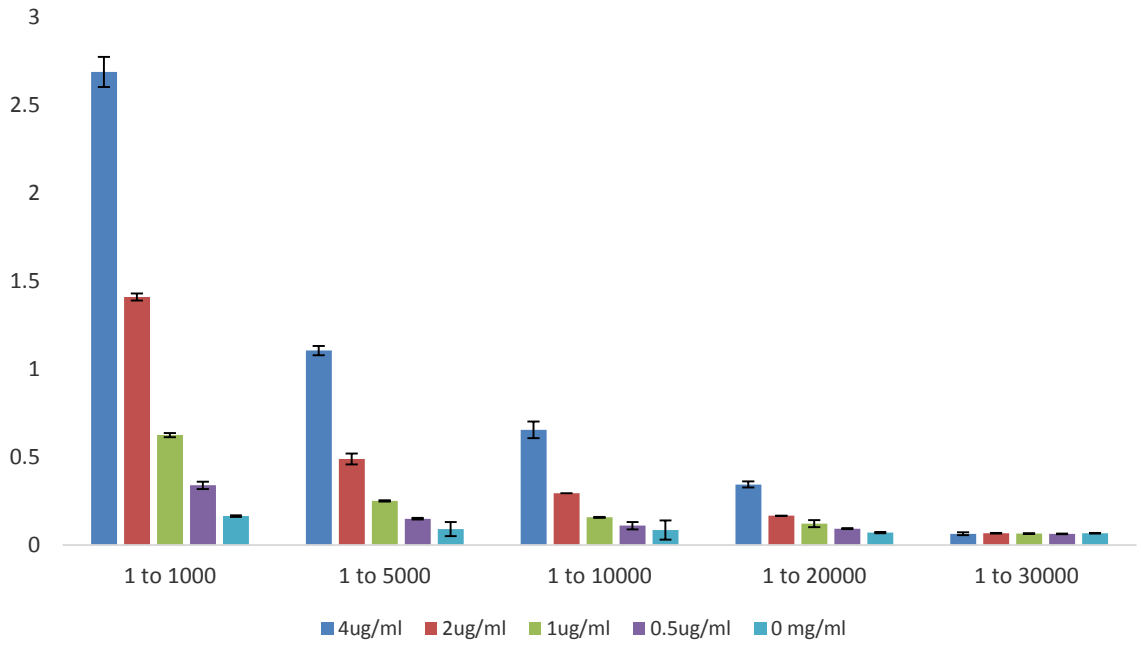


Figure 4-8 Figure 4.8 Bar graph showing the interaction between anti-human IgG and keratin with antibody dilutions ranging between 1:30000 and 1:1000 and capture keratin at between 4µg/ml and 0µg/ml.

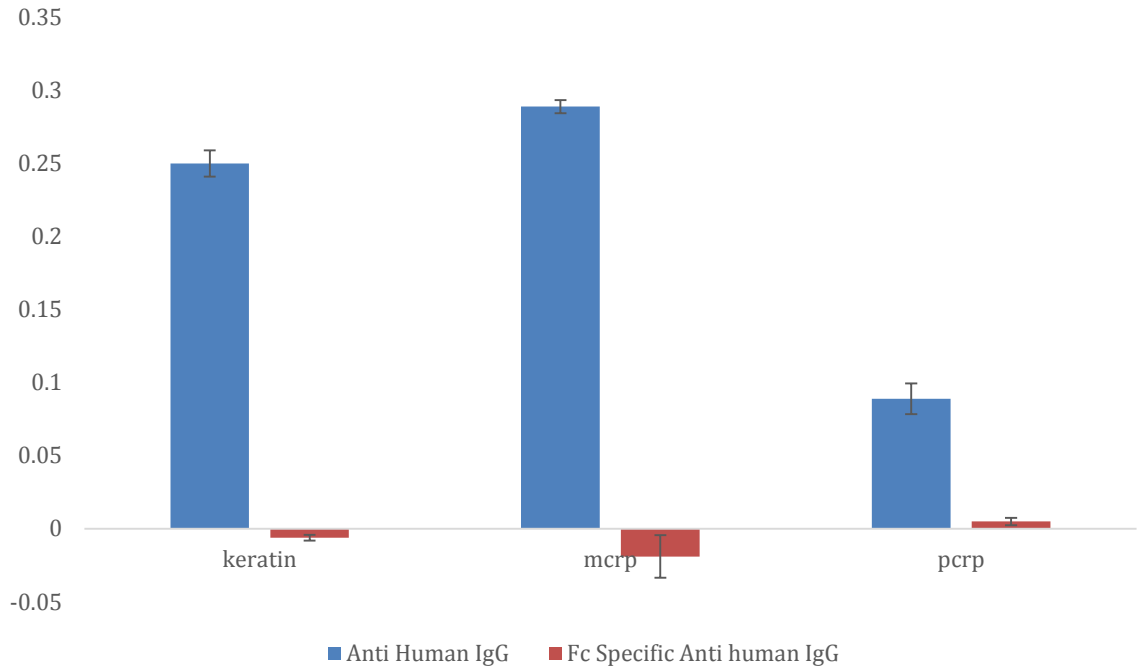


Figure 4-9 Bar graph showing the interaction between keratin, mCRP, pCRP and either anti-human IgG or Fc specific anti-human IgG. Plates were coated with 1µg protein and challenged with a 1:5000 dilution of antibody.

4.3.5 Effects of Sodium Azide on Clone 8

It is possible that differences between the observed binding of various antibodies to mCRP and pCRP may be caused by sodium azide present within the pentameric CRP solution supplied by Scripps. Sodium azide is known to interact with peroxidase conjugated antibodies and is not present within the monomeric sample; this may have been responsible for reduced interactions when using pCRP within assays. However as can be seen in figure 4.10 the resulting difference is not significant, although once again the binding of anti-human IgG to mCRP is higher than that to pCRP.

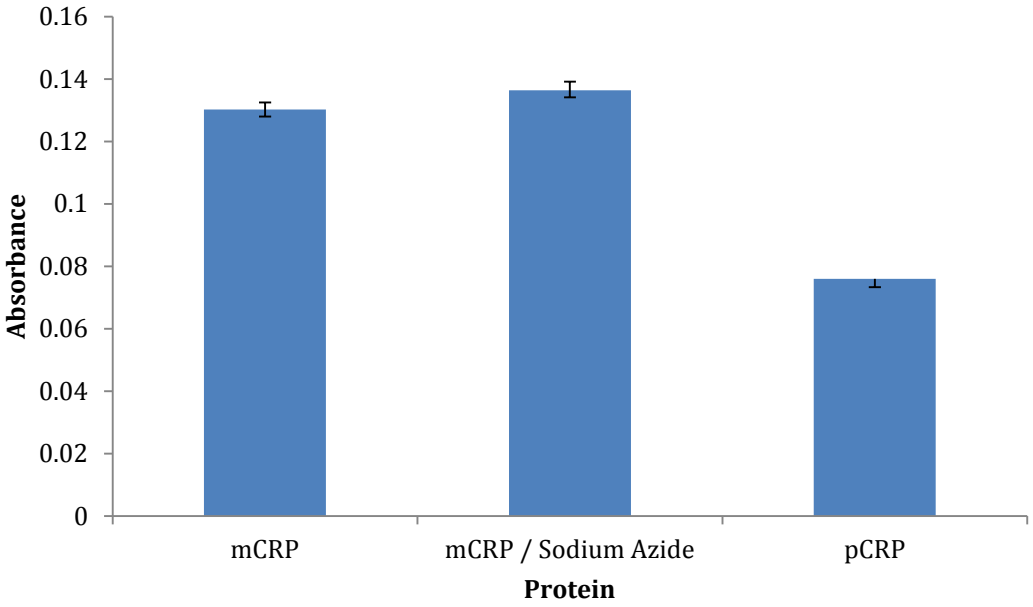


Figure 4-10 Bar graph showing the effect of sodium azide on CRP/IgG interactions. Wells were incubated with 1µg CRP in a bicarbonate buffer overnight followed by 1:30000 dilution of goat anti-human IgG. Pentameric CRP is supplied in buffer containing sodium azide at 0 .1% protein concentrations. To assess the effects of this on the peroxidase conjugated antibody an equivalent amount of sodium azide was added to mCRP. No significant effect was found between anti-IgG binding signal to mCRP with or without the addition of sodium azide. There is a significant difference between anti-human IgG interactions with mCRP as opposed to pCRP.

4.3.6 CRP DuoSet ELISA specificity

In order to test for serum pCRP, a commercial ELISA kit was tested for specificity against both mCRP and pCRP. Figure 4.11 shows the absorbances against concentration for both monomeric and pentameric CRP. Whilst pCRP shows a clear correlation between concentration and absorbance, mCRP is not detected by the kit.

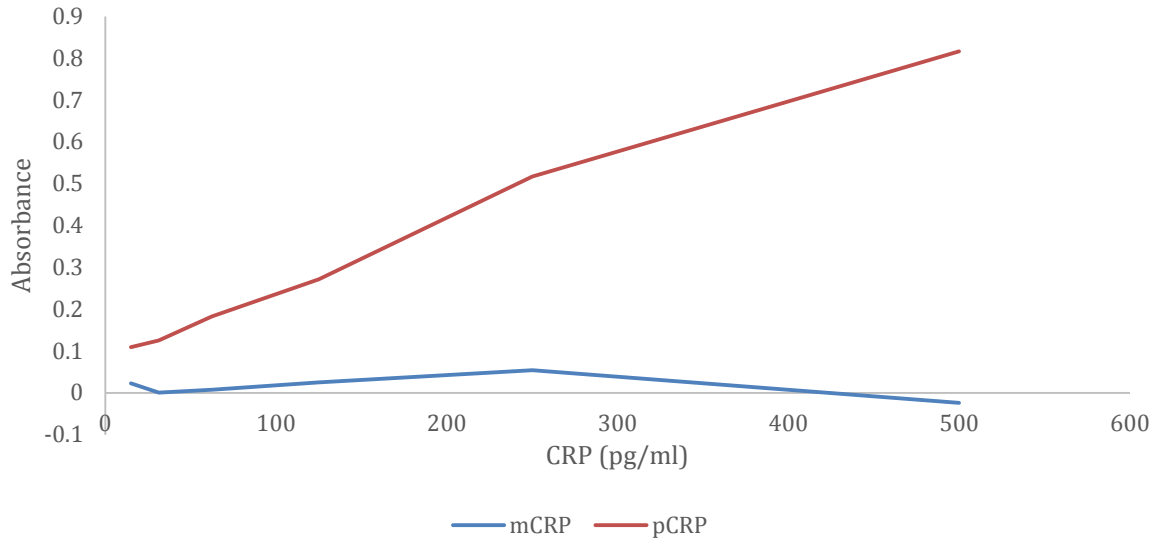


Figure 4-11 Line graph showing the specificity of the R&D Systems CRP DuoSet ELISA kit for pCRP and mCRP. Whilst a correlation is present between the detection of pCRP with rising absorbance with concentration, there has been no detection of mCRP.

Patient samples were tested within the R&D Systems kit to optimise sample dilutions. Dilutions of patient samples between 1:1000 and 1:128,000 were trialed together with varied capture and detection antibody dilutions. The optimum conditions taken forward were a patient sample dilution of 1:16000 and a control sample dilution of 1:1000 in reagent diluent (Table 2.8).

4.4 Discussion

4.4.1 Antibody Specificity

In order to design a robust protocol capable of detecting and differentiating between both mCRP and pCRP, together with their associated auto-antibodies, it was important to ensure antibodies were specific for the desired protein and any additional interactions were avoided. With this in mind initial investigations considered the primary detection antibodies selected for both the ELISA and western blotting analysis. The first antibody considered was the commercially available Clone 8 (Sigma) which not only detected monomeric CRP, it appeared to have a higher affinity to this form than that of the pentamer, this can be seen in the western blot shown in Figure 4.1. The monomeric band has a deeper intensity than the pentamer although both contained the same amount of loaded protein, indicating a higher affinity for the monomeric form of CRP than the pentameric.

Whilst the monomer appears in the location expected there is also a third and fourth band visible between the monomer and pentamer, it is suggested that this may be dimer or trimer formation, although which surface interactions are playing a part are unknown. The presence of such conformations have also been noted within a study by Li *et al*., (2016), although they were able to show several conformations of CRP within western blotting however not the monomeric form. The knowledge that Clone 8 recognised both forms of CRP eliminated it from ELISA serum detection studies as it was important to differentiate between the two isoforms of CRP. Specific anti-CRP antibodies were sourced that would allow recognition of individual isoforms of CRP within both ELISA and western blotting. These antibodies (3H12, 1D6 and 8C10) were tested against both mCRP and pCRP within a western blot (Figure 4.2) and confirmed to be specific for the CRP conformation

required. Both 3H12 and 1D6 provided a strong signal against mCRP and pCRP respectively; however 8C10 was not found to be as effective and was therefore not used further.

4.4.2 Interactions between Anti-IgG and CRP

Whilst optimising ELISA protocols an unexpected interaction between anti-human IgG and both mCRP and pCRP became apparent. Research has shown that the risk of cross reactivity is elevated in proteins with a high level of homology amongst species, which may also include CRP; for instance, Delgado *et al*. (2003) reported that anti-human CD36, anti-human CD41a and anti-human CD62P antibodies all cross reacted with porcine platelets (Delgado *et al*., 2003). It has also been reported that mCRP binds immunoglobulins non-specifically. In their 2012 paper Boncler *et al*. tested binding of antibodies directed against human serum albumin (HSA) and IgG to mCRP and additionally to IgG, fibronectin and C1q. They found that anti-HSA and anti-IgG bound to mCRP, however there was a stronger interactions between mCRP and human IgG, murine anti-human CD16 and murine anti-human CD32 (Boncler *et al*., 2003).

Additionally anti-HSA antibodies bound not only to HSA but also to fibrinogen, IgG, fibronectin, C1q and mCRP, this binding was only observed in higher concentrations of antibody (10µg/ml). These results reflected the findings in our study which also showed greater binding at higher antibody concentrations, although Figures 4.4 shows considerable binding at both 2.5 and 5µg/ml. Interactions between mCRP and both anti-human and anti-mouse IgG were also reported (Boncler *et al*., 2003), although again only at mCRP concentrations of 10µg/ml. The interaction of mCRP and immobilised murine antibodies directed towards human Fc receptors CD16 and CD32 and immobilised human IgG was also assessed with considerable binding of mCRP to all three. Whilst our results show

limited interactions between pCRP and anti-human and anti-mouse IgG, Boguslawski *et al*. (2007) reported interactions with only mCRP, once again only at higher concentrations of CRP and antibody. It is clear that mCRP and to a lesser extent pCRP interact with anti-IgG, however it is difficult to determine whether it results from cross-reactivity of the antibody, or binding of the antibody by CRP. In order to ensure the particular IgG tested was not less specific than required, it was tested against keratin. Keratin was initially chosen as it was available within the laboratory group and is also subject of auto-antibody activity within inflammatory diseases (Boguslawski *et al*., 2007).

Anti-keratin antibodies have been found in normal human sera and that of patients with RA, Sjogren's syndrome, cutaneous lesions, liver disease and lymphoproliferative disorders (Borg *et al*., 1993; Mattey *et al*., 1993). It was initially surprising to see an interaction between keratin and goat anti-human IgG antibody. As with mCRP the interaction was only apparent at high antibody and protein concentrations as seen in Figure 4.8 and as with both pCRP and mCRP, no interaction was found with the Fc specific anti-human IgG (Figure 4.9). It is known that anti-IgG binds keratin within autoimmune disease and that such interactions between anti-IgG and keratin were not in fact as unusual as first considered (Endo *et al*., 2004; Young *et al*., 1979; Mallya *et al*., 1983; Lin *et al*., 2014), therefore it was considered that the antibodies were not less specific than required.

Whilst the interaction between anti-IgG and CRP was accepted as having minimal impact within the ELISA assay designed for detection of CRP or associated auto-antibodies, it was considered interesting and was therefore further investigated. Both IgG and CRP bind through Fc gamma receptors, pCRP binds to FcγRI, FcγRIIa and FcγRIIb (CD64, CD32A & CD32B), mCRP is thought to bind FcγRIIb and FcγRIII (CD32B & CD16) (Li *et al* 2004; Shih *et al* 2009). IgG also binds Fc gamma Rs (Ravetch & Bolland, 2001) and therefore it was considered possible that the recognition of mCRP by anti-IgG

may have been due to a shared homology within the Fc binding sites. However, Figures 4.7 and 4.9 indicate that this is not the case as there was no interactions found between anti-Fc specific IgG and either mCRP or pCRP. Although our result has not been able to clarify how or where the interactions take place, they may be useful within the body to reduce circulating levels of mCRP or to aid removal of damaged or denatured proteins.

The difference between the interaction between the secondary antibodies and either mCRP or pCRP could not be explained and is an interesting area of future research. However, within our own experiment it was decided to ensure the potential effect of sodium azide upon peroxide was not influencing our results. Horseradish Peroxidase (HRP) is commonly used as a conjugate within detection antibodies and sodium azide (SA) used as a chemical preservative within some biological compounds and solutions. It is also known that SA can inhibit the actions of HRP via its inhibition of the HRP-H_2O_2-hydrogen donor system (Weinryb, 1968; Ortiz de Montellano *et al*., 1988). The pCRP solution supplied by Scripps Labs is within a buffer containing 0.01% SA, whereas the mCRP solution did not. The effect of SA on mCRP was therefore tested to ensure the lower results gained within the IgG interaction studies were not as a result of SA inhibition. As can be seen within Figure 4.10 this was not the case as no significant difference between mCRP with or without 0.01% SA was found.

Although it was important to eliminate any unwanted interactions within the ELISA assay, it was considered that the CRP and antibody dilutions intended for use within the experiments would not be unduly affected. For instance, the secondary anti-mouse IgG used was at a 1:30000 dilution and mCRP or pCRP used to coat the plates was 2µg/ml. Interaction studies showed limited interactions at these levels (Figure 4.4), with mCRP absorbance reaching only 0.09 and pCRP 0.018. All assays were run with matched control

wells which were also subject to the same interactions between CRP and anti-IgG, removing the risk of the interaction influencing the results.

4.4.3 Interactions between Antibodies, SAP and PTX3

It was also important to ensure that the chosen antibodies did not recognise closely related pentraxins which may also be present within serum. SAP and PTX3 were therefore both tested against the antibodies and none of the capture or detection antibodies were found to interact with them (Figure 4.3). The antibodies chosen were therefore all (with the exception of 8D10, which did not produce a strong enough signal), considered appropriate for use within further optimisation of protocols and in all future experimentation.

4.4.4 Optimisation of Sandwich ELISA

The R&D ELISA Duoset used within the pCRP detection assays was tested to ensure that all CRP detected was in fact pCRP and not mCRP. As can be seen in figure 4.11, no recognition of mCRP was found at any concentration and therefore the kit was optimised for use within the pCRP detection assays. Patient samples and antibodies were optimised to allow accurate and reproducible results within the assay system and as such patient samples were diluted between 1:1000 and 1:1280000. The range that accommodated high to low serum concentrations of pCRP was 1:16000 for patient and 1:1000 for control samples (Table 2.8). In addition to this the capture antibody concentration was halved.

4.4.5 Optimisation of Competitive ELISA

During study design of auto-antibody detection assays, within both patient and control samples, it was necessary to ensure the assay reduced interactions and confounding variables with other agents. Human serum antibodies may interact with the blocking agents

or capture antibodies used within ELISA and western blotting procedures (Waritani *et al*., 2017, Hilger *et al*., 2001). Patient and healthy control serum may contain levels of antibodies directed against dietary antigens such as BSA, with studies reporting between 50-55% of patient samples and up to 62% of controls anti-BSA positive (Mogues *et al*., 2005; Noriaki Tamura, 1982; Sjowall *et al*., 2011). Rheumatoid factors can also interfere with immunoassay results, due possibly to reactions against secondary detection antibodies within the assay, or by interaction with immune complexes immobilised on the plate (Tarkowski *et al*., 1983). In addition to these factors, the serum could not be bound directly to the plate as the pCRP contained within it could dissociate into mCRP before the assay began, thus affecting results. This conformational change was avoided by the use of a capture antibody or agent for both pCRP and mCRP assays.

Several studies have stabilised pCRP by pre-coating the ELISA plate with phosphocholine bound keyhole limpet haemocyanin (PCh-KLH), which allows pentamer binding when in the presence of 2mM $CaCl_2$ (Potempa *et al*., 2015; Wang, 2012;). This method was adopted within the assays seeking to profile anti-pCRP IgG, IgA and IgM, with a goat anti-CRP antibody used to capture mCRP. However the danger of potential interactions between the BSA blocking step and capture antibodies remained and therefore a competitive ELISA system was developed

4.5 Conclusion

Following optimisation steps, capture and detection antibodies were assessed for their specificity and found to be suitable to correctly identify either mCRP or pCRP, additionally no interaction was found between these antibodies and either PTX3 or SAP. Considerable steps were taken in order to minimise or remove all other possible interactions between the assay components and either antibodies or auto-antibodies.

Chapter 5 - Detection of CRP within human serum

5.1 Introduction

5.1.1 Detection and Measurement of CRP within Human Serum

The detection and quantification of CRP concentrations within serum or plasma has long been a feature of routine blood tests, as a measure of non-specific inflammation or infection within a patient. Higher than an accepted normal levels, (>3 mg/L), often indicates an underlying diseased state, inflammation or autoimmune linked condition (Pepys & Hirschfield, 2003; Ucer *et al*., 2008; Araújo *et al*., 2009; Salazar *et al*., 2014).

Whilst detection of higher than normal levels of CRP indicates a non-healthy state, the actual cause requires further diagnosis through more specific testing. Diagnosis of RA can be determined through blood tests for high levels of CRP in association with other recognised diagnostic factors such as elevated erythrocyte sedimentation rate (ESR) or the presence of rheumatoid factor or anti-citrullinated peptide (anti-CCP) antibodies. MRI and ultrasound tests may be undertaken to track the progression of joint damage over time (Davis *et al*., 2012) and symmetrical joint pain and damage is often assessed by Disease Activity Score (DAS) of either a 28 or 66 joint count (Houssien *et al*., 1999; Scott *et al*., 2000; van Riel & Renskers, 2016) of which DAS28 is routinely used for diagnostic purposes. Within clinical settings the detection of CRP has been performed by ELISA systems which rely on the specificity of antibodies and their detection of the native pentameric form of the protein. Increased sensitivity has been accomplished using high sensitivity ELISA assays, again reliant on antibody specificity. Upon acceptance that there could in fact be additional forms of CRP found within the serum or tissue, the question of accuracy and implied meaning of previous testing could be called into question, both within clinical and research settings. Findings generated over the last 20 plus years may

have assumed detection of native pentameric CRP, however, could possibly have identified monomeric or a combination of the two forms instead. A system that cannot consistently detect and differentiate between multiple forms may add to conflicting evidence for the either pro or anti-inflammatory nature of the protein. RA disease activity is consistently found higher in cases of high confirmed CRP levels (Otterness, 1994). Interestingly it is not just upon flare that CRP levels are found to be raised, a study in 2004 by Nielen *et al*, via analysis of CRP levels within blood donor populations, found that CRP levels were increased up to five years before the onset of RA symptoms, with two years pre onset being the most commonly found period of detected increased (Nielen *et al*., 2004) It is clear that the accurate detection of CRP is an important diagnostic tool. Determination of both monomeric and pentameric forms has begun within some disease models such as atherosclerosis and kidney disease, but is not used clinically within diagnosis or prognosis.

Patient CRP serum levels are routinely analysed within both clinical and research settings, often via immuno-detection methods reliant upon antibody recognition of CRP. Within a clinical setting these techniques need to be rapid and efficient and are now often automated, for example the National Health Service (NHS), who are now less reliant on more costly and time consuming manual techniques such as traditional ELISA now commonly use automated systems which allow higher throughput, such as latex-enhanced immunoturbidimetry (Koivunen and Krogsrud, 2006; Siemens, 2014). This is not always the case within a research setting where many individuals and groups are still reliant on standard ELISA protocols carried out manually, often in kit form, but also developed specifically using a combination of self-raised antibodies. Even minor deviations from a baseline serum level can indicate inflammation or infection connected to disease, therefore the development of high-sensitivity CRP tests has aided detection of these subtle variations substantially. Within clinical settings it is not always possible to determine which form of

CRP is being detected, Table 5.1 summarises some common hs-CRP tests used within clinical laboratories and illustrates the range of detection limits in addition to the lack of determination of which form of CRP has been detected.

Table 5.1 A table summarising the range of clinical CRP tests performed, including advantages and disadvantage. Table adapted from (Roberts *et al*., 2001). Additional information from (Deegan *et al*., 2003; Luo *et al*., 2012).

Clinical Test Description	Methodology	Limit of detection, μg/L	Advantages / Disadvantages	mCRP or pCRP detection	Manufacturer
N High Sensitivity CRP	Immunonephelometric	20.0	Fast, accurate, affordable Require trained personnel	Not specified	Dade Behring (Dade)
Pure Auto S CRP	Immunoturbidimetric	40.0	Fast, cost effective, sensitive	Not specified	Daiichi
CRP-Latex (II) High Sensitive Application	Immunoturbidimetric	30.0	Fast, cost effective, sensitive	Not specified	Denka Seiken (Denka)
CRP	Immunoluminometric	20.0	Fast, cost effective, sensitive	Not specified	Diagnostic Products Corporation (DPC)
HS-CRP	Immunoturbidimetric	5.00	Fast, cost effective, sensitive	Not specified	Iatron
K-Assay CRP (I)	Immunoturbidimetric	320	Fast, cost effective, sensitive	Not specified	Kamiya
CRP (Latex) Sensitive Application	Immunoturbidimetric	80.0	Fast, cost effective, sensitive	Not specified	Olympus
Tina-quant CRP (Latex) US	Immunoturbidimetric	210	Fast, cost effective, sensitive	Not specified	Roche
CRP-UL	Immunoturbidimetric	60.0	Fast, cost effective, sensitive	Not specified	Wako
Hs-CRP	Quantum dot-labelled immunosorbent	0.06	Rapid, sensitive, low cost and high-throughput	Not specified	Luo *et al*., 2003

High-sensitivity CRP testing is based upon a system of light scattering immunoassays in which an antigen: antibody complex can be detected within a solution. A study of 491 healthy blood donors by (Price *et al*., 1999), reported median CRP values of 2.40 mg/l males and 2.20 mg/l females with a 95th percentage range of 1.20 -5.20 μg/mL male and 0.40-5.40 μg/mL female using light scattering immunoassays. In a more recent study, a quantum dot-labelled immunosorbent assay was developed allowing sensitive and rapid detection of CRP, this assay allowed detection of CRP within a range of 1.56–400 ng/ml at a limit of detection at 0.46 ng/ml and quantification limit at 1.53 ng/ml (Lv *et al*., 2017). This novel detection method is whilst fast, still reliant on predictable interactions with antibodies specific to CRP; in their case mouse anti-CRP purchased from Abcam, but allows a detection method of far greater sensitivity than many traditional ELISA systems.

It is clear that procedures are being developed rapidly enabling faster and more accurate/sensitive detection of CRP, but there is little or no distinction within a clinical setting defining the isoform of CRP, with detection currently being assigned to native pentamer. At research level, groups studying monomeric CRP have developed individual protocols based upon sourced antibodies directed towards epitopes exposed on the monomer but not the pentamer as ways to differentiate isoforms (Kresl, Potempa and Anderson, 1998; Khreiss *et al*., 2004; S.-R. Ji *et al*., 2006, 2009). It is very possible historical research studies have used antibodies now known to detect both forms of CRP, including Clone 8 (Sigma), and drawn conclusions of the effects or presence of CRP without distinction of which isoform(s) are present.

5.1.2 Serum Monomeric CRP in Health and Disease

The ability to produce and detect monomeric CRP dissociated from the native pentameric form, within *in vitro* systems, via various different production protocols has

been illustrated in Chapter 3. Documentary evidence indicates a growing number of studies which also identify the dissociated form of CRP *in vivo* within human tissue and less frequently serum, occurring most commonly within inflammatory linked diseases (S.-R. Ji *et al*., 2006; Ji *et al*., 2009; Zhang *et al*., 2009). The biological and physiological relevance of mCRP is yet to be fully understood, however it is frequently suggested to be pro-inflammatory in nature due to its association within inflammatory and autoimmune disease.

Whilst the biological relevance and exact method of production of mCRP is yet to be fully understood, there is a pool of research indicating association with phosphocholine (PC) and cell membranes allows rapid dissociation of the pentamer towards a monomeric form (S.-R. Ji *et al*., 2006; Eisenhardt, Thiele, *et al*., 2009; Ji *et al*., 2009; Slevin & Krupinski, 2009; Habersberger *et al*., 2012; Molins *et al*., 2016). In their 2006 paper, Ji *et al* showed that calcium-dependent binding of pCRP to membranes, including cell membranes and low density liposomes, allowed a rapid although partial dissociation of the pentamer and structural changes towards an isoform allowing exposure of novel inter-subunit antigenic sites. This partial dissociation retained pentameric conformation in a relaxed form and was termed mCRP(m). The properties of this isoform allowed significantly enhanced complement fixation and when detached from the membrane was observed to form mCRP. Within their study they further documented the ability of mCRP to be solubilised (which they termed mCRP(s)), and to apply strong stimulatory effects to endothelial cells (Ji *et al*., 2006). Effects upon endothelial cells linked to mCRP include an upregulation of IL-8, monocyte chemoattractant protein, E-selectin together with ICAM-1 and VCAM-1 the cell to cell adhesion molecules (Ji *et al*., 2006).

Few studies have shown a soluble form of mCRP, which is neither associated nor bound with tissue, cells or fragments of cells such as the microparticles (MP) derived from

endothelial cells as described in Crawford *et al*'s 2016 study, or the membrane bound form mCRP(m) described by Ji *et al*. previously. In 2013 Habersberger *et al*. (2012) suggested that their research on activated platelets indicated a role for MP within the transportation of membrane bound mCRP throughout the body. They suggest a model whereby pCRP dissociated upon contact and binding to MP shed by activated platelets which then transport the monomeric form throughout the body in response to cardiovascular events such as myocardial infarction. In many of these studies the role of mCRP has been suggested to be pro-inflammatory in nature, such as Crawford *et al*'s 2016 finding that mCPR instigated pro-inflammatory endothelial shedding, whereas pCRP induced anti-inflammatory effects via regulation and reduction of inflammatory immune cells.

One study that has detected solubilised mCRP within circulation is that of Wang *et al*. (2015) who suggested this form of the protein was present within the serum of patients who recently had suffered acute myocardial infarction (AMI). The study showed levels of plasma mCRP elevated within AMI patients in contrast to those suffering from unstable/stable angina pectoris or control groups (0.021mg/l ± 0.0016). Interestingly, not only did they detect plasma mCRP, but nine of the AMI group who died prior to a 30 day follow up had significantly elevated plasma mCRP (0.0367 mg/l ± 0.01 vs 0.0194mg/l ± 0.00143, P = 0.02) when compared to those who survived, indicating a possible future AMI diagnostic value to mCRP testing. Increased mCRP levels were also observed in 6 patients with high identified levels of pCRP, their plasma mCRP levels ranged from 0.0077 to 0.1227mg/l. This study ensured the mCRP measured was present within serum tested rather than an artefact of the ELISA protocol, by coating a specific capture antibody to the ELISA plate, rather than coating serum directly to the plate. This method prevents spontaneous dissociation of CRP upon immobile surface binding seen within other studies.

As shown, within the field of mCRP research, diseases linked to the cardiovascular system are perhaps the most frequently studied. Supporting the findings of Wang *et al*., (2015) researchers within another group have initiated patent proceedings suggesting serum mCRP identification may provide a useful diagnostic tool detecting early preclinical stages of atherosclerosis (Kiefer *et al*., 2005 Patent number US2006057642-A1).

Aside from the cardiovascular system, several other diseases have been identified as having mCRP co-localised to diseased tissue or within circulation. The ocular system has emerged as one such disease frequently associating mCRP to pathology. Age-related macular degeneration has been associated with detectable levels of mCRP elevated within tissue taken from genotyped human donor eyes (Chirco *et al*.,, 2016). Those homozygous for the CFH (Y402H) high risk allele showed choriocapillaris and Bruch's membrane associated with elevated mCRP, in contrast to genotypes of low risk. RNA analysis of expressed inflammatory genes within cultured choroidal endothelial cells showed increased migration rate and monolayer permeability when treated with mCRP in comparison to those challenged with pCRP or medium alone. Altered expression of ICAM-1 and CA4 were reported within organ cultures upon treatment of mCRP (Chirco *et al*., 2016). Further to this, Molins *et al* suggested that mCRP and not pCRP present within retinal tissue disrupted the outer blood-retinal barrier, via functional impairment to the retinal pigment epithelium (RPE), from which it is formed. They suggested that mCRP increased paracellular permeability and caused disruption to ZO-1, a tight junction protein in addition to occludin a protein found within RPE cells. These effects were prevented by the presence of corticosteroids or anti-VEGF drugs (Molins *et al*., 2017). Table 5.2 details the levels of mCRP and pCRP found within a variety of disease states where reported.

Table 5.2 A summary table of mCRP and pCRP levels detected within key diseases where known. AMD (age related maculardegeneration, AMI (acute myocardial infaction), NR (not reported).

Disease	mCRP mg/l	pCRPmg/l	Source
Healthy	-	Median 1.3mg/l (range 0.75-2.22mg/l)	(Pearson *et al*., 2003) (Meyer, 2010)
AMI	0.021mg/l (range 0.019-0.036mg/l)	NR	(Wang *et al*., 2015)
Not defined	0.88mg/l (range 0.04 -3.52mg/l)	23.3mg/l (range 2.9-66.8mg/l)	(Williams 2017)
Alzheimer's	Tissue bound	NR	(Strang *et al*., 2012; Slevin *et al*., 2003)
Ischemic Stroke	Tissue bound	NR	(Strang *et al*., 2012; Slevin *et al*., 2003)
cholecystitis, lung squamous cell carcinoma, mucinous gastric adenocarcinoma, renal cell carcinoma	Tissue bound	NR	(Shi et al., 2014)
RA	-	Median 5.3 mg/L	(Graf *et al*., 2009)
Inactive Lupus SLE non serositis	NR NR	mean 0.96 mg/l, (range 0 – 12mg/l) Median 1.37 mg/l	(Kakai *et al*., 2003) (Enocsson *et al*., 2014)
Active Lupus SLE serositis	NR NR	Mean 7.1 mg/l, (range 0 – 36) Median 2.98 mg/l	(Kakai *et al*., 2003) (Enocsson *et al*., 2014)
Coronary Heart Disease	NR	1.75 ±5.3mg/l	(Danesh *et al*., 2004)
Myocardial infacation	Tissue bound	NR	(Habersberger *et al*., 2012)
AMD	Tissue bound	NR	(Chirco *et al*.,, 2016).

Brain tissue from patients, post-acute ischemic stroke, has been shown to have high levels of deposited monomeric CRP within functional neuron and glial cells tissue. This deposit, suggested to be expressed within the brain extracellular matrix, was determined to be an agent in part responsible for neurodegeneration and abnormal vascular development associated post stroke by (Slevin *et al*., 2010). The same group further detected monomeric CRP at higher levels adjacent to infarcted areas, within Alzheimer's disease (AD) affected brain tissue, possibly linking mCRP to vascular damage within the brain

(Slevin *et al*., 2017). Elevated mCRP levels were found within tissue of the frontal cortex regions of AD affected brains when contrasted against non-diseased brain tissue by Strang *et al*., *(*2012). In this study they were also able to illustrate the ability of amyloid plaques to dissociate pentameric to monomeric CRP (Strang *et al*., 2012).

As shown, few studies have detected soluble mCRP within serum, it is as yet unclear whether mCRP circulates freely within the blood or if as indicated by some studies it is bound MP, or both, with most attributing it to co-localisation with diseased tissue or circulating microparticles. In addition to Wang *et al*'s 2015 paper, Williams (2017) research detailed the detection, isolation and quantification of soluble mCRP within serum samples from a cohort of patients identified as having high baseline pCRP levels. Serum CRP was isolated by PC affinity column and mCRP separated via FPLC, enabling quantification by ELISA. In this study the solubilised mCRP detection levels were between 0.04 mg/l and 3.52 mg/l, however this followed concentration of the FLPC fractions (Williams, 2017).

In their 2016 study, Crawford *et al* investigated plasma levels of endothelial cell microparticles found located in association with mCRP with results indicating a correlation to increased peripheral artery disease. This study also observed soluble free mCRP and suggested differing biological effects dependent on the manner in which it circulated the blood stream (Crawford *et al*., 2016).

There is greater focus and wealth of research associated with auto-antibodies directed towards mCRP present within SLE patient serum than that of mCRP itself. Whilst pentameric CRP has long been associated with RA pathogenicity, and a monomeric CRP isoform, or auto-antibodies directed towards it, associated with SLE pathogenicity - there is little research investigating the presence of monomeric CRP within RA patients. A study by Shi *et al*., *(*2014) looked at the association of mCRP with lesions in differing disease

models. The tissue sites chose included skin tissue of a discoid lupus erythematosus patient in the active phase showing mCRP but not pCRP presence and knee joint synovium tissues of a rheumatoid arthritis patient which also showed the presence of mCRP and not pCRP within the cytoplasm of synovial cells. It was suggested the implication of this finding were cellular uptake of mCRP. In addition to these results tissue taken and stained from patients with ulcerative colitis, Hashimoto's thyroiditis, and lupus nephritis also showed negligible pCRP but the presence of mCRP, with suggested features of cell and arteriole associated expression. It is interesting that these tissues are taken from various locations across the body regardless of tissue type indicate co-localisation of mCRP and not pCRP within inflammatory sites, suggesting mCRP is perhaps the predominant isoform found within local inflammatory processes (P. Shi *et al*., 2014)(Shi *et al*., 2014).

5.1.3 Experimental Aims

To enable meaningful quantification and analysis of mCRP and pCRP within RA patient serum it was essential to develop an effective protocol for the identification of CRP isoforms. The overall aim was to design an ELISA assay capable of accurately identifying and quantifying isoforms of CRP where present within RA patient serum. The identification of such would allow greater understanding of RA and the role of both pentameric and monomeric CRP play within this inflammatory disease. Rheumatoid arthritis is characterised by high levels of serum CRP, which are elevated during flare and reduced in response to corticosteroid treatment. Little research has occurred investigating the conformational nature of elevated CRP and if, as in several other diseases, it is actually found to be at least in part monomeric. An ELISA assay capable of overcoming the suggested spontaneous dissociation of pentameric CRP must be developed, allowing accurate differentiation between serum mCRP and pCRP. The successful development of

this assay will allow full and rigorous investigation as to whether monomeric CRP was present within serum and if it was found to be higher or lower within the RA patient or control group serum. This comparison to non-inflammatory disease control serum may aid further study as to whether monomeric CRP is pro or anti-inflammatory in nature.

5.2 Materials and Methods

5.2.1 Ethical Application

Prior to collection of patient samples and experimental procedure took place, both peer review and ethical research committee approval was sought. This oversight ensured the soundness of the study aims and assessed risk to both participants and researcher. The review process and ethical application are detailed within section 2.3.1.

5.2.2 Acquisition of Human Serum Samples

Upon recruitment of a patient to the study and a signed and dated consent form provided, a qualified phlebotomist at the Haywood Rheumatology Hospital drew venous blood, following their internal clinical care protocols. Samples were collected and processed as detailed within section 2.3.2.

5.2.3 ELISA Quantification of Human Serum Samples

Detection of pentameric CRP within patient serum samples was determined by ELISA using a commercially available sandwich ELISA assay kit (R&D systems Human CRP DuoSet ELISA) as described within section 2.3.8. Monomeric CRP was detected by an optimised sandwich ELISA using a specific anti-human mCRP detection antibody also described within section 2.3.8.

5.2.5 Western Blot Analysis of Human CRP

Native PAGE gels were run as described in Chapter 2, section 2.3.7, with buffers and components described within Tables 2.4, 2.5, 2.6 and 2.7. A representative set of samples were chosen from both the RA and control sample sets in order to confirm ELISA positive and negative results in both monomeric and pentameric assays. In order to allow

mCRP to run cleanly within the gel, the method described in section 2.3.7 using 1/20th SDS within both the sample and running buffer was used. Serum samples were diluted 1:100 pCRP and 1:100 mCRP in deionised water and added to an equal volume of 2x sample buffer (containing 1/20th SDS). The western blot protocol and reagents used were as described in section 2.3.7, with the following amendments. Following the blocking step and once thoroughly washed, the primary antibodies diluted in BLOTTO were added. For mCRP detection, primary antibody 3H12- mouse anti-human mCRP at a concentration of 1:50 and for pCRP detection, Clone 8 mouse anti-human CRP (Sigma) at a concentration of 1:2000, were added. Both antibodies were diluted in BLOTTO and the membrane was then incubated overnight upon a rocker at 4^{o}C.

Following wash steps the conjugated secondary antibody, rabbit anti-mouse IgG at a concentration of 1:30,000 was incubated for 1 hour at room temperature before once more washing prior to development with ECL reagent.

5.2.4 Statistical Analysis

Statistical analysis was carried out as described in section 2.3.10. During initial statistical analysis it became apparent that the male cohort data was affecting analysis and in many cases the combination of male and female data sets produced bimodal data making further analysis based on comparison of mean or median data unreliable. It was therefore necessary to subcategorise the data by gender into male and female groups before further detailed statistical analysis

5.3 Results

5.3.1 Experimental Design

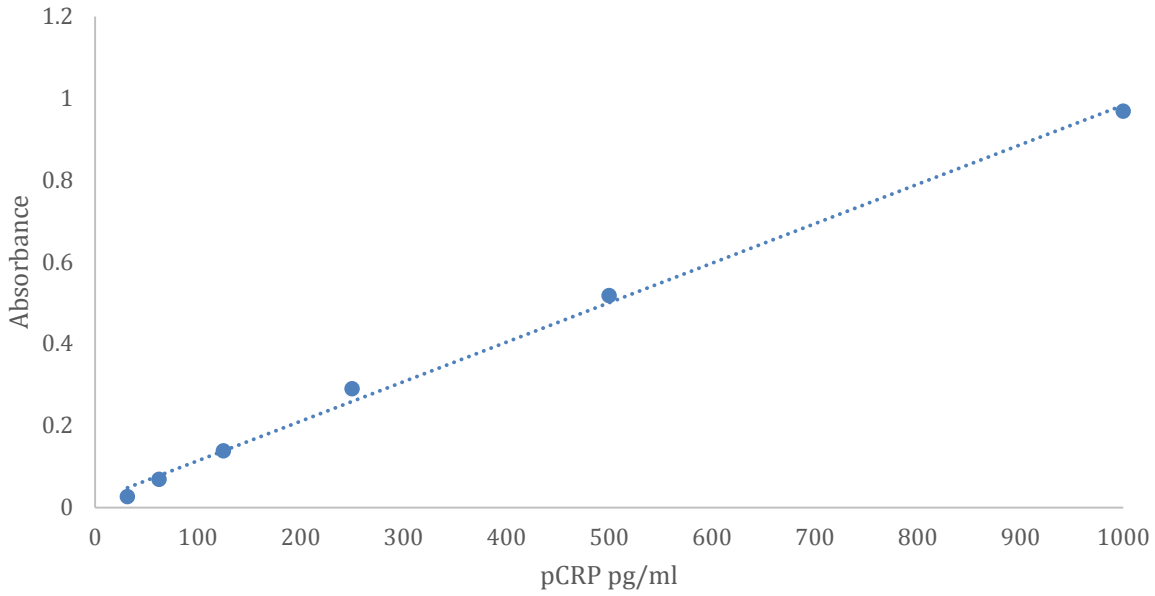
All patients were recruited from a routine monitoring clinic at Haywood Rheumatology Hospital. Patients with an existing and confirmed seropositive RA diagnosis were identified from the monitoring clinic list and only those with a medically required routine blood test were approached with an explanation of the study remit and purpose. RA diagnosis had been previously documented via DAS28 score which consists of an assessment of the number of swollen joints out of 28 tested, the number of tender joints (of the 28), blood tests for both CRP levels and erythrocyte sedimentation rate (ESR) and lastly a 'global assessment of health' indicated by the patient. In addition to DAS28 scores, seropositive patients were positive for the presence of RFs and anti-CCP antibodies. Due to the nature of the clinic it was not possible to predict the number of suitable patients available within the time available for sample collection.

The primary outcome for this study was to determine whether mCRP could be distinguished from pCRP and identified within human RA patient serum. It was possible to recruit 30 patient samples and 30 control samples within the time available.

5.3.2 Enzyme Linked Immunosorbent Assay Analysis of Human CRP

In order to quantify both monomeric and pentameric CRP levels, if present, within serum samples an ELISA system was developed. Analysis by ELISA allows a sensitive detection of low levels of protein in a specific manner. Each plate was processed in triplicate with samples also triplicated upon the plate, including calibration proteins added in order to produce individual calibrations for each assay. A representative example of both monomeric and pentameric calibration graphs are illustrated in figure 5.1.

(a.)



(b.)

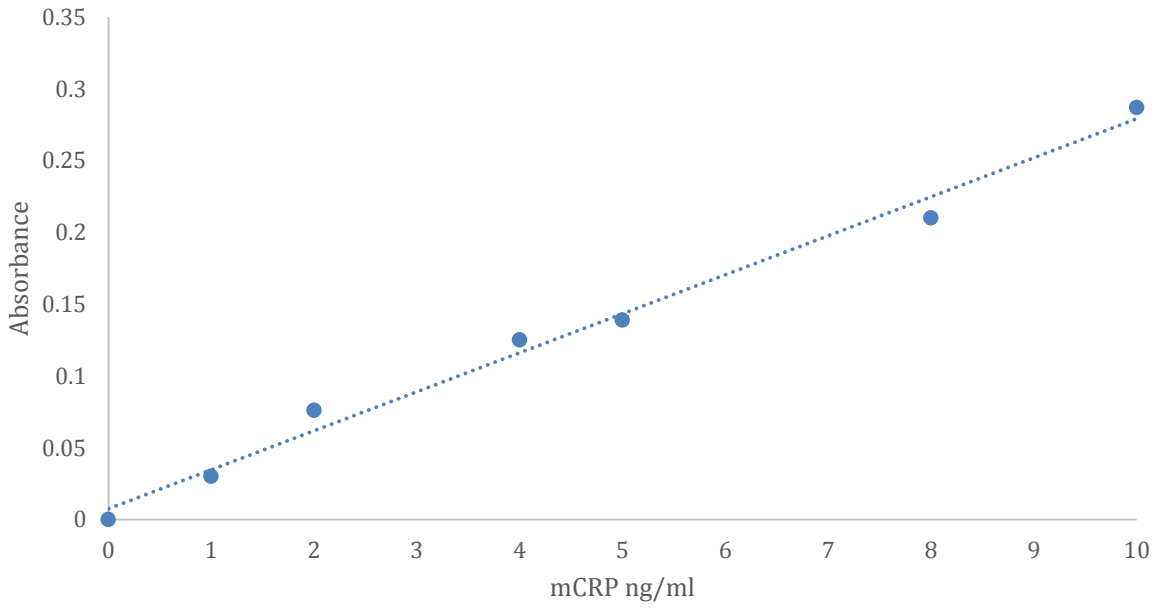


Figure 5-1 The calibration graph for CRP ELISA assays. (a.) pCRP with a range between 0 to 1000pg/ml. The line equation is $y = 0.001x + 0.0177$ and the $R^2 = 0.9968$. b) The calibration graph for mCRP with a range of concentrations between 0 to 10ng/ml. The line equation is $y = 0.0272x + 0.0075$ and the $R^2 = 0.9892$.

Figure 5.1a and 5.1b illustrate representative calibration graphs for both mCRP and pCRP assays. The protein standards used were human pCRP purchased from Scripps Labs and mCRP produced *in vitro* using 2M urea dissociated pCRP as described in sections

3.2.2 and 3.3.2. The R^2 indicates a positive correlation between absorbance and concentration indicating reliability within the calibration equation and therefore calculated protein values. The line equations generated from each calibration were used to determine concentrations of mCRP and pCRP respectively. The range of concentrations chosen for the mCRP calibration (0.00 to 10.00ng/ml) allowed detection of patient samples with a detection limit of 0.26ng/ml equating to a patient concentration of 0.027mg/l. The range of concentrations chosen for the pCRP calibration (0.00 to 1000pg/ml) allowed detection of patient samples with a detection limit of 17.68 pg/ml, equating to patient concentrations of 0.028 mg/l and control concentrations of 0.035mg/l. Each plate contained a positive control which tested positive for either monomeric or pentameric CRP and a negative control which tested negative (results not illustrated).

Results of the ELISA quantification of CRP, show that neither the RA nor control groups were found to contain samples categorised as elevated within the mCRP assay, when compared to the calculated threshold (≥ 0.143; mean + 2SD in 30 normal controls), although a greater frequency of samples with mCRP detected were found within the RA as opposed to control groups (Figures 5.2 and 5.3). As seen in Figure 5.2 all samples bar one, were found to contain detectible levels of mCRP with the highest recorded value at 0.133mg/l (sample RA23) and the lowest positive value 0.053 mg/l (sample RA20). Results of the pCPR ELISA once again showed a significant difference in terms of pCRP concentration found between the entire mixed gender RA and control group samples as determined by Mann Whitney ($P < 0.001$, Figures 5.4 and 5.5). Further analysis of results is shown within Table 5.3 which details the median (IQR), range and calculated threshold for each group.

Table 5.3 A summary table illustrating calculated median (IQR), threshold and range (mg/l) for CRP detection assays as determined by ELISA.

Group	Median (IQR)	Threshold mg/l (Mean + 2SD)	% Samples with Detectible CRP	Proportion of Samples above Threshold (mean control ± 2SD)	Range mg/l
RA mCRP	0.098 (0.080-0.105)	0.143	96.6% (29/30)	0 of 30	0.053-0.133
Control mCRP	0.085 (0.06-0.09)	0.143	80% (24/30	0 of 30	0.058-0.131
RA pCRP	22.02 (7.35-29.44)	2.924	100% (30/30)	27 of 30	1.08-83.68
Control pCRP	0.285 (0.118-0.947)	2.924	96.6% (29/30)	1 of 30	0.06-5.41

The higher than normal levels of pCRP in one of the control group (C14) was unexpected as this group was determined not to have an underlying inflammatory condition upon consent.

However during initial statistical analysis it became apparent that the male cohort data was affecting analysis and in many cases the combination of male and female data sets produced bimodal data making further analysis based on whole group comparison of mean or median data unreliable. It was therefore necessary to subcategorise the data by gender into male and female groups before further detailed statistical analysis. The control group comprised of 7 males and 23 females and the patient group of 8 males and 22 females (female median 0.086, IQR 0.07499-0.093, male median 0.00, IQR 0.00). Once adjusted for gender there was no longer a significant difference found between mCRP levels within the separated male and female, RA and control groups (female $P > 0.067$, male $P > 0.09$).

Due to the small sample size it is difficult to predict whether this finding would be observed within the wider population, although power analysis suggests the sample size for

females is adequate (female group sample size suggested minimum 9, power 0.852). However the male population is suggested to require a sample size of 50 to achieve a power level of 0.8. These results when taken in conjunction with the fact that no samples in either RA or control groups exceed the higher mCRP threshold level, suggest a lack of significant difference between the RA and control group despite combined data findings. It is however necessary to determine the concentration levels across a much larger population, in order to assess whether the female value of $P > 0.067$ and male value of $P > 0.09$ become significant.

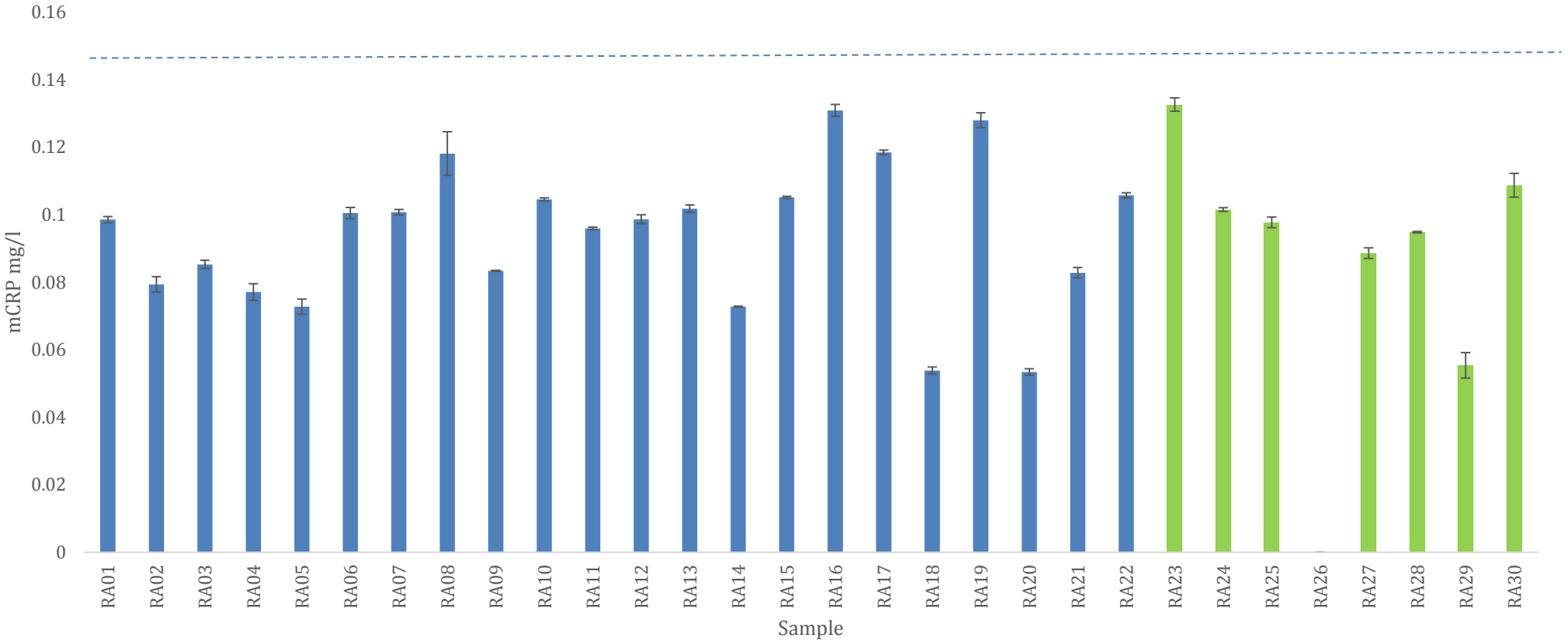


Figure 5-2 Bar graph displaying monomeric human CRP concentrations within 30 RA group serum samples as determined by ELISA assay. The concentration for each sample is expressed in terms of mg/l. Samples were initially diluted 1:100 and measured in triplicate over 3 separate ELISA assays, data represents the calculated mean ± SEM. Capture antibody dilutions were 1:10,000, Primary antibody dilutions (mAb 3H12) 1:50 and secondary conjugated antibody 1:30,000. Error column represent the SEM for each sample RA group *n*=30. Samples RA23 – RA 30, highlighted green column = male, blue column = female. The dashed line represents upper normal mCRP level (≥0.143; mean + 2SD normal samples) *n*=3.

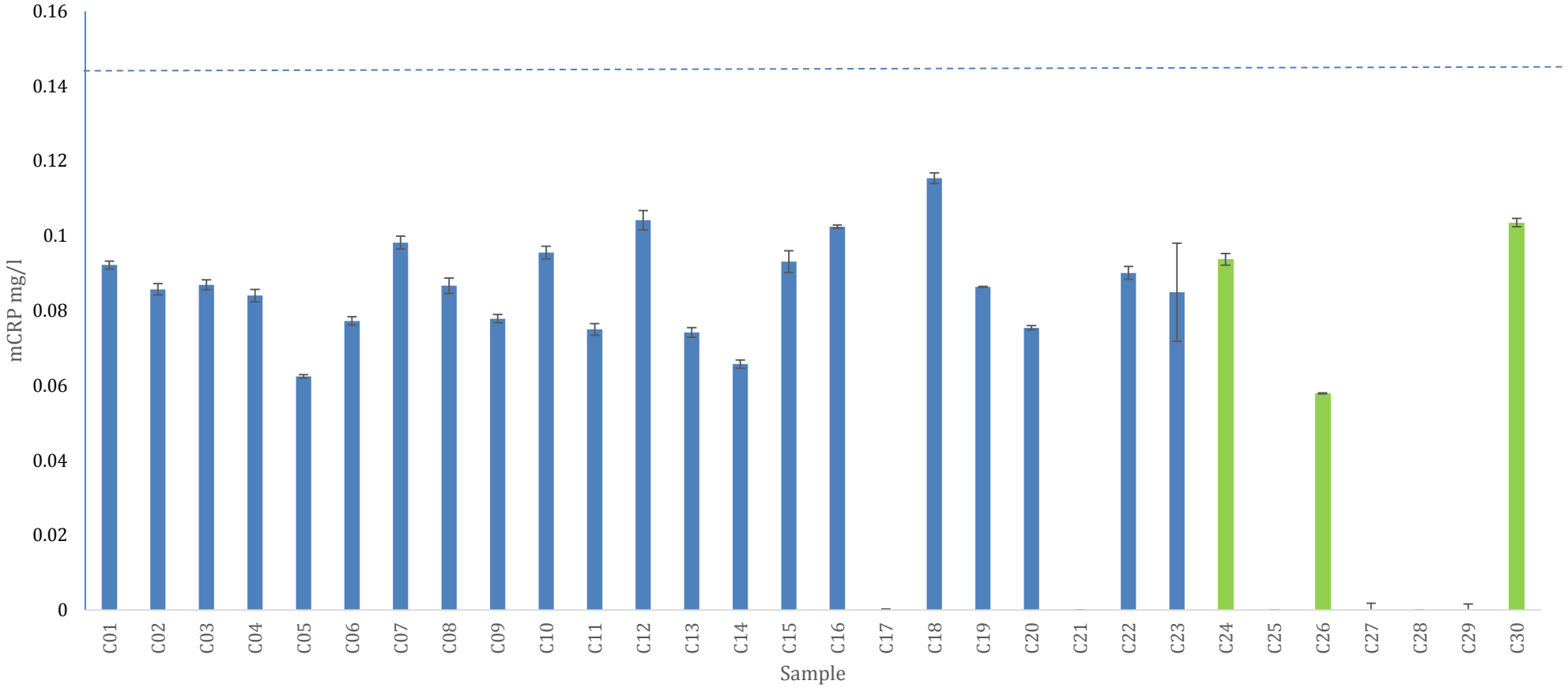


Figure 5-3 A bar graph displaying monomeric human CRP concentrations within 30 control group serum samples as determined by ELISA assay The concentration for each sample is expressed in terms of mg/l. Samples were initially diluted 1:100 and measured in triplicate over 3 separate ELISA assays, data represents the calculated mean ± SEM. Capture antibody dilutions were 1:10,000, Primary antibody dilutions (mAb 3H12)1:50 and secondary conjugated antibody 1:30,000. Error bars represent the SEM for each sample. Control group *n*=30. Samples C23 - C30 highlighted green column = male, blue column = female. The dashed line represents upper normal mCRP level (≥0.143; mean + 2SD normal samples), n=3.

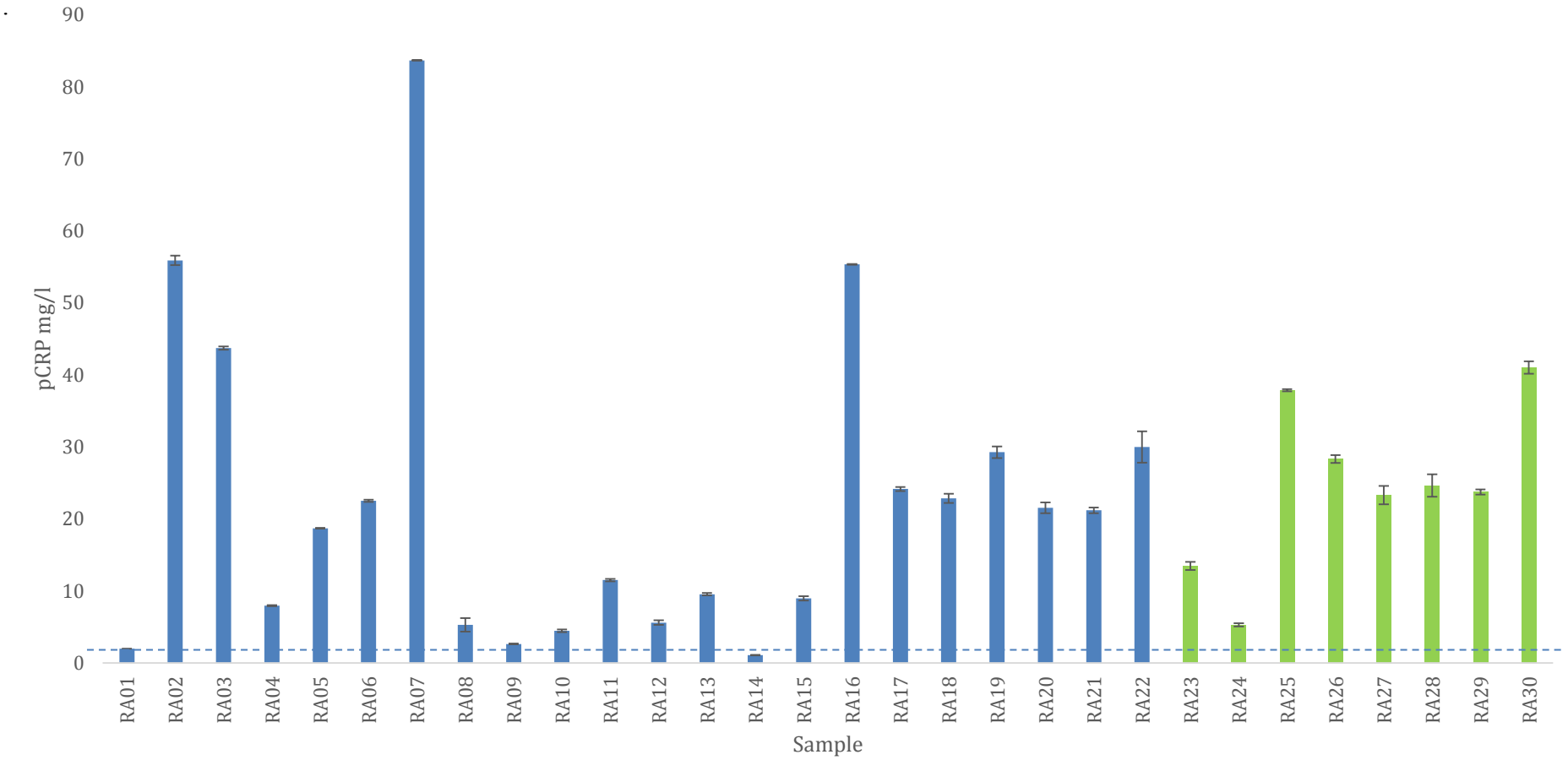


Figure 5-4 A bar graph displaying pentameric human CRP concentrations within 30 RA group serum samples as determined by ELISA assay. The concentration for each sample is expressed in terms of mg/l. Samples were initially diluted 1:16000 and measured in triplicate wells, replicated in 3 individual ELISA assays, data represents the calculated mean and error bars represent ± SEM for each sample. RA group *n*= 30. Samples RA23 – RA 30, highlighted green column = male, blue = female. The dashed line represents the above normal pCRP threshold (≥2.924; mean + 2SD normal samples), n=3.

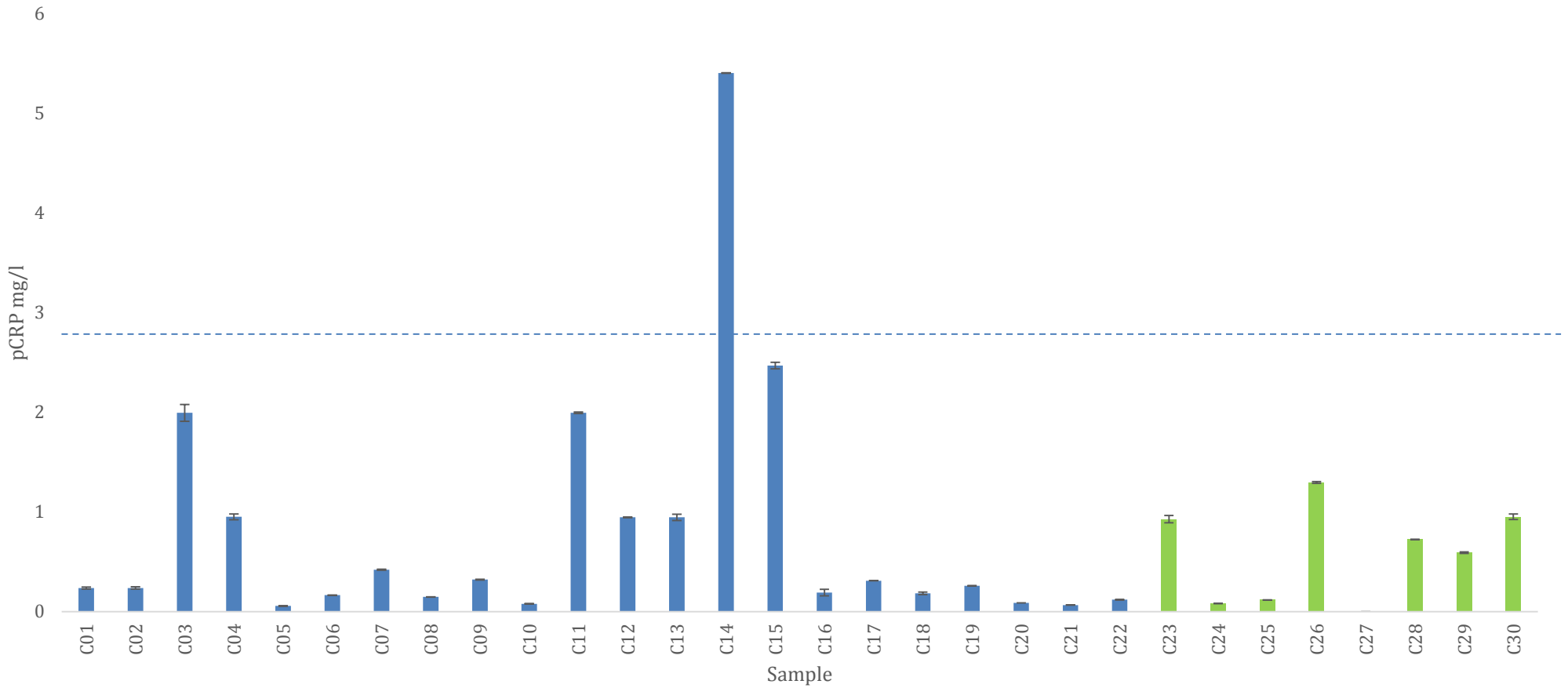


Figure 5-5 A bar graph displaying pentameric human CRP concentrations within 30 control group serum samples as determined by ELISA assay. The concentration for each sample is expressed in terms of mg/l. Samples were initially diluted 1:1000 and measured in triplicate wells, replicated across 3 individual ELISA assays, data represents the calculated mean. Error bars represent ± SEM for each sample. Control group *n*=30. Samples C23 - C30 highlighted green column = male, blue = female. The dashed line represents the above normal threshold (≥2.924; mean + 2SD normal samples), *n*=3.

5.3.3 Western Blot Analysis of Human CRP

In order to further confirm the presence of CRP within the samples, western blot analysis was carried out. All RA samples within the pCRP assay and those above an absorbance value of 0.1mgl/l within the mCRP assay were tested. Samples were first diluted 1:100 pCRP and 1:100 mCRP assay, in deionised H_20. A mouse monoclonal anti-human mCRP antibody (3H12), specific to the monomeric form of CRP, (Dr L. Potempa, College of Pharmacy, Roosevelt University, Illinois), was used in order to test samples for mCRP. A commercially available human CRP specific monoclonal antibody (Clone 8, Sigma), was used to determine the presence of pCRP. The Clone 8 antibody was pre-tested for detection of both forms of CRP with *in vitro* produced mCRP and human pCRP (Scripps Lab) and found to recognise both, see section 4.3.1. Thus indicating the recognised epitope for this antibody is conserved upon both isoforms of CRP, with mCRP retaining key structural integrity when compared with pCRP, this antibody detects both forms of CRP. Whilst monomeric CRP produced *in vitro* may not be structurally identical to potential forms found within human serum, it can be assumed that the epitope antibody 3H12 recognises, the terminal fragment (residues 147-206) of proteinase-cleaved CRP, will be apparent on *in vivo* samples too.

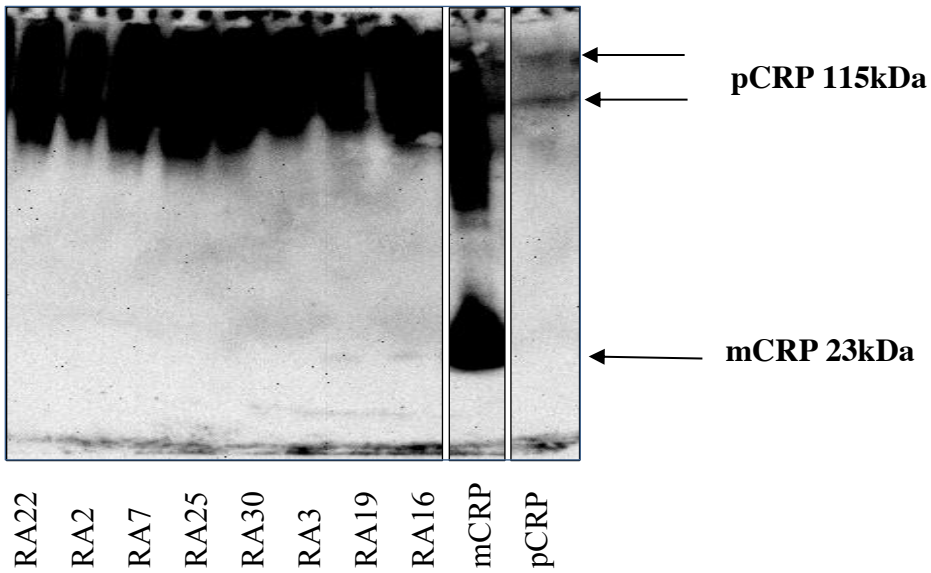


Figure 5-6 A pCRP and mCRP detection western blot image showing a representative group of RA samples run on a precast gradient gel developed over 45 minutes. Patient samples from left to right (lanes 1-8), lane 9 is mCRP control (0.5µg), and lane 10 pCRP (0.5µg). Primary antibodies 3H12 (1:50 dilution) and Clone 8 (1:2000 dilution) were followed by secondary rabbit anti-mouse IgG (1:30000 dilution), *n*=2.

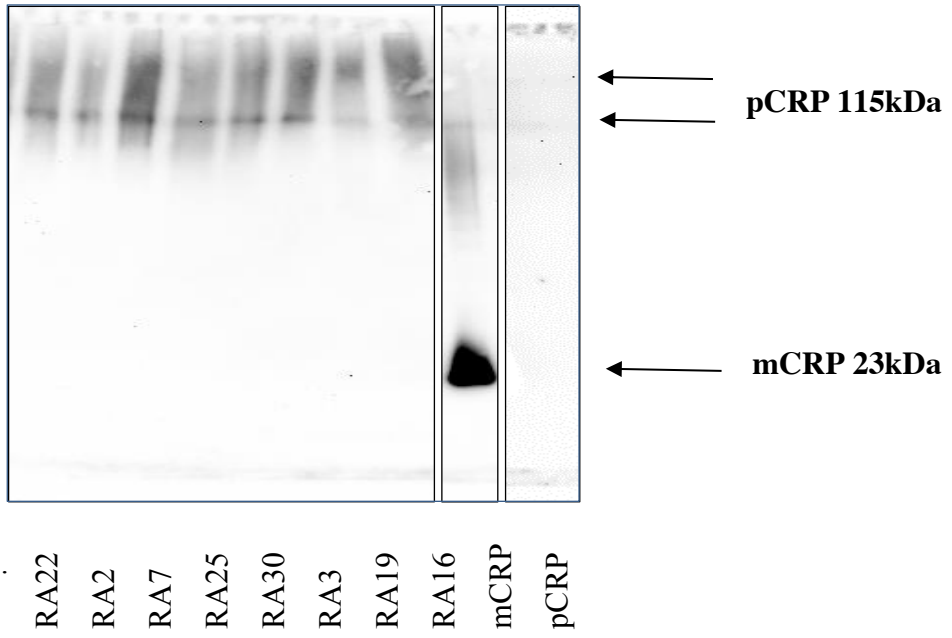


Figure 5-7 A pCRP and mCRP western blot image showing a representative group of samples run on a pre-cast gradient gel, developed over 2 minutes. Patient samples from left to right (lanes 1-8), lane 9 is mCRP control (0.5µg), and lane 10 pCRP(0.5µg). Primary antibodies 3H12 (1:50 dilution) and Clone 8 (1:2000 dilution) were followed by secondary rabbit anti-mouse IgG (1:30000 dilution), *n*=2.

All samples within the pCRP assay and those above an ELISA concentration of 0.1mgl/l within the mCRP assay were subjected to native gel western blot analysis in order to confirm the presence of mCRP. Figures 5.6 and 5.7 show duplicated representative western blots of RA samples, developed over different times to allow the mCRP band to develop (as can be seen in Figure 5.6 and not 5.7). The blot tested for the presence of both mCRP and pCRP. Samples RA22, RA2, RA7, RA25, RA30, RA3, RA19 and RA16 were chosen. The levels of mCRP identified within the ELISA assay were very low and therefore not all previously identified as positive for mCRP were detected within the Western Blot analysis. Lane 9 of Figures 5.6 and 5.7, contains an *in vitro* produced monomeric control produced within the 2M urea dissociation trial described previously in Chapter 3. The monomeric band is consistent with that of mCRP when run in a gel system containing $1/20^{th}$ normal SDS amounts within both the sample and running buffer, as described within 2.3.6. Lane 10 of both Figures 5.6 and 5.7 contain 0.5µg pCRP (Scripps), which shows a double band within the gradient gel. It was found that within non gradient gels an amount of pCRP remains within the stacking gel and this is presumed due to mobility problems, and to be responsible for the dual bands seen within these gels. Size exclusion chromatography had previously confirmed the size of the pCRP supplied by Scripps to be 115kDa. It is evident that mCRP is apparent in lanes 7 and 8 (samples RA19 and RA16), however they were very faint.

5.3.4 The Correlation between Human Monomeric and Pentameric CRP

It would perhaps make sense that samples which contain higher levels of pentameric CRP may also contain higher levels of monomeric CRP and the same for samples with lower pCRP and therefore lower mCRP. In order to investigate a possible correlation between levels of pCRP and mCRP within both the RA and control groups, the ratio of each protein was analysed as a group and individually per sample. There was no significant correlation found

between mCRP and pCRP within either the RA or control groups ($P > 0.709$, $P > 0.646$ respectively). Figures 5.8a) and b) illustrate the lack of correlation between mCRP and pCRP in both sample groups. It is clear that whilst the control group contains one participant with higher than normal levels of pCRP, this was not matched by higher mCRP concentrations.

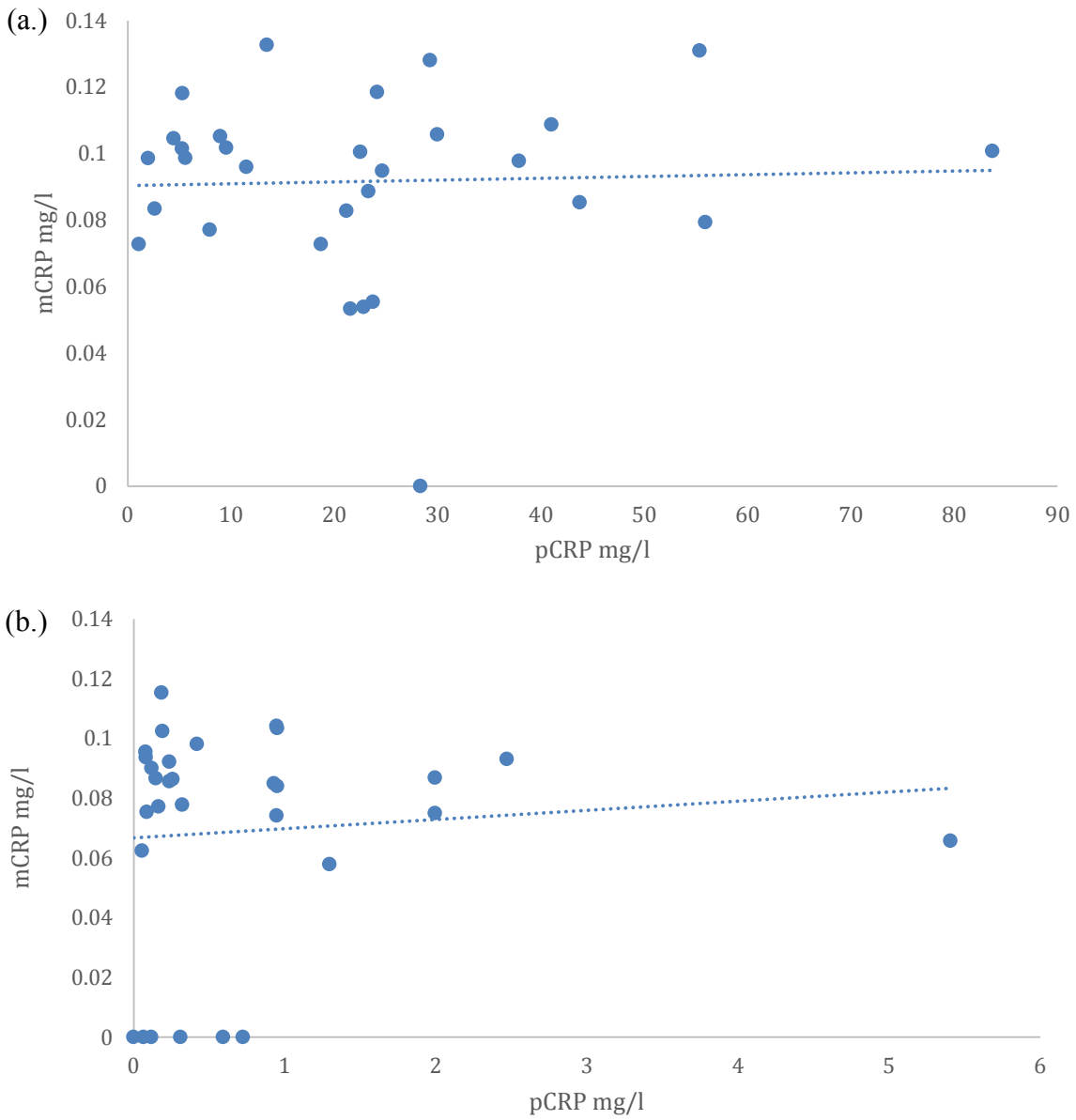


Figure 5-8 Scatter plot illustrating the correlation between human pentameric and monomeric CRP within serum samples as determined by ELISA (a) RA patient group. The line equation is y = 5E-05x + 0.0904, with R^2 = 0.0015 showing no correlation between levels of monomeric and pentameric CRP P > 0.709. (b) Control group. The line equation is y =.0031x + 0.0667 with R^2 = 0.0081 showing a weak positive correlation between levels of monomeric and pentameric CRP P > 0.646. The concentration for each sample is expressed in terms of mg/l. Samples were initially diluted 1:100 for mCRP assays and 1:1000 control / 1:16,000 RA within pCRP assays, measured in triplicate wells, and replicated across 3 individual ELISA assays.

Individual sample ratios can be seen in figures 5.9 and 5.10 which also illustrate the lack of correlation between pCRP and mCRP within each of the RA and control group

samples. It does not appear within the RA group, apart perhaps from samples RA16 and RA7, that higher amounts of mCRP are found within samples containing higher concentrations of pCRP, nor are there lower levels of mCRP in samples containing lower pCRP (Figure 5.9). Within the control group slightly greater parity is seen in samples with lower levels of CRP, for instance samples C17, C21, C25 and C27 all of which have minimal amounts of both pCRP and mCRP. It is less clear within samples of higher CRP concentration whereby C15 and C14 are closest to possessing high levels of both CRP conformations (Fig 5.10).

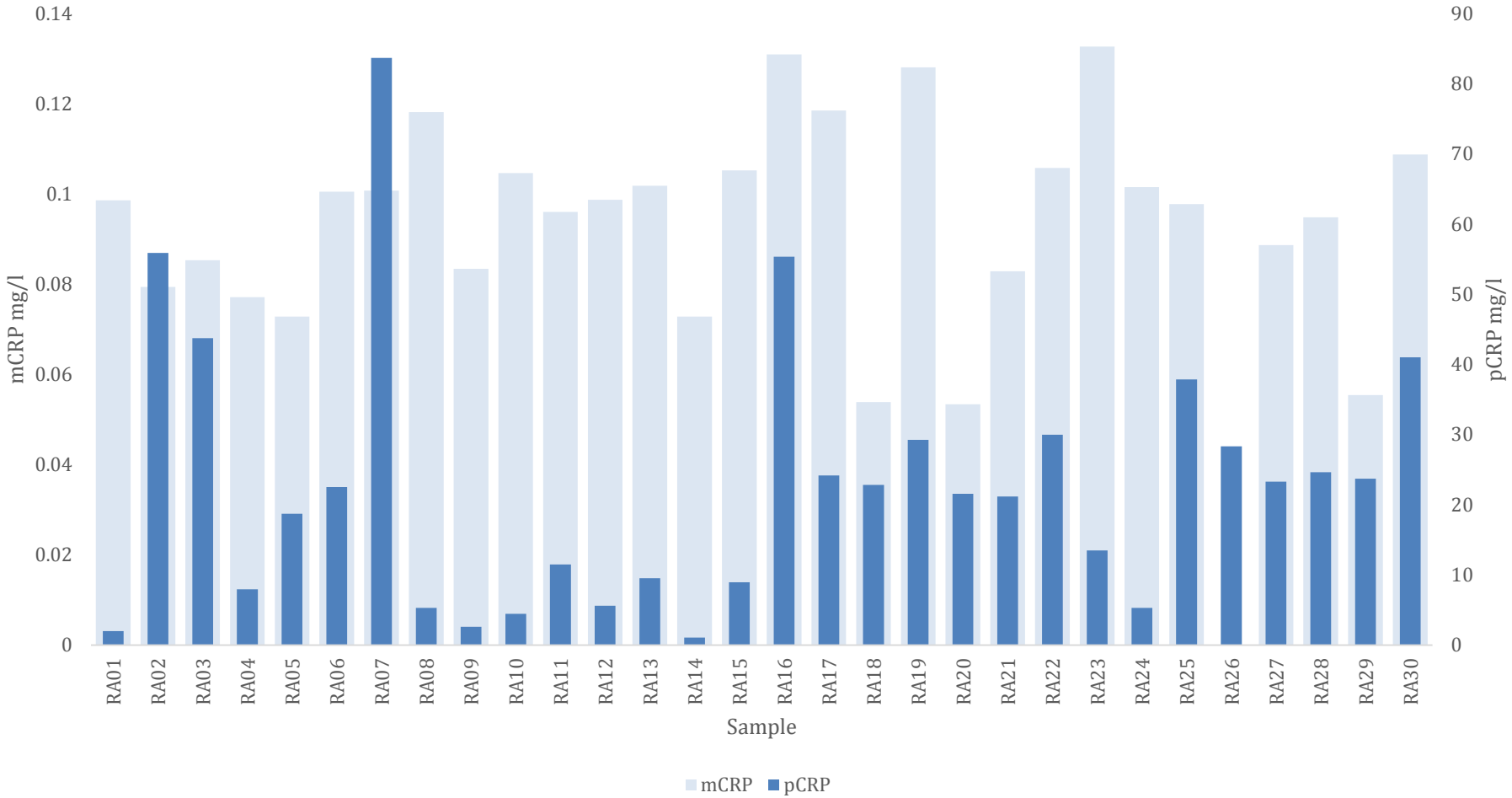


Figure 5-9 A Bar graph showing the ratio of pCRP/mCRP per patient sample within the RA patient group serum samples as determined by ELISA assay. The concentration for each sample is expressed in terms of mg/l. Samples were initially diluted 1:100 for mCRP assays and 1:16000 for pCRP assays and measured in triplicate wells, replicated across 3 individual ELISA assays. Data represents the calculated mean *n*=3.

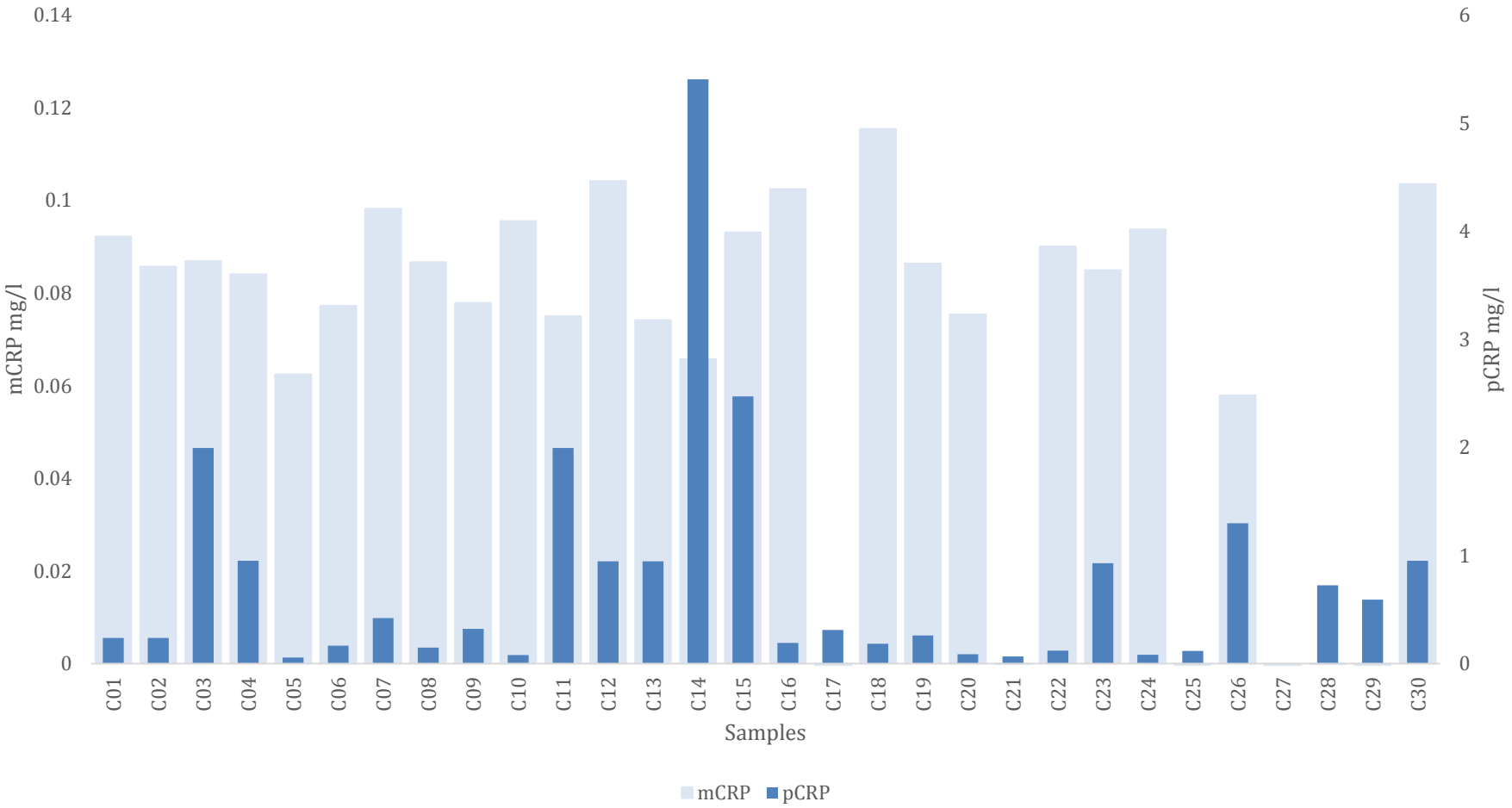


Figure 5-10 A bar graph showing the ratio of pCRP/mCRP per sample within Control group serum samples as determined by ELISA assay. The concentration for each sample is expressed in terms of mg/l.. Samples were initially diluted 1:100 for mCRP assays and 1:1000 for pCRP assays and measured in triplicate wells, replicated across 3 individual ELISA assays. Data represents the calculated mean, *n*=3.

Whilst there are few apparent individual sample correlations between monomeric and pentameric CRP, it is evident that mCRP forms a higher ratio of pCRP within the control group (5.91%), than within the RA group (0.27%) (Figure 5.11 a. and b.). This is despite 73% lower mean concentrations of mCRP within the control group than that found within the RA group. However the mean concentrations of pCRP within the control group is only 3.23% of that found within the RA group due to the much higher levels of pCRP within those samples. It is therefore likely this disparity is affected more by the significant reduction in pCRP than increase in mCRP. Whilst the levels of mCRP found within RA and control groups are low, they are significantly different to each other $P < 0.01$ (Figure 5.11c), with levels appearing independent of the level of pCRP found within each group. It is however apparent that the number of samples within the control group found to have no detectable mCRP (n=6) when compared to the RA group (n=1) may be responsible for the significant difference in levels determined, affecting the mean concentration across the group. However the remaining positive samples are generally of a lower concentration than those in the RA group, for example there are less samples within the control group which exceed 0.1mg/l (n=4), when compare to the RA group (n=12).

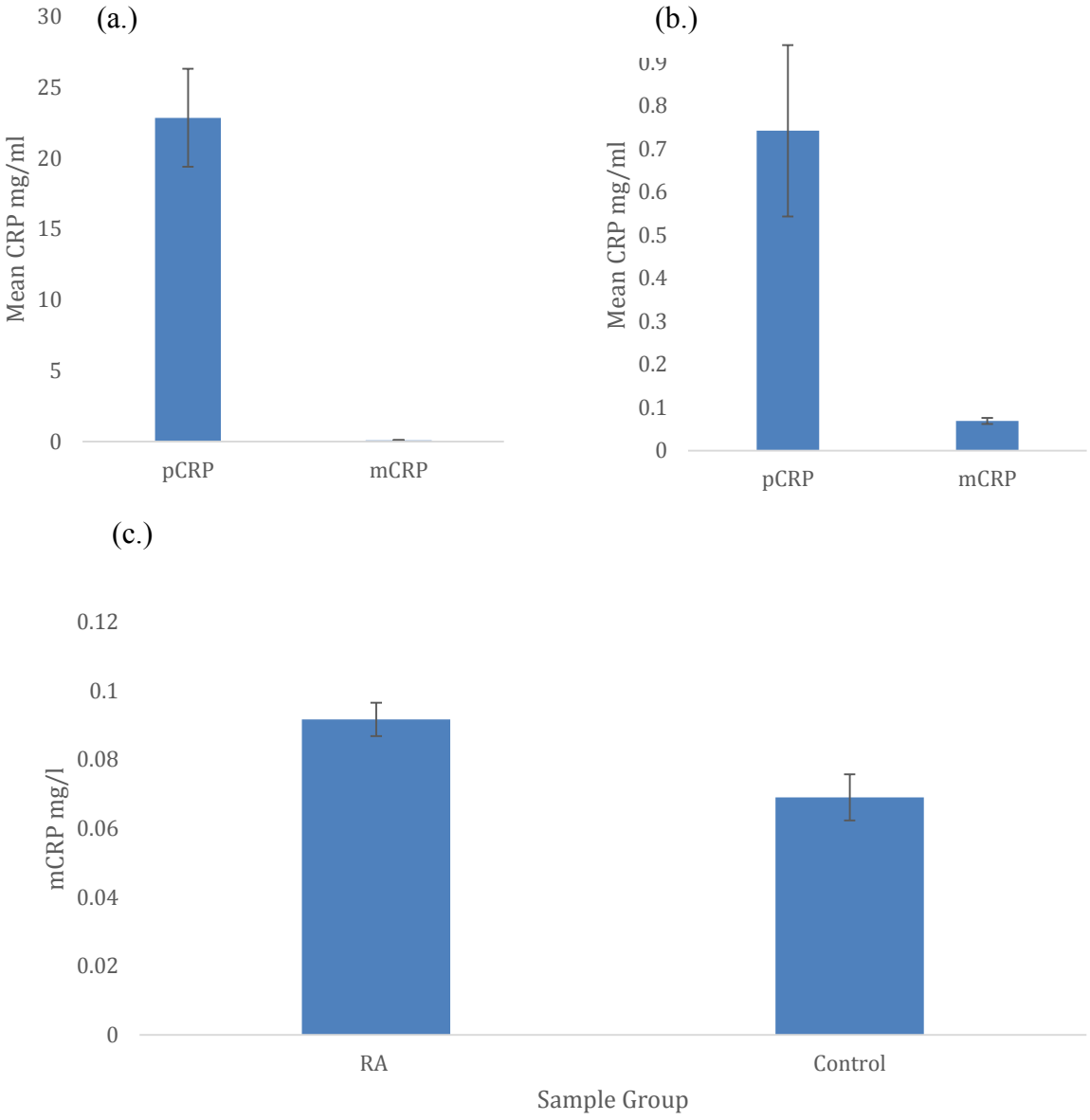


Figure 5-11 A bar graph illustrating the difference in mean concentrations between mCRP and pCRP, in RA and control groups. (a) Illustrates the difference between mean levels of pCPR and mCRP within the RA group of which mCRP formed only 0.27% of pCRP levels. (b) Illustrates the difference between the mean levels of pCRP and mCRP within the control group of which mCRP formed 5.91% of pCRP levels ± SEM. c) Illustrated the difference between mean levels of mCRP within the RA and control groups $P < 0.01$, $n=3$.

5.3.5 The Effect of Gender on Serum CRP Levels

In order to ensure no bias was present towards the effects of gender between the samples, groups were subcategorised to carry out analysis by sex. It was evident that a significant difference was found between RA and control mCRP levels within the entire

cohort, but Figures 5.2 and 5.3 shows the effect of splitting the group (blue bars represent female and green bars male samples). A lower proportion of male subjects in the control group were found to have mCRP within their serum (42.85%, *n*=7) than females (91.3%, *n*=23), indicating control males are less likely to have circulating mCRP than females. Within the RA group this deference is less pronounced (males with mCRP 87.5%, *n*=8, females 100%, *n*=22).

The calculated upper normal range threshold for male subjects was 0.0725 mg/l (mean + 2SD normal samples); two of the control samples together with six of the RA samples are above this level suggesting higher levels of mCRP present. When the frequency of elevated mCRP male samples was compared by X^2 analysis this was found not to be significantly different ($P > 0.072$). Although the small sample size (male RA *n*=8, control *n*=7) makes it difficult to assign any influence to sex, and the inference drawn from these results can not be projected upon the wider population. However there is a visual difference between the profiles illustrated, suggesting possible sex related differences within mCRP concentrations. Analysis between female RA and control group mCRP concentrations found no significant difference ($P > 0.068$), there was also no significant difference between male RA and control groups ($P > 0.093$).

5.3.6 The Effect of Age on Serum CRP Levels

Whilst it is apparent that no significant correlation exists between the individual sample concentrations of monomeric and pentameric CRP, further analysis sought to reveal whether age bore influence over the CRP levels within samples. A Spearman's rank correlation was carried out enabling assessment of age as a variable in both RA and control groups. The RA group ages ranged between 24 and 79 years (mean 62.7 years), with 90 % or participants aged 50 years or above. The control group ages fell between 26 and 79 years

(mean 46 years), with 44% aged 50 or above. As can be seen in figures 5.12 a) and b) there is no significant correlation between age and mCRP in either the RA or control groups, although a slight trend towards lower mCRP levels with age is visible. There is a difference between the RA and control groups when pCRP serum concentrations are considered. Figures 5.12 c) and d) show the effect between age and pCPR levels, which are slightly reduced as age progresses in the RA group and slightly raised within the control group, however neither of these correlations are significant $P > 0.05$.

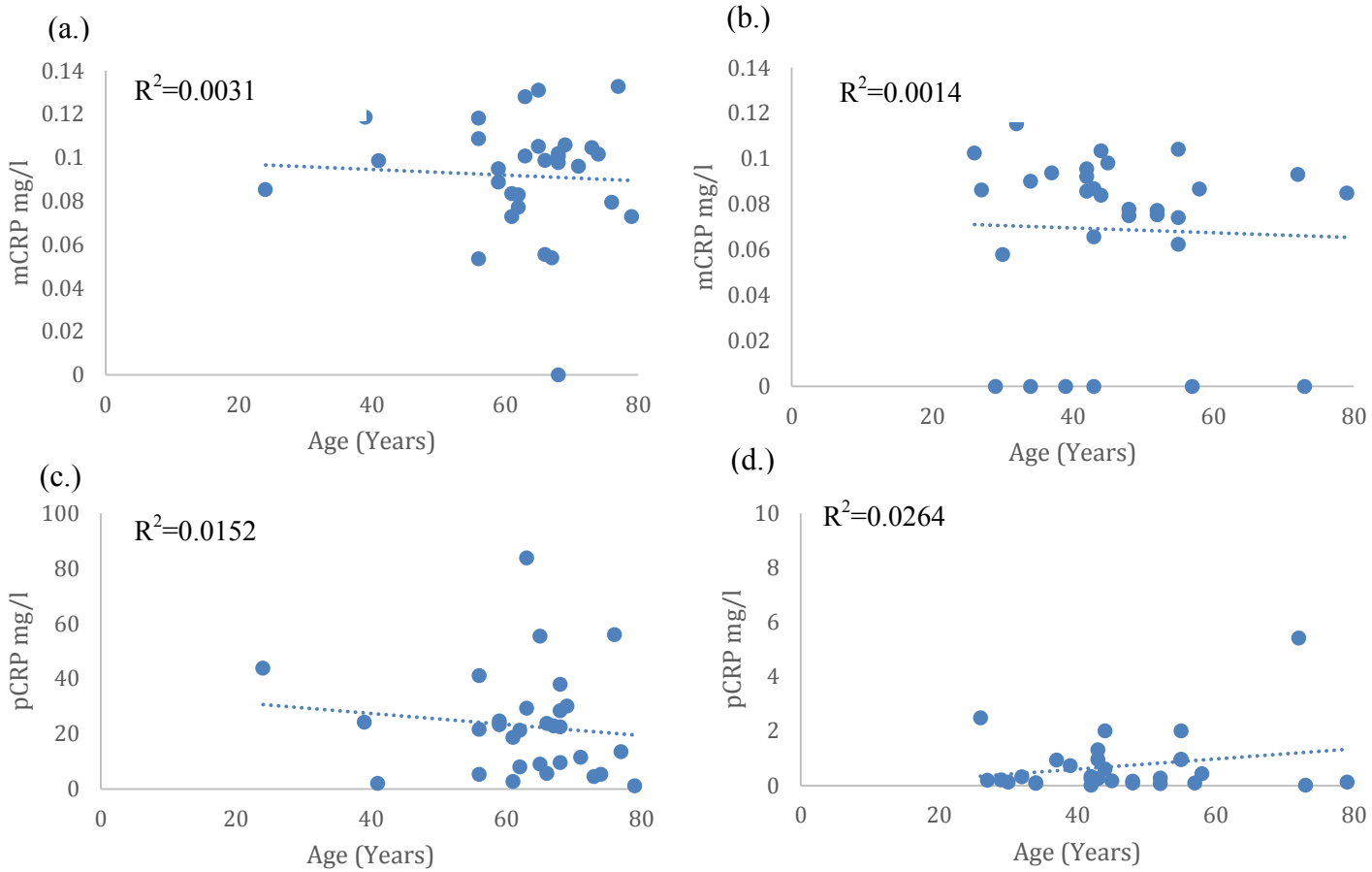


Figure 5.12 A scatter plot illustrating the correlation between CRP mg/l and age as determined by ELISA. a) mCRP vs Age RA group. b) mCRP vs Age control group. c) pCRP vs Age RA group d) pCRP vs Age control group. A Spearman's rank correlation showed no significant correlation $P > 0.05$ between age and concentration of CRP in all groups. $n = 30$, mean age RA group 62.73 years, mean age control group 46 years.

Whilst Figure 5.12 considered the correlation of CRP and age across the entire RA and control group, in order to determine if sex influenced the data, these groups were further split into male and female cohorts. Although the male group size is too small to draw significant inference (RA group n = 8, mean age 65.88, range 56 -77 years; control group n = 7, mean age 46.14, range 30 -73 years), it is interesting to note that where results for mCRP in the RA group split by sex, Figure 5.13a and c, are broadly similar in profile to the combined male/female RA results seen in figure 5.12a and c. When results for pCRP are considered, levels for males appear to drop steeply with age, although not significantly $P > 0.08$ in contrast to female results which show a slight and non-significant decline, (Figure 5.12b and 5.12d). The trend shown in Figure 5.13d is heavily influenced by just 2 males aged >75 years, with lower pCRP than others in that group, however their equivalent concentrations of mCRP are not reduced in a similar manner. The profile for male RA group serum mCRP concentrations (Figure 5.13c) remains similar in nature to that of the combined gender RA group (figure 5.12c). Therefore whilst age appears to have influenced the level of pCRP in RA group males, it has not had the same effect on the equivalent concentration of mCRP. The profile of age and serum level of female mCRP and pCRP are little changed (Figure 5.13a and b) from those in the combined gender group (Figure 5.12a and b); although as the predominant group within the original combined gender group it is unsurprising that these remained similar. A slight but insignificant rise in mCRP is apparent in older males and also in females, with both sexes sharing a trend of declining pCRP in older participants.

Within the control group a different observation can be considered as the concentration of mCRP rather than pCRP is more directly impacted. A sharp, but insignificant drop in concentration levels of mCRP within the male sub group ($P > 0.26$) and to a lesser extent pCRP (figure 5.13c and d) was observed as age advances, however this was not found

to be the case within the female sub group (figure 5.14a and b), wherein both mCRP and pCRP concentrations were found to rise with age ($P > 0.328$).

It appears that age may influence the concentration and profile of CRP differently between males and females and again this changes between the RA and Control groups. Male and female subsets both see a slight rise in mCRP and reduction in pCRP levels with age within the RA group. Within control samples the correlation of mCRP vs age results diverge, with female levels of mCRP rising with age and males falling, this is mirrored within the pCRP results.

It appears that as non RA women age their levels of both mCRP and pCRP increase slightly whereas the level in males may decrease. When combined, the control population shows a gradual increase in pCRP with age and little change to mCRP levels; however the RA population appears to show a slight reduction in both.

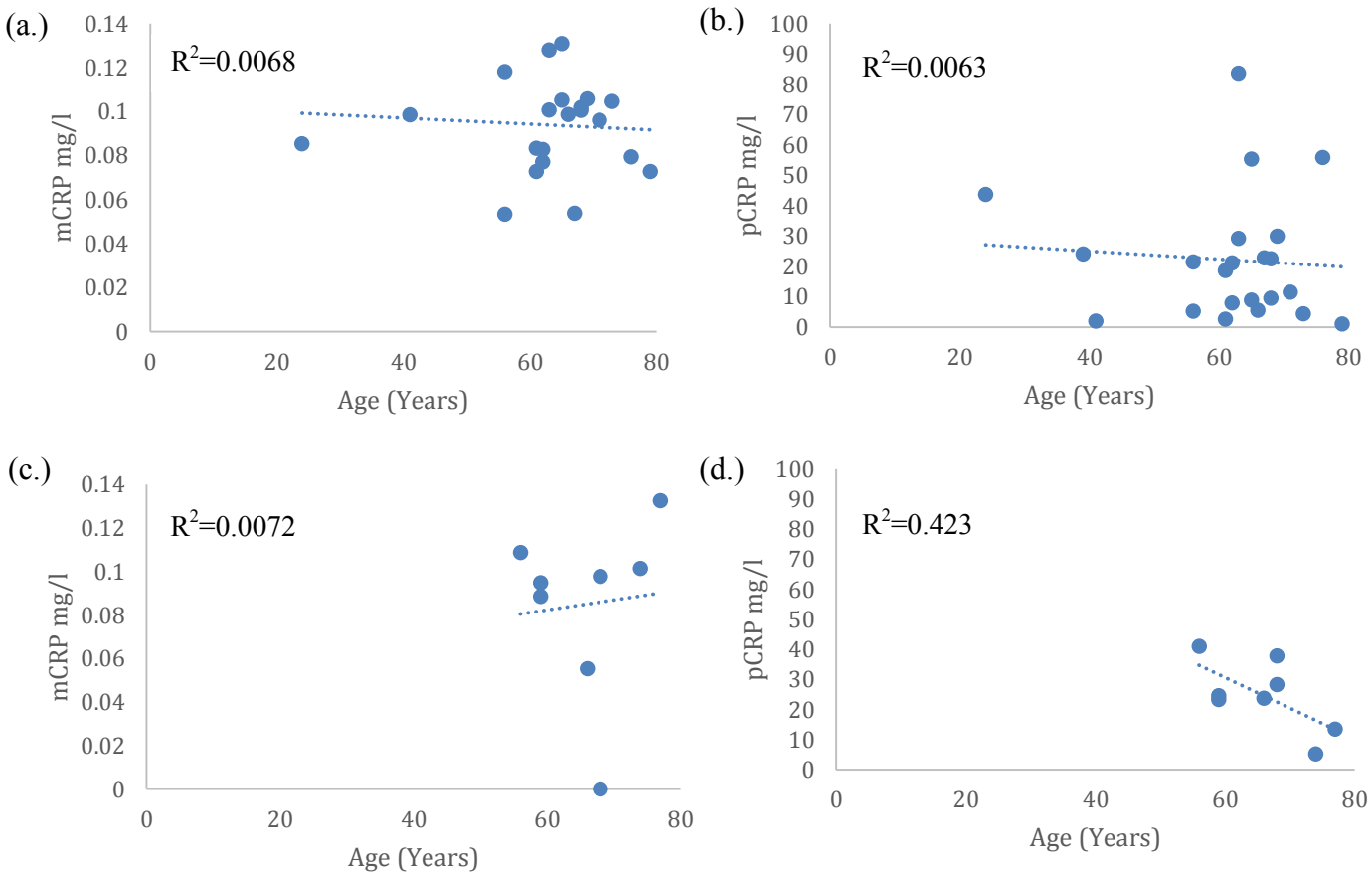


Figure 5.13 Scatter plots illustrating the correlation between serum CRP mg/l and sex in the RA group as determined by ELISA. a) The correlation between mCRP and age in females. b) The correlation between pCRP and age in females. c) The correlation between mCRP and age in males and d) The correlation between pCRP and age in males. *n*=8 male samples, mean age 65.88 years, *n*=22 females, mean age 62.26 years.

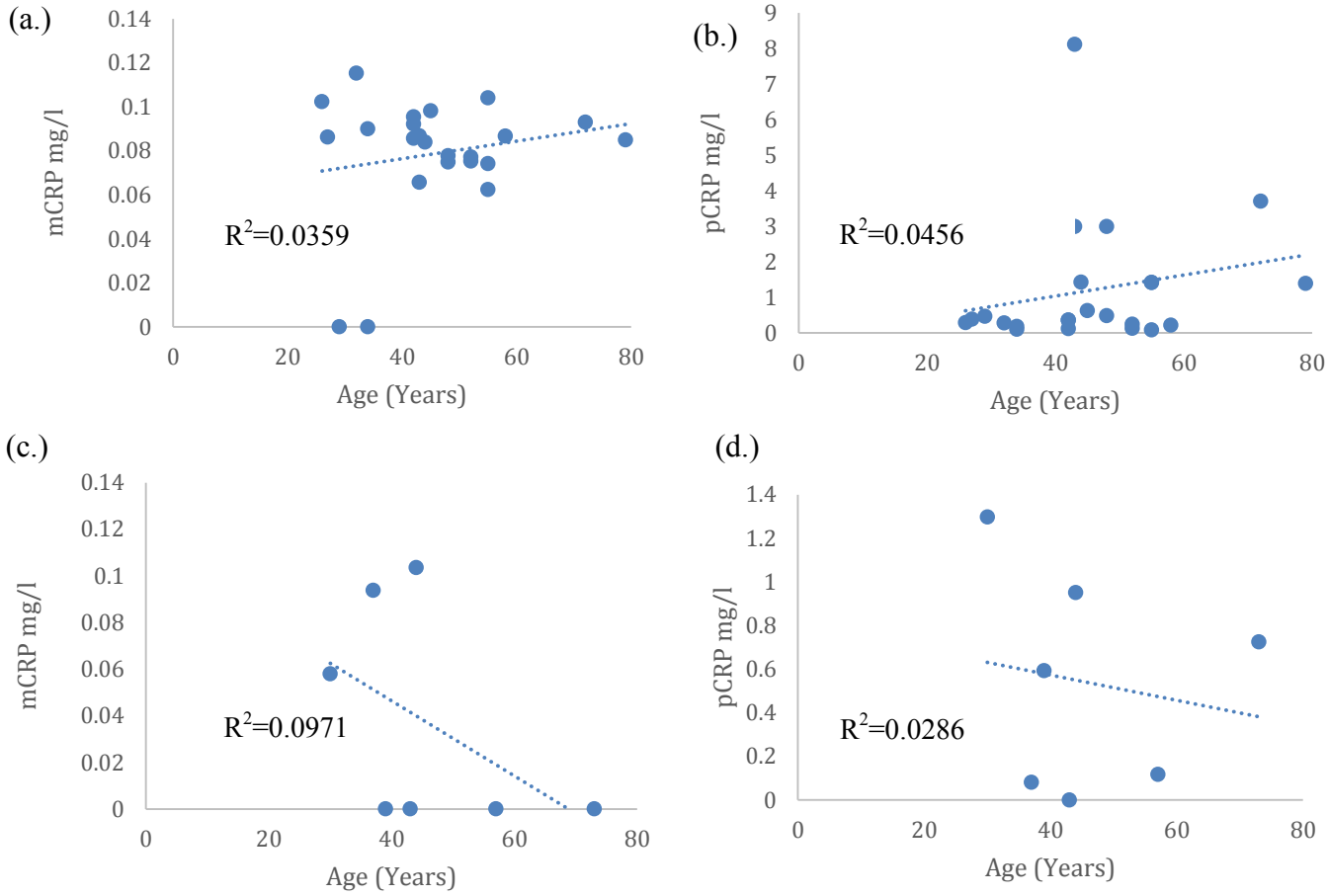


Figure 5.14 Scatter plot illustrating the correlation between serum CRP mg/l and sex in the control group as determined by ELISA. a) The correlation between mCRP and age in females. b) The correlation between pCRP and age in females. c) The correlation between mCPR and age in males. d) The correlation between pCRP and age in males. *n*=8 male samples, mean age 65.88 years, *n*=22 females, mean age 62.26 years.

5.4 Discussion

5.4.1 Serum mCRP and pCRP Detection in RA and Control Samples

ELISA analysis was carried out upon all samples within both RA patient and control sample groups and confirmed positive levels of pCRP in all 30 of the RA and all 30 of the control samples. Statistical analysis prior to splitting the cohorts into gender subsets, found a significant difference ($P < 0.001$) in concentration levels of pCRP detected between the RA and control groups, (RA group pCRP mean 22.85mg/l, control group pCRP mean 0.742mg/l). Detectable levels of mCRP were found in 29 of the 30 RA samples and 24 of the 30 control samples, (RA group mCRP mean 0.092mg/l, control group mean 0.069mg/l) once again a significant difference was found $P < 0.01$. Comparison between the groups highlighted some unexpected results, although the levels of pentameric CRP were significantly lower within the control group than RA and broadly in line with reported normal range of 0.1-3mg/l (Pearson *et al*., 2003), there was less difference detected between the monomeric levels quantified within the two groups. This suggests that although the concentration levels of pCRP are universally higher than mCRP across all samples, a clear increase is observed within the RA group. There is less difference within mCRP concentration between an RA state and non RA control state, although significant. The difference found may have been driven by the higher number of negative control samples (n=6) when compared to RA (n=1), however there are fewer samples abover 0.1mg/l within the control group (n=4) than the Ra (n= 12), suggesting the level of mCRP within positive control samples is generally lower than that found within the RA group. The level of mCRP determined within our study only considers that detected within the serum and is not representative of that which may be found within the synovial tissue itself. There is evidence of mCRP co-localised within inflammed tissue of many

disease states including the myocardium of acute coronary syndrome patients (Wang *et al*., 2015), the relevant brain regions of patients suffering post stroke inflammation (Slevin *et al*., 2015) and the choroids of donors with a high risk of age-related macular degeneration (Chirco *et al*., 2016). It is therefore possible that mCRP may occure in the locality of inflammed tissue within RA patients and that a greater difference between these subjects and a matched control polupation could be found.

5.4.2 Western Blot Analysis

Western blot analysis confirmed the presence of pCRP within all 30 patient samples, which corresponded to ELISA results, aligning to the band profile of pCRP as shown in Figures 5.6 and 5.7, lanes 10 and 9 respectively. The stock pCRP purchased from Scripps had previously been determined as 115kDa following size exclusion chromatography and this together with antibody recognition within the western blot and ELISA led to confidence that these bands were pCRP. Corresponding bands in the position expected of mCRP (23kDa) were not confirmed within all patient samples. ELISA determined the presence of mCRP within 29 of the 30 RA samples, however at very low amounts $\leq$ 0.132mg/l. The western blot was able to confirm a band at 23kDa for both RA16 and RA19 (Figure 5.6), however the blot had to be exposed for 45 minutes in order for the bands to become visible, a second image showing a reduced time (Figure 5.7) could not positively identify either samples for mCRP content. An ELISA is able to detect much lower concentrations of protein than a western blot and this is evident within the results presented, highlighting the difference in sensitivity between these two techniques. The amount of loaded protein per technique for sample RA23, identified as containing the highest concentration of mCRP, was 13ng/well within the ELISA and 13pg per lane within the western blot.

5.4.3 Correlation between pCRP and mCRP

ELISA results show that not only were mCRP levels significantly lower than those of pCRP but that the distribution does not correlate uniformly to pCRP across the samples. However, mCRP has been detected within the serum, and the levels determined appear to support previous findings by Wang *et al*., *(*2015) who detected levels of mCRP within serum between 0.019 mg/l and 0.037 mg/l. These concentrations were considerably lower than the higher range reported by Williams (2017), who demonstrated between 0.04mg/l and 3.52mg/l mCRP within samples taken from patients identified with high sera pCRP. The mCRP within Williams' study was first purified from serum by affinity chromatography and further concentrated and this concentration step may explain the difference in detected concentration between this and our study result (Williams, 2017).

A correlation between concentrations of mCRP and pCRP within individual samples, had one been found, forming reduced but corresponding concentrations of mCRP to those of pCRP, may have implied the soluble mCRP detected within our serum samples were of pentameric origin resulting from degradation or dissociation. However this was not the case as no correlation was detected between RA ($P > 0.708$), or control ($P > 0.646$) sample mCRP and pCRP levels. It is possible that elements of the mCRP identified within both patient and control samples may have originated from circulating pentameric CRP through local dissociation, which has been well described within recent literature (Ji *et al* ., 2009, Thiele *et al*., 2014, Eisenhardt *et al*., 2009). CRP is produced within hepatic cells following inflammatory cytokine signalling, but CRP has also been shown to be produced systemically, following local production within a variety of cells, including epithelial cells of the respiratory tract, renal epithelium (Gould *et al*., 2001, Jabs *et al*., 2003), human coronary artery smooth muscle cells, human adipocytes (Calabro et al., 2005), inflamed human striated muscle, atherosclerotic plaque and infarcted myocardium (Thiele *et al*.,

2014). CRP mRNA has been identified within both normal and plaque tissue of atherosclerosis patients (Yasojima *et al*., 2001).

Previous literature has suggested circulating pCRP dissociation can occur via membrane binding and insertion, with *Pneumococcal* C-polysaccharide a phosphocholine containing molecule, polylysine and the lysophosphatidylcholine exposed on activated cell membranes and activated platelets each described as capable of inducing pCRP dissociation upon binding (Ji *et al*., 2009, Wang and Sui 2001, Thiele *et al*., 2014, Eisenhardt *et al*., 2009). Additionally Strang *et al*., *(*2012) reported dissociation via amyloid plaques within cortical inflammation in Alzheimer's disease. Whilst pCRP primarily binds cells via FcγRIIA (CD32), mCRP has been associated with binding of Fcγ-RIII (CD16) (Bharadwaj *et al*., 1999, Devaraj *et al*., 2005), however it is also suggested that membrane insertion via cholesterol rich lipid rafts may be a key area for anchorage (Eisenhardt *et al*., 2009, Li *et al*., 2014,). This theory is supported by Ji *et al*., *(*2009) who further suggested pCRP dissociation occurs during CRP interaction with cholesterol during membrane insertion.

During periods of acute or chronic inflammation associated with RA prognosis, it is likely that high levels of circulating CRP are in constant contact with activated cell membranes or platelets, with dissociation possible within the local inflammatory environment. A hydrophobic microenvironment has also been suggested as a key factor in CRP dissociation; mCRP produced via dissociation *in vitro* is less soluble in nature than the pentamer but retains its ability to bind phosphocholine (Williams, 2017), therefore the slightly hydrophobic environment of cell membranes may enhance possible mechanical means leading to the rapid subunit dissociation described previously in literature.

Whilst dissociation of circulating or locally expressed pentameric CRP within the inflammatory environment of RA goes some way to explain the presence of mCRP within

the serum of RA suffers, it does not provide a complete picture. Levels of monomer do not correlate with pentamer in either RA or control subjects, with an uneven distribution across each group. There is not therefore a universally plausible method or mechanism capable of supporting the wide variety of monomer concentrations observed across the sample groups. Additional factors may influence structural changes to pCRP or possibly expression of monomeric rather than pentameric CRP may be responsible. However, the feature uniting many studies into the presence of mCRP within diseased tissue or serum is that of inflammation, be it within amyloid plaques of Alzheimer's, atherosclerosis or RA itself.

Rheumatoid arthritis as an inflammatory disease is associated with high levels of comorbidity. In their 2013 cross-sectional study of 4586 patients, Dougados *et al*., (2014) described the most frequently associated diseases as depression, 15%; asthma, 6.6%; cardiovascular events (myocardial infarction, stroke), 6%; solid malignancies (excluding basal cell carcinoma), 4.5%; chronic obstructive pulmonary disease, 3.5% (Dougados *et al*., 2014).

The pathophysiological conditions often associated with many of these diseases may well provide the conditions required for either increased pCRP expression or possible dissociation, with inflammatory conditions including atherosclerosis and heart disease correlated to high levels of CRP. Activated platelets and endothelial cells together with circulating microparticles are a common pathological occurrence within these diseases and are known to dissociate CRP (Eisenhardt, Habersberger, Murphy, *et al*., 2009). Identified inflammatory conditions may be responsible for mCRP occurring within a patient sample, but not samples of the control group. It is possible that participants of both the control and RA groups have additional underlying or undiagnosed disease, which may account for an independent increase of either CRP form, or count towards synergistic effects from combined factors.

The presence of mCRP within the control group, even at statistically lower levels than those of the RA participants cannot therefore be fully explained by inflammatory environments or high levels of circulating pentameric CRP, in fact 93% of control participants fell within the normal pCRP range. However, studies have suggested that RA can be predicted within non-symptomatic / undiagnosed people several years before symptoms present. It is possible that some participants within this control group were exhibiting early signs of future inflammatory or autoimmune disease yet to be diagnosed. RA itself can be predicted several years in advance by the presence of certain proteins within the blood, although CRP is not one of them. A study in 2006, analysing blood donor samples donated before symptoms of RA became apparent, identified 93 people from a cohort of 138 who more than two years later went on to develop rheumatoid arthritis and whose blood contained anti-CCP antibodies, indicating a risk of future disease (Berglin *et al*., 2006). In another study 83 patients with confirmed RA, identified as previously donating blood between one and five years before symptoms of RA presented, were found to have anti-CCP antibodies and RFs within the samples, predating the onset of RA by several years (Rantapaa-Dahlgvist *et al*., 2003).

These studies illustrate that a number of auto-antibodies appear in the preclinical phase of RA. Almost half of the RA patients identified had serologic abnormalities several years before the onset of symptoms. It is possible that some features of autoimmune diseases are a factor within dissociation of CRP, even within a presumed control population. It is however likely that just as pCRP has a recognised ‘normal’ baseline level below 3mg/l (Pearson *et al*., 2003) that mCRP may exhibit an equivalent ‘normal’ level, and that the levels identified within the majority of the control group of less than 0.143mg/l fall within this range.

Although no experimental evidence is available to suggest oxidation plays a role in pentameric CRP dissociation, it is theoretically possible within the inflammatory confines of an RA joint, that these conditions may in isolation or in synergy with as yet unknown action lead to CRP dissociation. A key feature of the inflammatory environment within an RA joint is an increase in reactive oxygen species and oxidative stress involved within the pathogenesis of the disease (Biemond *et al*., 1984, Ozturk *et al*., 1999). The environment created by the highly reactive chemical species involved within oxidation has the potential to damage lipids, proteins and DNA within joint tissue. Lipid peroxidation, protein oxidation, DNA damage and impairment of the body's enzymatic and non-enzymatic antioxidant defence system within RA patients were all recently reported (Mateen *et al*., 2016).

Key protein oxidation markers (significantly increased protein carbonyl and decreased sulfhydryl group levels) were found by Mateen *et al*., (2016) within the RA cohort plasma, alongside significantly low levels of non-enzymatic antioxidants (reduced glutathione and vitamin C). It is possible the oxidative environment found within RA joints may destabilise the pentameric structure of CRP, making it more vulnerable to previously mentioned dissociation factors. Each CRP subunit contains an intra-subunit disulphide bond formed between Cys36 and Cys97 during oxidative folding. The subunit first folds spontaneously following protein expression and the correctly folded protein drives formation of the internal disulphide bond which is dependent upon binding of a stabilising calcium atom. The correctly folded subunits are then assembled around a central pore and held together with individually weak, but collectively strong, salt bridges and hydrophobic forces. It is possible that oxidative stress derived from high levels of reactive oxygen species is responsible for damage to the subunit and therefore the stability of pentameric CRP. The effects of a highly oxidative environment upon protein stability can be seen

within superoxide dismutase (SOD) which is itself vulnerable to oxidative attack within RA pathology. SOD forms a stable homodimer and binds with either zinc or copper ions, important for its function and also tertiary and quaternary structure (Drazen *et al*., 2016). In their 2016 study they were able to illustrate that oxidative stress could lead to impaired dimerization through oxidative modifications of interfacial residues (Drazen *et al*., 2016).

Alterations to the body's normal physiological state within disease progression may ensure the essential conditions required for mCRP creation. One factor within CRP dissociation is pH, with the normal physiological range tightly regulated to fall within pH 7.35-7.45. Whilst pH4 trials within our own experimentation were able to elicit dissociation of CRP in combination with calcium chelation, it is not physiologically possible that this extreme environment exists extracellularly, although sites of inflammation are known to become more acidic in nature extracellular due to acidification considered due to lactate secretion from anaerobic glycolysis (Riemann *et al*., 2015, Rajamaki *et al*., 2013, Yasumasa Kato *et al*., 2013). CRP is able to bind a wider range of ligands within acidic than neutral or alkaline conditions and this in itself may facilitate the binding required for dissociation (Hammond, 2010; Salazar *et al*., 2014). Although pH alone may not be responsible for monomeric CRP, it could become important in addition to a combination of other described factors including mechanical action of lipid raft binding or oxidation.

5.4.4 Age and Gender Effects on CRP Prevalence and Concentration

Our data suggests that whilst it could be expected that as a population ages their level of serum CRP may increase, due in part to underlying health issues or inflammation, this was not found to be the case consistently across all the sample groups within this study as seen in Figure 5.12. RA can occur at any age but is more frequently diagnosed in people

aged 40-50 years (Haris, 1989;, Krane and Simon, 1986). There is a weak negative correlation between age and mCRP within the control figure 5.12b, and between both mCRP and pCRP within the RA group (Figures 5.12a and c respectively), however pCRP levels do rise with age within the control group (Figure 5.12d), albeit insignificantly, partially supporting this theory.

It is possible that the difference in pCRP and age correlations between the RA and control groups arises due to the use of anti-inflammatory drugs within the RA group, with these responsible for a reduction in CRP level due to differences within the category of drug and usage period. It was proposed in a study by Innala *et al*., (2014), that dependent upon the age at diagnosis, either young onset RA (YORA) or late onset RA (LORA), different drug intervention pathways may be implemented. For instance LORA was found to be treated more frequently with corticosteroids and less frequently with methotrexate or biologics. YORA was more frequently associated with DMARD treatment. Methotrexate (a DMARD) can have less effect on CRP, although only pCRP levels were monitored in historical research, (Aletaha and Smolen, 2002), than moderate to high levels of steroid treatments such as prednisolone, which have been found to significantly reduce patient CRP levels, again however, unknown effects within mCRP concentrations (den Uyl *et al*., 2011; McConkey *et al*., 1973; Mysler *et al*., 2004). Evidence within these studies suggest that during treatment, CRP levels should reduce, due to the combination of RA drugs used, unless serum samples are taken during periods of RA flare. However, this appears not to consistently be the case. A study by Siemons *et al*. (2014) reported a significant association between ESR and CRP levels in RA, both of which increased with age ($p < 0.001$). This is supported by Zhoa *et al*., (2010) who also reported within their study based on a Chinese population that a correlation between CRP and age existed. Their study was representative of the generally lower levels of CRP found within Asian populations, which tend to be one

third to half that of Caucasian populations (Zhoa *et al*., 2010). The median level of CRP within Zhoa *et al's* study was 0.55 mg/l (0.51 mg/l female and 0.61 mg/l male), levels found within the serum increased with age, from 0.42gm/l in the 18-29 age group, 0.64 mg/l in the 50-59 age group, 0.82mg/l in the 60-69 age group and reached 0.98 mg/l within the 70-80 age group. The rise in CRP during aging was supported by Meyer in both males and females (Meyer, 2010), but was in contrast to another study which reported no correlation between CRP levels with age or sex (Feldman & Sbong 2014).

Within our study, no correlation between age and CRP concentration was detected within the RA group in concordance with Felman & Sbong's research (figure 5.14c), however figure 5.12d illustrates a slight increase in the control group pCRP concentrations as age increases. This is however a small sample size in comparison Zhao *et al*'s study which drew data from 3133 participants. Their study used an unknown combination of antibodies with plasma analysed by immunonephelometry, it is therefore possible that only either pCRP alone, or a combination of both mCRP and pCRP were detected dependant on the antibody specificity used.

Physiological changes occurring during the aging process may be partly responsible for this finding. Proinflammatory cytokine IL-6, known to instigate CRP expression and generally expressed at low levels, rises in response to inflammation or trauma and is down regulated by either oestrogen or testosterone. As the general population ages, menopause or andropause occur and levels of the downregulating hormones drop, thus allowing higher levels of IL-6 to be expressed. It is possible that the increased levels of CRP found within the control population during aging occurs partly in response to the effective reduction of both oestrogen and testosterone, resulting in higher levels of IL-6 known to instigate CRP expression (Daynes *et al*., 1993).

Whilst our study was in general concordance with the age related changes to CRP levels reported within literature, differences between CRP concentrations in male and female sub groups were not in universal alignment. Higher control group levels of pCRP were recorded in the female group (mean 0.80mg/l), in comparison to the male group (mean 0.54mg/l). Many studies, especially those based on Asian populations report higher CRP levels within males (Zhoa et al., 2010), however in Western populations studies have found the reverse with higher levels of CRP in females (Meyer 2010). Within our study the RA male group pCRP mean levels were higher than females, (male 24.70mg/l, female 21.79mg/l), which is supported by Siemons *et al*., (2014) who reported that in early RA men had 1.2 times higher CRP levels than women. There is a significant difference ($P < 0.035$), between the frequency of samples with detectable mCRP in the male and female control group, of seven male samples three were negative for mCRP (42.9%), with only two of 23 females negative (8.7%). There was no significant difference between male or female occurrence of mCRP within the RA group, one of the seven control males was negative for pCRP (14.3%), as opposed to none of the 23 females.

When considered further and samples assessed by both sex and age, it is apparent that some differences exists between the way male and female serum levels of CRP respond to ageing. Whilst both male and female mCRP levels within the RA group show a limited increase as they grow older (figure 5.13a and c) and both show declining pCRP values with age (figure5.13b and d), pCRP concentrations within the male group show a more pronounced decline (figure 5.13d). The two eldest subjects within this male group skew the pCRP results as they have much lower concentrations than other members of that group. Conversely their mCRP levels are amongst the highest within that group (figure 5.13c), suggesting mCRP and pCRP could be affected differently and therefore independently of each other, age and possibly also RA drugs or pro-inflammatory

cytokines such as IL-6. Within the control group mCRP and pCRP rise slightly in the female subset with age, however both isoforms of CRP reduce with age in the male group, mCRP falling to a greater extent.

Whilst the RA group male and female subsets follow similar profiles, with little difference between the sexes in either the mCRP or pCRP response to age (figure 5.13a-d), this differs within the control group. The responses of control male and female subsets to CRP against age diverge, with female levels of both CRP isoforms rising and male levels falling. These differences between the male and female groups are interesting when the incidence of RA diagnosis in women, reported to be four to five times higher than males in the under age 50 group and two times higher in the 60-70 group (Kvien *et al*., 2006), is considered. However, the sample number of males and females within this study does not fully reflect these ratios and any significance within these results are further undermined by the small number of subjects within each group. It is an area which invites further work with a larger sample group to discover whether this observation holds true for the wider population.

Whilst many studies are in agreement that mCRP exists *in vivo*, it is often difficult to compare their findings due to irregularities within research methods. Studies have indicated that pCRP can spontaneously dissociate upon binding to a fixed surface such as an ELISA plate (Eisenhardt, 2011; Sjowall *et al*., 2012, 2013). This variable was avoided within our study by using capture antibodies within the ELISA protocol, thus retaining conformation of CRP isoforms within a sample. However, some previous studies have used a methodology whereby CRP is bound directly to the plate, suggesting the identification of CRP isoforms within these studies may be unreliable. It is also possible that some antibodies used within previous studies may have been capable of recognising both CRP conformations for instance Clone 8 (Sigma), or pCRP alone, therefore

preventing the identification and quantification of possible mCRP within a sample. It is not unreasonable to consider that in some cases existing research may have misidentified which form of CRP they reported on.

It is possible that some of the confusion between the pro or anti-inflammatory effects of CRP reported within literature is resultant from the lack of specific detection properties within methods used to determine study results. The manner in which mCRP circulates the system, either freely soluble or bound to microparticles for instance, and the sensitivity of different detection methods can obviously affect results determined. As described previously, not only is it possible that some studies fail to address the nonspecific nature of commercially available antibodies used within their assay, but also the method itself may not identify both forms of CRP reliably and selectively when measuring. Crawford *et al*., (2016) considered this point when contrasting results gained from a high-sensitivity CRP assay which did not detect any form of CRP bound to microparticles, and flow cytometry which did. This group also contrasted the effectiveness of the same commercially available CRP ELISA kit (R&D systems) used within our research, and also found it unable to detect monomeric CRP, detecting the pentameric form only. A Sekisui automated hsCRP assay again failed to measure mCRP within an assay indicating how a variety of techniques available do not detected the presence of mCRP (Crawford et al., 2016). It is likely that many clinical or research settings are unable to detect mCRP effectively and if this form of CRP is shown within future RA studies to be important within disease progression or diagnosis, a reliable and accurate system of quantification should be developed and used.

5.4.2 Conclusion

Whilst the existence of monomeric CRP has previously been reported within scientific literature generally associated with diseased tissue or membrane bound, research results described within this chapter indicate its presence within the serum of both RA patient and control samples. This soluble form of mCRP has successfully been identified in 29 of the 30 patient samples as well as 24 of 30 control samples and quantified by ELISA analysis. It has been shown that pCRP sample concentration does not influence mCRP concentration and no significant correlation between these two values was apparent. Age alone does not influence the presence of either isoform of CRP within serum samples of the RA or control group. However, there appears to be a weak but not significant interaction when age and sex combined are taken into account, with older control group males displaying lower levels of both monomeric and pentameric CRP, and older RA group males showing reduced pentameric CRP

Little research has taken place within rheumatoid arthritis considering the effects or presence of a monomeric form of CRP. To our knowledge there are no studies which consider the effect of age upon the level of mCRP within this disease nor have quantified its presence. The levels of monomeric CRP identified are low and indicate a baseline level present within the general population in addition to a small but significant increase in level within this inflammatory autoimmune disease. It is clear that a consistent approach and careful consideration of ELISA protocol and antibody choice are important within both a research and diagnostic setting in order to correctly identify CRP isoforms present in serum.

Although these results have not been correlated to disease severity and progression there are clear differences in RA patients when compared to controls, in the concentration of pCRP and mCRP when both age and sex are taken into account, although due to sample

size these results are not significant in nature. Our research indicated that mCRP is present in RA patients and suggests the importance of developing an accurate system of mCRP determination, allowing fast and reproducible measurement within a clinical setting.

5.4.5 Future Work

Future work should focus on the link between mCRP concentrations and disease severity and progression within RA to elucidate whether its effect are pro or anti-inflammatory in nature and also if the suggested baseline level is maintained. These findings should also be correlated with the profile, class and dose of RA prescriptive drugs to further assess these factors. Whilst it is clear that mCRP is present within RA patient serum it is unclear as to the origin of the protein, be it due to pCRP dissociation within the local inflammatory conditions, systemic circulation or through local expression of the monomer rather than pentamer. Studies investigating the origin of monomeric CRP may help further our understanding of both the protein and RA disease progression. Our study has investigated mCRP within serum, but synovial fluid may prove an interesting focus area for future examination due to the milieu of inflammatory and immune proteins found within it.

The sample size within this study has not allowed all findings to be suggestive of those within a larger population and therefore a continuation of this pilot research within a larger population is important. Males represented just over 25% of the population within this study, they are therefore underrepresented against the wider population, future research should ensure a relevant proportion of male subjects are included in order to examine further the differences between male and female CRP levels. If this picture is repeated in a larger sample size the influence of sex may aid in future understanding of the causes and risk factors for this autoimmune disease.

Chapter 6 - Detection of Anti-CRP Auto-antibodies within RA Serum

6.1 Introduction

6.1.1 Overview

As a well characterised autoimmune disease, serum auto-antibodies play a key diagnostic role within the identification and development of RA. Some of these auto-antibodies are well characterised and understood, however the presence of mCRP and auto-antibodies against it have not been well studied within this disease. Within this study the presence of serum IgA, IgM and IgG auto-antibodies directed against either monomeric or pentameric CRP were studied within the 30 RA and 30 control subjects in order to explore the relationships between these proteins and either CRP isoform and investigate their potential association with RA. Auto-antibodies are antibodies that react with self-antigens, which may comprise of proteins, carbohydrates, nucleic acids or lipids. The body, during normal antibody production has a failsafe system that determines whether the antibodies produced by B cells following the presentation of an antigen, have self-recognition properties or not. If they are determined to be auto-antibodies they are destroyed. Put simply a lymphocyte undergoes a process whereby the lymphocyte-antigen interaction instigates a process ending in either the physiological elimination (negative selection) of that T or B cell or in clonal anergy, in which the lymphocyte is functionally inactivated. (Nossal,1994). This process when working effectively prevents autoimmunity, however in some cases an antigen-lymphocyte interaction may not illicit full immunological tolerance. Although a weak recognition of a self-antigen may not in all cases initially cause a response within the body, this may change should the physiological concentration of that

antigen rise. In this case a B or T cell that would previously have ignored the self-antigen may now begin to respond to it (Nossal, 1994).

Within autoimmune disease the system ensuring deletion or silencing of B cells generating self-recognition antibodies breaks down and antibodies with the ability to recognise the body's own proteins are allowed to travel through the body. Organ specific autoimmune diseases, including myasthenia gravis, thyroiditis, type 1 diabetes mellitus, primary biliary cirrhosis or pemphigus (Elkon and Casali 2008, Lesage and Goodnow 2001), are typified by antibody binding of an injury to a target organ. It is suggested that these auto-antibodies may be initially stimulated by inflammation within the target organ, or due to molecular mimicry (Cusick et al., 2013). Within systemic autoimmune diseases such as RA and SLE, the auto-antibody reacts with non-cell-type specific molecules such as phospholipids, nucleoprotein and cell surface antigens (Elkon and Casali, 2008), therefore capable of affecting multiple organs. Some auto-antibodies instigate complement together leading to inflammation, as seen in the complement mediated damaged to kidneys of patients with systemic lupus and lupus nephritis and foetal loss associated with antiphospholipid syndrome (Cervera *et al*., 2002; Cochrane and Koffler, 1973; Wendell *et al*., 1999). However the presence of auto-antibodies in the healthy population has also attracted attention and it is now suggested that these may play a protective role, perhaps as a mechanism for debris clearance (Nagele *et al*., 2012). Auto-antibodies are however, a key feature of disease pathogenesis and diagnosis within RA, this chapter seeks to investigate whether mCRP is a target of self-directed antibodies.

6.1.2 Antibody Generation

Antibodies that react with self-antigens also occur in normal healthy individuals and are termed natural antibodies. This class is composed predominantly of IgM

antibodies, which are produced during a primary antibody response in the absence of activation by a specific antigen and are polyreactive, they bind to several antigens. These antibodies possess broad specificity but a low to moderate affinity to self and foreign antigens and are an important bridge between the innate and acquired immune system components (Boes, 2000, Elkon and Casali 2008). Natural antibodies have been studied for over two decades and much of this research has focused on natural IgM antibodies, originally studied in experimental animals deprived of any potential antigens and exhibiting similar reactivity profiles (Haury *et al*., 1997; Hooijkaas *et al*., 1984,). Natural antibodies may play a useful and conserved role within the removal of cell debris during inflammation and those directed against pro-inflammatory cytokines may protect against uncontrolled inflammatory cascades and are shown to be universally present in human serum (Madi *et al*., 2009).

Antigen specific antibodies are secreted by plasma cells in response to antigen presentation by cells such as macrophages and dendritic cells, both of which recognise a pathogen through a range of pattern recognition receptors. Stimulated B cells then undergo somatic hypermutation (Berek *et al*., 1991; Kelsoe *et al*., 1991), followed by clonal selection, becoming a long lived and antigen specific antibody secreting cells (Tarlington and Smith, 2000). Where natural or antigen specific antibodies are the product of a functioning immune system and unmutated gene expression, high-affinity and somatically mutated IgG auto-antibodies can lead to pathological events within the body including disruption of the homeostatic pathways relating to cell clearance, signalling or cell effector functions (Elkon and Casali 2008).

6.1.3 IgM, IgG and IgA Antibodies

The immunoglobulins IgM, IgG, and IgA are involved in a range of immunological processed and functions with roles as diverse as protection against microbial infection, immune homeostasis, humoral immunity and detection of cancer formation. IgM is the largest antibody due to formation of the pentameric structure taking it to 990 kDa in size, it is involved in early immune response and when in serum can indicate a recent infection, it is also commonly found within healthy individuals (Madi *et al*., 2009). This class of antibody is produced by the long lived and self-renewing B-1 subset of B cells, which are positively selected for when presented with a self-antigen (Boes, 2000; Madi *et al*., 2009). IgM antibodies tend to be more polyreactive than other antibody classes, this allows IgM secreting B cells to respond very quickly to a large variety of antigens. These antibodies are relatively low affinity and are also commonly known as natural antibodies, playing a role in both the first line of defence and immunoregulation (Boes, 2000). Although low affinity, they have high avidity, due to their pentameric nature and therefore multimeric interaction with an antigen. Functionally IgM opsonises an antigen for destruction and complement fixation (Boes, 2000).

IgG is the most abundant antibody within the human immune system representing approximately 75% of serum antibodies and the most common found within circulation and extracellular fluid. There are four subclasses of IgG (IgG1, IgG2, IgG3 and IgG4) which are labelled in highest to lowest rank order of their levels within serum of healthy individuals. Binding a variety of pathogens including bacteria and viruses IgG protects the body via a range of mechanisms leading to pathogen elimination, including agglutination and opsonization initiating phagocytosis and activation of the classical complement pathway (Schroeder and Cavacini, 2010). A key feature of IgG is its ability to leave the serum and enter infected tissue due to its small size (150 kDa). IgG predominantly engages

within the secondary immune response following class switching and is secreted as a monomer playing a part in antibody dependent cell mediated cytotoxicity (ADCC) and intracellular antibody-mediated proteolysis and is involved in type II and III hypersensitivity reactions.

IgA is the second most common human immunoglobulin, with between 3-5g secreted by the intestinal lumen daily forming 13% of total immunoglobulins it is secreted in milk, tears, saliva and mucous (Placzek *et al*., 2018). In addition to secretory IgA, circulatory IgG is also present within the serum, however, the circulatory serum isoform of IgA is of very low levels. This antibody is resistant to digestion and can, when aggregated, activate the complement pathway. IgA is able to bind Fc receptors.. Structurally the antibody is found as either a monomer within serum or as a polymeric (dimer) form in secretions (Pillemer *et al*., 1987, Singh *et al*., 2014). It is termed either IgA2 which is found in higher concentrations within secretions or IgA1 which comprises 85% of serum IgA, with serum concentrations of IgA found between 1-4mg/ml (Singh *et al*., 2014).

This antibody is known as a poor activator of immune complement (Singh et al., 2014). Auto-antibodies to IgA are relatively common within autoimmune disease as are deficiencies in IgA which also lead to selective IgA deficiency. IgA deficient patients can have problems within blood transfusion reactions due to their interactions with IgA in the blood. Evidence suggests that normal levels of IgA are age-dependent and that these levels vary according to ethnicity and gender (Buckley and Dees, 1967; Steihm and Fudenberg, 1966). IgA is important within immune function of the mucous membranes and is especially evident in mucosal tissues including the respiratory and gastrointestinal tract.

6.1.4 Auto-antibodies within RA

Rheumatoid arthritis has been associated with several auto-antibodies including, rheumatoid factors (RF), anti-keratin auto-antibodies (AKA), anti-filaggrin antibodies (AFA) and anti-perinuclear factor (APF) (Goldbach-Mansky *et al*., 2000). RFs are identified as the prevalence of auto-antibodies directed against the Fc region of IgG antibodies and other auto-antibodies. These are often IgM in origin but IgG and IgA are also identified, with the presence and quantity of RF factors used diagnostically to indicate serologically positive RA (Meyer, 2010; Pollard, 2006). Within RA, anti-citrullinated protein antibodies (ACPA) and RF are predominantly IgM or IgG isotype with little evidence to suggest IgA plays a diagnostic role. However a study in 2006 illustrated that 44.7% of RA patients were IgA-RF positive. IgA antibodies were found in approximately 50% of the tested patients and this study suggested both IgA-RF and IgA-ACPA appeared to have diagnostic value (Sieghart *et al*., 2016). Citrullinated proteins are formed during an inflammatory state when arginine residues are enzymatically converted to citrulline. Cyclic citrullinated peptide (CCP) is formed when two serine residues of filaggrin are enzymatically converted to cysteines, which then undergo disulphide bonding resulting in a circular form (Pollard, 2010). Anti-cyclic citrullinated peptide (anti-CCP) antibodies are also a feature of RA diagnosis. Clinically these auto-antibodies are significant within RA diagnosis, the specificity of anti-CCP within RA is between 89 - 98% (Bas, 2002; Goldbach-Mansky, 2000; Klareskog *et al*., 2006; Schellekens, 2000; Simon, 1993; Suzuki, 2003), AKA (94%) and IgM RF (74%), (Bas 2002). Interestingly the study by Bas *et al*. (2002) found that although anti-CCP antibodies had the highest level of specificity within RA diagnosis, IgM RF was a better predictor of disease severity. These RFs are found in 60-80% of RA patients, but can also be detected in a range of other diseases (Pollard, 2006).

Where detection of RFs is the only clinical diagnostic in seropositive RA, SLE can be characterised by the prevalence of auto-antibodies within patient serum to a variety of self-proteins, in addition to nucleic acids and phospholipids, with over 100 different identified targets (Meyer, 2010). The identification and quantification of some highly specific self-directed antibodies such as anti-CCP auto-antibodies are therefore valuable tools used within the confirmation of autoimmune diseases. IgA rheumatoid factor (IgA-RF) and IgA containing circulating immune complexes (IgA-RF complexed to IgG) have also been identified as significantly raised in RA compared to control groups (Pillemer *et al*., 1987). IgA antibodies directed against both cytokeratin-18 and epidermal keratin have been identified as significantly raised in patients with RA compared to controls or patients with OA (Borg *et al*., 1993).

6.1.5 Auto-antibodies Directed against C-reactive Protein

In addition to the collection of recognised auto-antibodies found present within RA, studies have emerged with identified autoantigenicity towards mCRP and suggest this may also play a role within disease prognosis and severity (Minatani *et al*., 2001; Rosenau and Schur, 2013). Whilst previously not strongly associated with RA disease determination, the anti-mCRP auto-antibodies are an important factor within the diagnosis of SLE, with both monomeric and pentameric CRP autoantigenicity recorded (Bell *et al*., 1998; Figueredo *et al*., 2006; Minatani *et al*., 2001; Rosenau and Schur, 2013; Sjowall and Wettero, 2007). In their 1998 paper, Bell *et al* performed inhibition studies which revealed anti-mCRP IgG auto-antibodies in the serum of four SLE patients tested ranging from 42% - 70% inhibition following preincubation of serum with 30µg/ml mCRP, whilst anti-pCRP auto-antibodies were present in only two of the four and at lower levels of inhibition (17% - 31% inhibition) (Bell *et al*., 1998). Whilst RA and SLE are both autoimmune inflammatory

diseases they present very differently with respect to CRP and its isoforms. Where RA is known for its high levels of pentameric CRP and low frequency of circulating auto-antibodies directed against it, for example between 23-29% of RA patients within Rosenau & Schur's study (2013), were found to be positive for anti-mCRP IgG (Minatani *et al*., 2001; Rosenau and Schur, 2013); SLE presents an opposite profile, with low serum CRP levels but anti mCRP auto-antibodies detected in between 40% to 78% of SLE patients (Figueredo *et al*., 2006; Meyer, 2010; Sjowall *et al*., 2004, ,), and all patients with active lupus nephritis during periods of flare (Sjowall *et al*., 2004).

The development of antigenicity is suggested to originate through impairment to the clearance of cellular debris and defects within the apoptotic pathway (Herrmann *et al*., 1998, Prechl, 2015; Shao *et al*., 2011,). CRP has a clearly defined role within recognition and clearance of apoptotic cells and therefore detection of auto-antibodies directed towards it may provide clues towards greater understanding of this disease.

6.1.6 CRP Auto-antibodies within SLE Patient Serum

Whilst an inflammatory state within many diseases including RA is associated with increased CRP serum concentrations, many SLE patients remain within a relatively normal range, albeit towards the higher end of that scale (Meyer,. 2010). Generally CRP levels only increase moderately during lupus flares, but the same patients are still able to exhibit an increase in CRP during bacterial infection for instance (Meyer, 2010). Whilst not characterised by high CRP levels, SLE patients are often found to have anti-CRP auto-antibodies within their serum. These auto-antibodies have been found within an average of 35 to 40% of SLE patients (Meyer, 2010; Sjowell *et al*., 2002) and were first reported in one of eight patients within a 1985 study (Robey *et al*., 1985). Bell *et al*. in 1998 reportedly found anti-CRP auto-antibodies in 78% of patient samples, but later studies dating from

2002 onwards have reported a frequency of between 10-51% of SLE patients affected (Bell *et al*., 1998, Figueredo *et al*., 2006; Kessel *et al*., 2009; Rosenau *et al*., 2006; Shoenfeld *et al*., 2007; Sjowell *et al*., 2002, 2004). The levels of CRP directed auto-antibodies was found to associate with SLE disease activity (Sjowell *et al*., 2004), suggesting biological and pathogenic functions within SLE and also significantly correlate to associated kidney disease (Figueredo *et al*., 2006; Sjowell *et al*., 2009; Tan *et al*., 2008). Using ELISA to determine levels, pCRP was coated overnight and patient sera used as primary antibody source followed by anti-human IgG antibodies. The form of CRP detected has been suggested to be monomeric rather than pentameric due to conformational changes exposing non-native intersubunit regions of pCRP upon binding to polystyrene surfaces (Potempa *et al*., 1987). These studies have not sought to identify pCRP targeted antibodies, nor have they sought to determine any other auto-antibody class in addition to IgG.

6.1.7 CRP Auto-antibodies within RA Patient Serum

Whilst levels of CRP are generally raised within RA in contrast to SLE, auto-antibodies to either pentameric or the modified monomeric form of CRP are reported with less frequency in RA than within SLE patients, it is a little researched area with greater focus directed towards SLE as a disease model. However a study by Rosenau and Schur in 2006 found evidence for anti-mCRP auto-antibodies within 22% of samples tested with an earlier paper reporting 29% (Minatani *et al*., 2001; Rosenau and Schur, 2006). In both cases the neo epitopes exposed during dissociation were found to be the targets of the CRP directed auto-antibodies. Studies considering auto-antibodies in SLE have found both IgG and IgM auto-antibodies directed against mCRP (Pradhan *et al*., 2013). Both Rosenau *et al*., (2006) and Minatani *et al*., (2001) considered the presence of IgG auto-antibodies and

to our knowledge there are no studies detailing the presence of either IgA or IgM auto-antibodies directed against CRP in either monomeric or pentameric form within RA.

6.1.8 CRP Dissociation upon Surface Binding

CRP is known to change in conformation when bound to the polystyrene surface of an ELISA plate, exposing neoepitopes and non-native structure. This method has been employed within studies to facilitate the examination of bound monomeric CRP (Sjowall *et al*., 2003, 2003). The native pentameric form is bound overnight onto the plate and loss of conformation occurs unless the plate has been pre-coated with phosphocholine bound keyhole limpet haemocyanin (PCh-KLH), which allows pentamer binding and stabilisation. Therefore an important consideration within ELISA experimental design is the behaviour of CRP upon binding to ELISA plates. If pCRP is used as a capture agent within antibody or auto-antibody detection tests, the stability of pentameric CRP must be fully understood in order to fully interpret experimental results.

6.1.9 ELISA Interactions

The ELISA test is incredibly useful in determining which proteins and antibodies are present within serum; however interactions between the serum and assay constituents are also possible. A research study in 2013 by Sjowall *et al* detailed an interaction found within RA patients to dietary proteins, with a common antigen found to be being bovine serum albumin (BSA). Many ELISA assay kits use BSA within a blocking or diluent step, or as an inert carrier to preserve biological function within storage. Results determined when BSA is used may prove unreliable should patient samples show sensitivity to this protein. In their study Sjowall *et al* found that 50% of RA patients and 62% of controls were anti-BSA positive (using a cut off of 0.1 net OD), although no significant difference was found within the groups (P=0.11) or between women and men (Sjowell *et al*., 2013).

It is not known whether anti-BSA antibodies are innate in nature or occur via immune system antigen presentation. The possibility that high anti-BSA antibody levels are implicated in diseases such as insulin dependent diabetes mellitus (IDDM) is reported, with cross reactivity between a BSA peptide sequence and pancreatic B-cell surface protein found (Karjalainen *et al*., 1992; Saukkonen *et al*., 1998). There is a possibility that past studies investigating autoimmune disease serum protein and auto-antibody levels may have determined increased anti BSA rather than the proteins of interest. Sjowall *et al*., *(2013)* justified a lack of blocking step within their assay as a method to avoid serum BSA interactions, although it is questionable as to whether non-specific binding occurred resulting from this decision. Rosenau and Schur chose to include BSA blocking criticising Sjowall's study robustness, although possibly introducing study bias due to binding of BSA (Rosenau,and Schur, 2006; Sjowall *et al*., 2013). These differences in study design may explain differences within results, as this could introduce inconsistency due to anti-BSA antibodies commonly occurring even within healthy individuals, therefore may be detected providing possible false positive results. A different study overcame these possible interactions by pre-incubating the serum on a BSA coated plate before adding it to a second plate coated in mCRP, thus removing anti-BSA antibodies present (Jakuszko *et al*., 2017). Figure 6.1 illustrates problems encountered within the competitive ELISA developed to identify auto-antibodies to both mCRP and pCRP assays including interactions with BSA and the steps taken to overcome them.

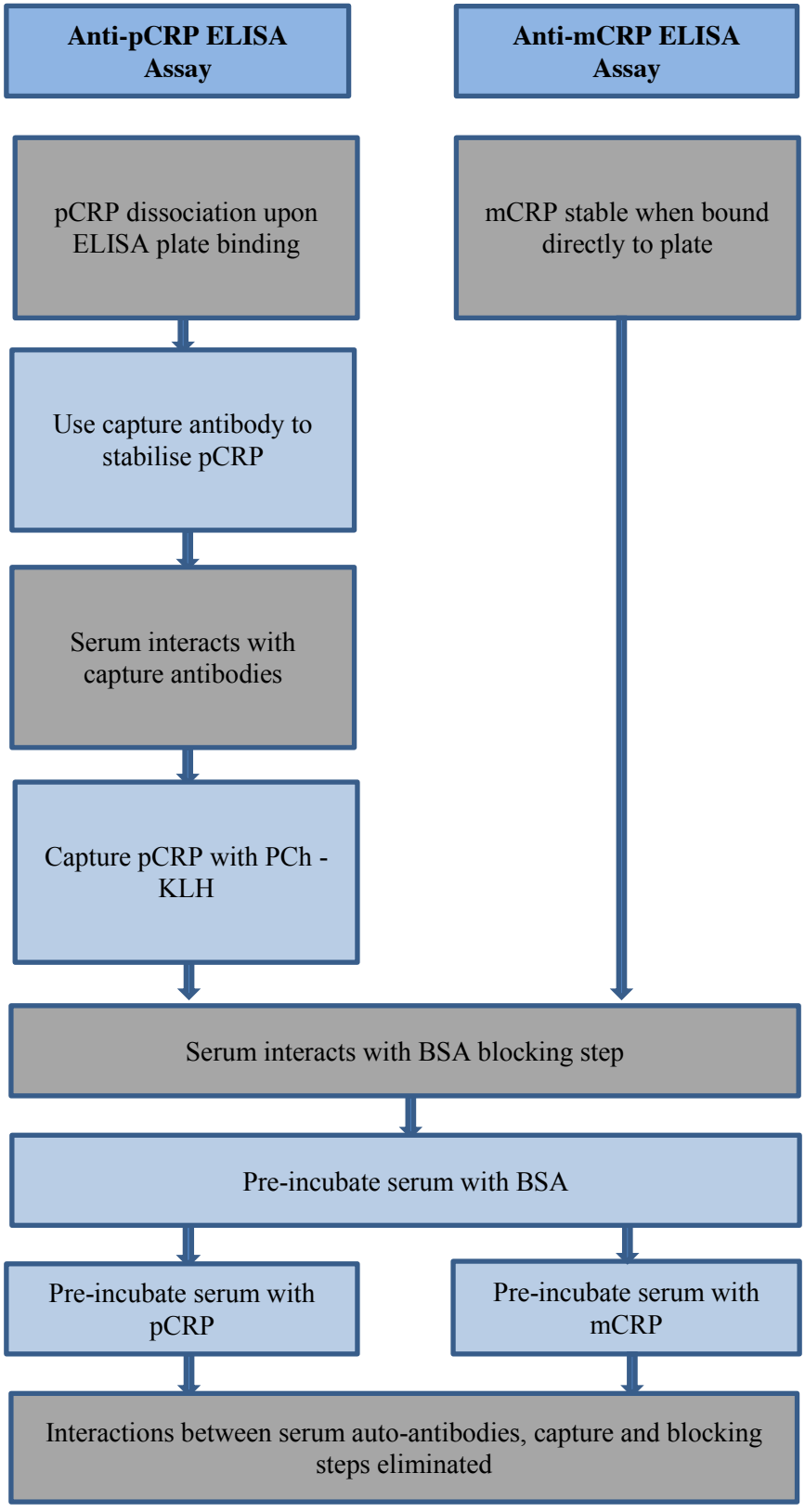


Figure 6-1 A flow diagram illustrating the obsticles encountered during measurement of anti-pCRP and mCRP auto-antibodies and the steps taken in order to eliminate them.

6.1.10 Experimental Aims

To enable meaningful quantification and analysis of auto-antibodies to mCRP and pCRP within RA patient serum, it was essential to develop an effective protocol for their identification. The aim of this chapter was to design an ELISA assay system which allowed accurate and reproducible determination of IgG, IgA and IgM auto-antibodies directed against both CRP conformations. The identification of such would allow greater understanding of RA and the role CRP auto-antibodies, in addition to those currently determined for diagnostic purposes, play within this inflammatory disease. Little research has occurred investigating auto-antibodies directed against CRP within RA, and those which have, sought to identify only those recognising mCRP not pCRP. The ELISA development must overcome the interactions between human serum and constituent parts of the assay including blocking solutions, in addition to ensuring the chosen CRP conformation is stable. The successful development of this assay will allow full and rigorous investigation as to whether CRP auto-antibodies are present within the serum and if levels are higher or lower within RA patient or control group serum. The comparison to non-inflammatory control group serum may aid further study as to whether monomeric CRP is pro or anti-inflammatory in nature.

6.2 Materials and Methods

6.2.1 Ethical Application

Prior to experimental collection of patient samples and any experimental procedure took place, both peer review and ethical research committee approval was sought. The details of this review are set out within section 2.3.1.

6.2.2 Preparation of Human Serum

Patient samples were collected and processed as described within section 2.3.2 and stored at -20^{o}C until required.

6.2.3 ELISA Analysis of Serum Anti-p CRP IgG, IgA and IgM Auto-antibodies

ELISA assays were carried out following the method in section 2.3.9 with reagents listed within Tables 2.9 and 2.10. Patient and control samples were arranged as detailed in Table 2.13.

6.2.4 ELISA Analysis of Serum Anti-mCRP IgG, IgA and IgM Auto-antibodies

ELISA assays were carried out following the method in section 2.3.9 with reagents listed within Tables 2.9 and 2.10. Patient and control samples were arranged as detailed in Table 2.12.

6.2.5 Western blot analysis of IgG/IgM/IgA Anti-Human mCRP and mCRP auto-antibodies

The method previously described in sections 2.2.6 and 2.3.7 was used to run the native gel. Each well was loaded with either 1μg of monomeric or pentameric CRP within

sample loading buffer to a maximum volume of 30µl within a pre-cast BioRad native gel (12%). Table 2.4, provides details of buffers and reagents used.

6.2.6 Statistical analysis

Samples were triplicated upon each plate and the mean of the closest two values taken. Each plate was processed in triplicate. Results were calculated by removing the absorbance of samples which had been pre-incubated with CRP from those that had not. The addition of either mCRP or pCRP allowed binding of any auto-antibodies present within the serum and prevented binding to the protein captured on the plate. The net difference represented binding of auto-antibodies present within the serum. The upper limit of the normal values for serum auto-antibody levels was defined as the mean ± 2SD of levels in the sera from the 30 healthy controls; samples elevated above this value were determined as high or elevated.

During initial statistical analysis it became apparent that the male cohort auto-antibody data was affecting analysis and in many cases the combination of male and female data sets produced bimodal data making analysis based on comparison of mean or median group data unreliable, in this case the groups were split by gender prior to detailed statistical analysis. Data also considers the number of patient or control samples found to contain auto-antibody levels above zero Absorbance with comparison of the number of elevated or above zero samples between groups analysed by Chi-squared analysis.

All statistical analysis undertaken within our research was generated by Minitab 18 and is detailed within section 2.3.10.

6.3 Results

6.3.1 Experimental Design

Overall experimental design details are as described within section 5.3.1, however the primary outcome for this area of study was to determine whether auto-antibodies directed against mCRP or pCRP were present within human RA patient serum.

6.3.2 ELISA Analysis of Human Anti-CRP Auto-antibodies

The analysis by ELISA allows detection of low levels of antibody present within serum in a specific manner. Studies have indicated an interaction between RA patient serum and dietary proteins, with a common antigen found to be bovine serum albumin (BSA) (Karjalainen *et al*., 1992; Saukkonen *et al*., 1998; Sjowall *et al*., 2013). This was also determined to be a problem early within our optimisation of ELISA protocols, as initial ELISA tests were complicated by interactions between the patient sample and both the BSA blocking agent and capture antibodies. It was therefore necessary to develop and use a competitive ELISA system, antibody dilutions were first optimised and the optimum assay taken forward for patient sample testing. Serum samples were also diluted and pre-incubated within BSA before adding to the plate, therefore eliminating as much BSA non-specific binding as possible. As previously described, pentameric CRP is unstable when in contact with a surface or membrane, with previous results showing a dissociation of pCRP during initial binding to an ELISA plate overnight and at reduced time periods. Due to this it was necessary to use an antibody capable of capturing the pCRP and maintaining it within its pentameric form. The capture antibody used to stabilise the pCRP conformation when bound to the ELISA plate was first assessed for effect. However, this led to the observation that anti-IgG present within serum samples was interacting with the chosen capture antibody, providing false positive results (results not shown). Initially a goat anti-

CRP antibody was used as a capture agent, however when interactions became apparent this was changed to phosphocholine bound keyhole limpet haemocyanin (PCh-KLH), pCRP is known to bind phosphocholine and has when conjugated to the larger KLH been used previously in studies to capture and stabilise CRP upon an ELISA plate (Potempa *et al*., 2015; Wang, 2012;). Within the anti-monomeric CRP auto-antibody detection assay, a capture antibody or agent was not necessary as mCRP could be bound directly to the plate without structural compromise.

When considered as a complete, mixed gender group the number of patients with elevated antibody levels (≥ mean + 2SD of normal control subjects) was, 10 of 30 (33%) for anti-mCRP IgG, 22 of 30 (73%) of anti-mCRP IgA, 8 of 30 (27%) of anti-mCRP IgM, 9 of 30 (30%) for anti-pCRP IgG, 3 of 30 (10%) of anti-pCRP IgA and 8 of 30 (27%) of anti-pCRP IgM. Table 6.1 summarises the mean ± SEM and percentage of samples with detectable auto-antibody presence for each of the sample groups and assays investigating IgG, IgM and IgA auto-antibodies directed towards mCRP and PCRP. This table also provides a comparative overview of the proportion of samples above threshold (control mean + 2SD) and calculated threshold. The spread of results varied greatly between control and RA groups and between those of the auto-antibodies themselves.

Table 6.1 A table summarising the mean ± SEM, % of samples containing auto-antibodies and proportion of samples elevated above the calculated upper threshold (≥ mean ± 2SD normal cohort), for each of the auto-antibody groups, split by RA (*n* = 30) and control (*n* = 30).

Auto-antibody	Mean ± SEM (Absorbance OD)	Threshold mg/l (Mean + 2SD)	% Samples with Detectible Autoantibodies	Proportion of Samples above Threshold (mean control ± 2SD)
Anti-mCRP IgG RA	0.077 ± 0.0139	0.100	66 (20 of 30)	10 of 30
Anti-mCRP IgG Control	0.024 ± 0.007	0.100	43 (13 of 30)	2 of 30
Anti-mCRP IgA RA	0.103 ± 0.015	0.039	93 (28 of 30)	22 of 30
Anti-mCRP IgA Control	0.006 ± 0.003	0.039	27 (8 of 30)	1 of 30
Anti-mCRP IgM RA	0.083 ± 0.016	0.149	87 (26 of 30)	8 of 30
Anti-mCRP IgM Control	0.053 ± 0.009	0.149	73 (22 of 30)	0 of 30
Anti-pCRP IgG RA	0.129 ± 0.017	0.205	93 (28 of 30)	9 of 30
Anti-pCRP IgG Control	0.072 ± 0.012	0.205	77 (23of 30)	1 of 30
Anti-pCRP IgA RA	0.039 ± 0.005	0.076	93 (28 of 30)	3 of 30
Anti-pCRP IgA Control	0.019 ± 0.005	0.076	53 (16 of 30)	3 of 30
Anti-pCRP IgM RA	0.101 ± 0.021	0.137	80 (24 of 30)	8 of 30
Anti-pCRP IgM Control	0.033 ± 0.01	0.137	47 (14 of 30)	3 of 30

Table 6.2 Laboratory parameters for RA and control samples. presented as median (IQR

Auto-antibody	Median (IQR)
Anti-mCRP IgG RA	0.054 (0.000-0.129)
Anti-mCRP IgG Control	0.000 (0.000-0.052)
Anti-mCRP IgA RA	0.096 (0.039-0.139)
Anti-mCRP IgA Control	0.000 (0.000-0.003)
Anti-mCRP IgM RA	0.031 (0.018-0.155)
Anti-mCRP IgM Control	0.046 (0.005-0.106)
Anti-pCRP IgG RA	0.095 (0.058-0.214)
Anti-pCRP IgG Control	0.065 (0.006-0.118)
Anti-pCRP IgA RA	0.037 (0.015-0.058)
Anti-pCRP IgA Control	0.001 (0.000-0.034)
Anti-pCRP IgM RA	0.046 (0.019-0.163)
Anti-pCRP IgM Control	0.003 (0.000-0.051)

As seen within Table 6.2 and Figure 6.2, median levels within four of the six controls, anti-mCRP IgG, anti mCRP IgA, anti-pCRP IgA and anti-pCRP IgM and are close to or zero (0.00, 0.00, 0.001 and 0.003 respectively), with many zero level samples found in each group. There was considerable truncation of data due to the number of zero

samples. Data apart from that of RA group IgA, was not normally distributed, therefore several analysis were performed using non-parametric tests as detailed within section 2.3.10. The nature of the data when subjected to analysis revealed differences between the male and female groups. These differences prevented direct statistical comparison of mean antibody values as a mixed gender group; therefore participants were split by gender before further analysis took place. Initial group data is illustrated within figures 6.2 to 6.8 and 6.13 to 6.18 showing the overall profile of auto-antibodies within both the RA and control sample groups. Analysis of data following the division of groups into male and female is presented in Figures 6.19 and Table 6.3.

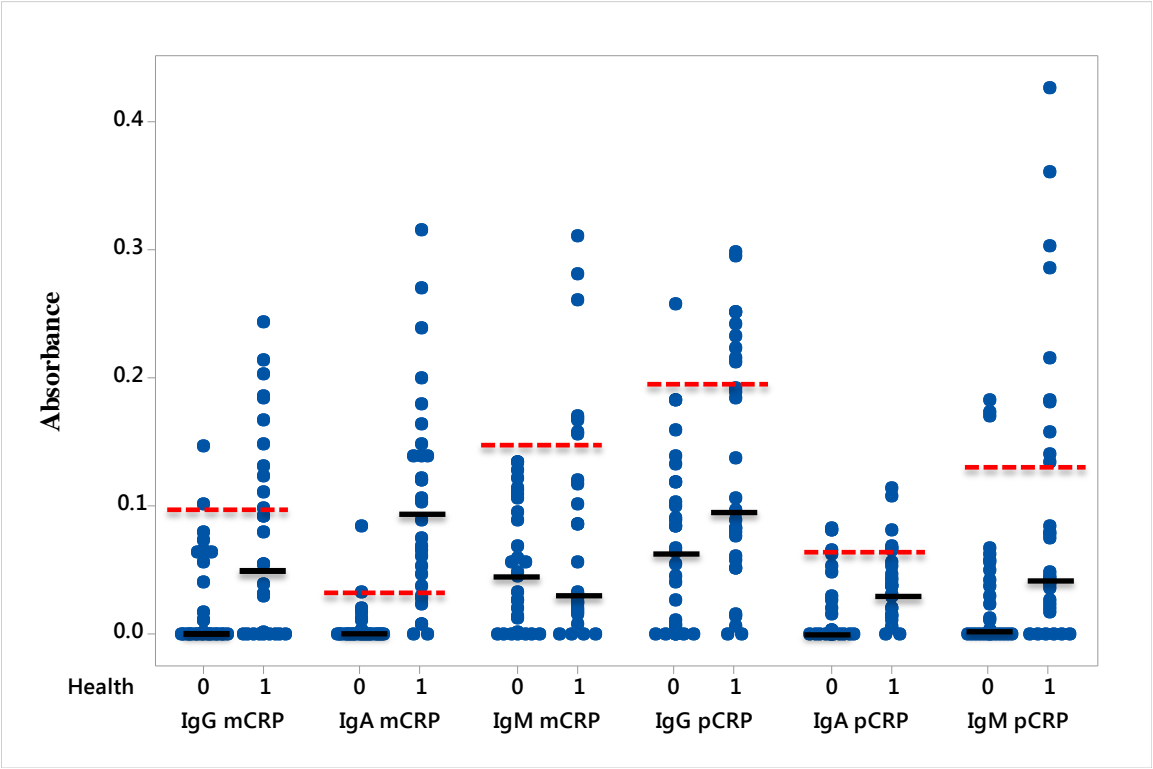


Figure 6-2 Serum levels of anti-mCRP and anti-pCRP antibody in control (0) and patient (1) samples, Absorbance at 450nm (less 550nm) per assay group. Horizontal bars indicate the median values. The dashed red lines indicate the upper limit of the normal range (mean ± 2SD value of normal).

Figures 6.3a and 6.3b display the level of IgG auto-antibodies directed towards mCRP within each of the 30 patient and 30 control samples as assessed by ELISA. Within the RA group (Figure 6.3a), 20 of the 30 samples (66%) were found to be have detectable

levels of anti-mCRP IgG auto-antibodies, with a mean absorbance value across the samples of 0.077 ± 0.0139. The absorbance of positive samples ranged between 0.244 (RA15) and the lowest of 0.029 (RA7). Within this group 10 samples were elevated above the upper limit cut off for normal levels of IgG auto-antibodies, an absorbance of 0.100 (≥ mean + 2SD of control subjects). Figure 6.3b displays absorbance within the control group samples, within this group 13 of the 30 (43%), were found to have detectable levels with a mean absorbance value across the group of 0.024 ± 0.007. The recorded absorbance of the group ranged between 0.147 (C05) and the lowest 0.0015 (C25), of these samples only 1 was elevated. The number of RA group samples with elevated levels of anti-mCRP IgG was found to be significantly higher than the control $P < 0.01$.

The difference seen between the patient and control groups was more pronounced within the anti-mCRP IgA group as can be seen in Figures 6.4a and 6.4b. Within the patient group (Figure 6.4a), 28 of the 30 samples (93%) were found to have detectable levels of anti-mCRP IgA auto-antibodies, with a mean absorbance value across the samples of 0.103 ± 0.015. The absorbance of positive samples ranged between 0.3154 (RA11) and the lowest of 0.008 (RA30), of the samples 22 were elevated above 0.039 (≥mean +2SD normal samples), in contrast to just one of the control samples. Within the control group 8 of the 30 (26.7%), were found to have detectable levels with a mean absorbance value across the group of 0.006 ± 0.03. The recorded absorbance of the group ranged between 0.0841 (C17) and the lowest 0.001 (C30). The number of RA subjects with elevated IgA auto-antibody levels was found to be significantly higher than that of the control $P < 0.001$. This difference is more pronounced than that between the anti-mCRP IgG groups.

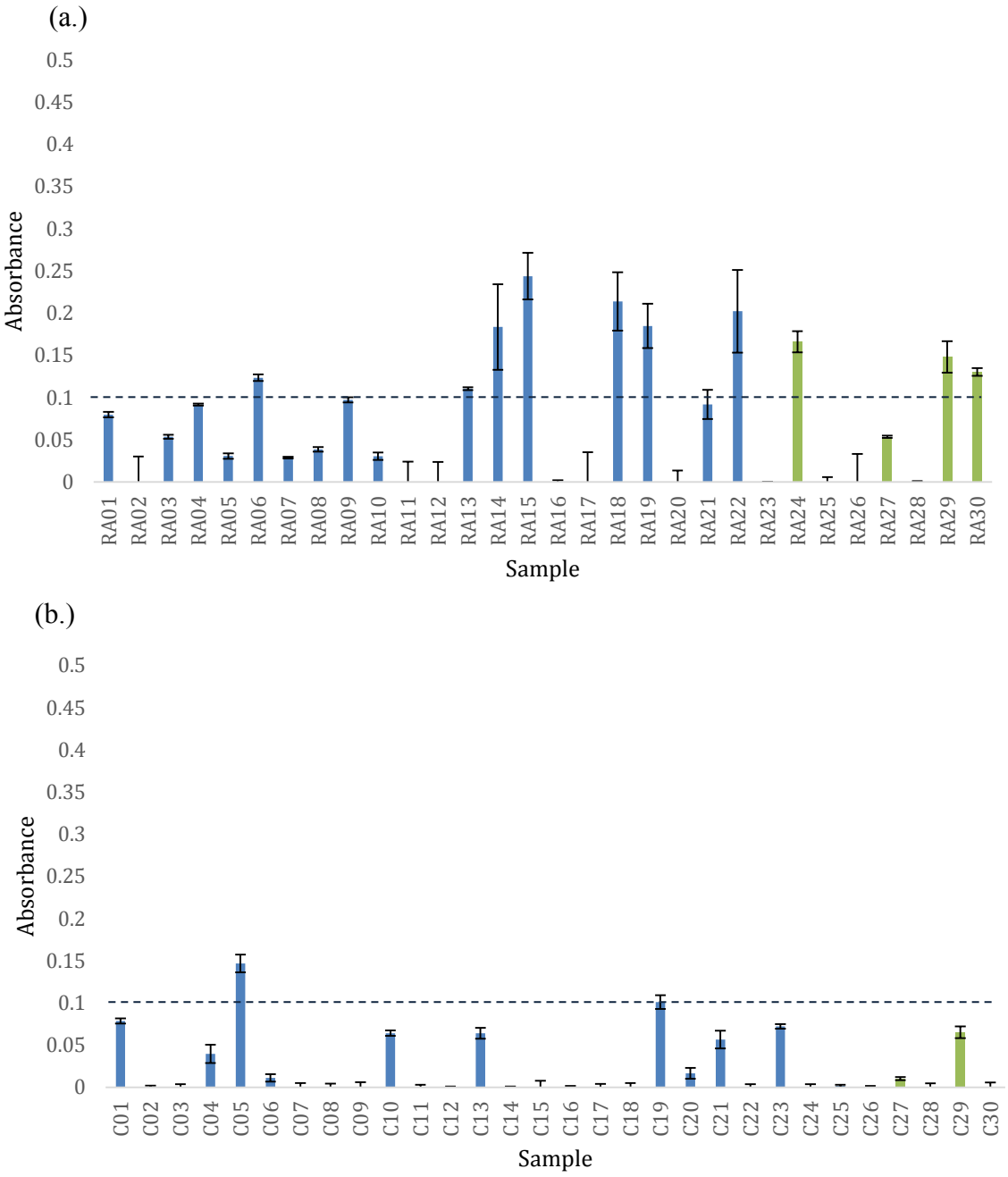


Figure 6-3 Bar graphs showing serum anti-mCRP IgG in both RA and control samples. (a.) RA group and (b.) control group samples. Auto-antibodies presented as Absorbance of sample. Data as determined by competitive ELISA (n = 3), following sample dilution of 1:100 in triplicate. The dashed line represents the upper normal limit, those above are considered elevated serum, (≥ mean ± 2SD (0.100 OD)). The number of elevated RA group samples (n = 10) is significantly different from the control group (n = 2), $P < 0.01$. Green columns (RA23- 30 C24-30) = male, blue = female..

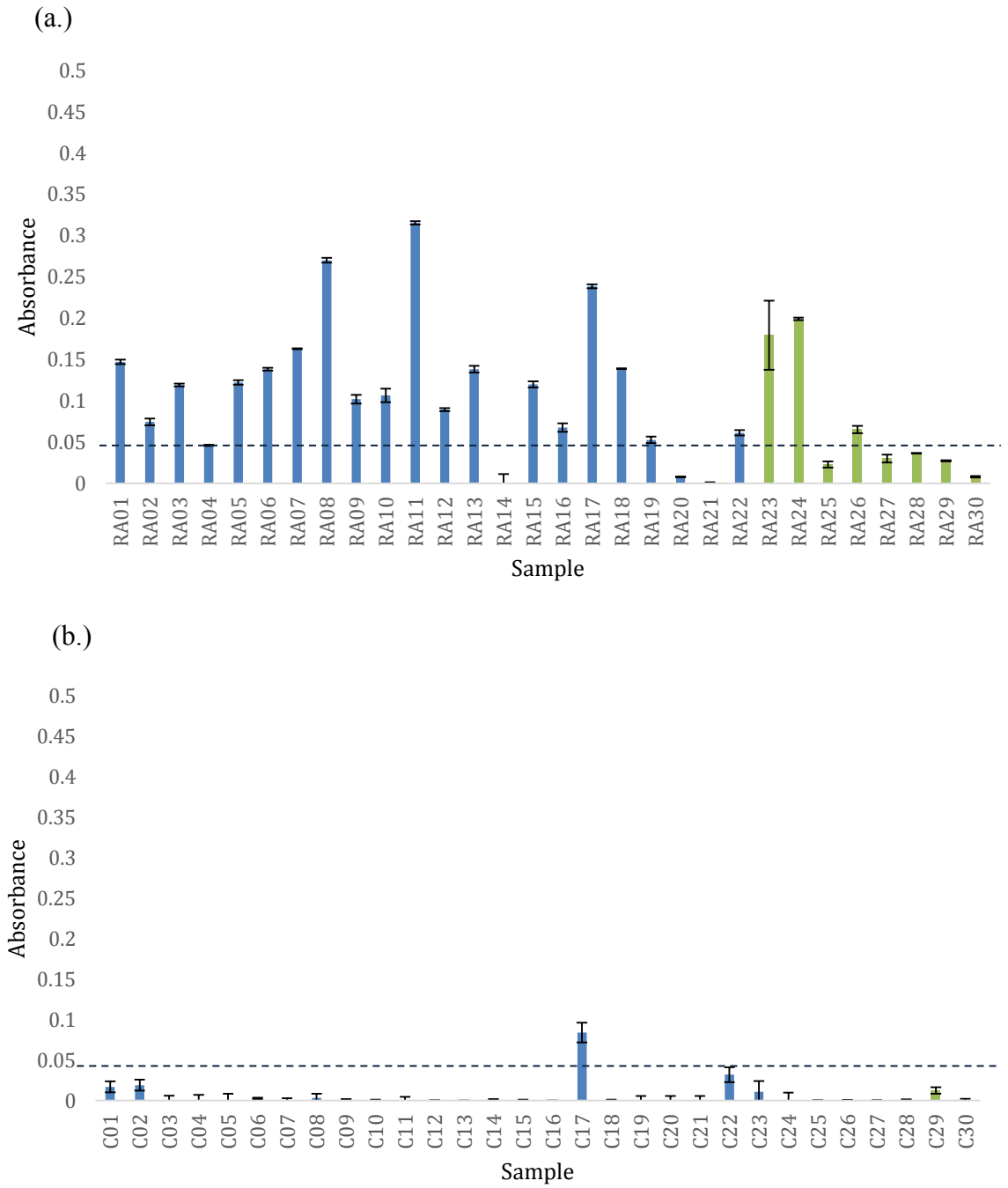


Figure 6-4 Bar graphs showing serum anti-mCRP IgA in both RA and control groups. (a.) RA group and (b.) control group samples. Data as determined by competitive ELISA, (*n* = 3), following sample dilution of 1:100 in triplicate. The dashed line represents the upper normal limit, those above are considered high, (≥ mean ± 2SD (0.039 OD)). The number of elevated RA group samples (n = 22) is significantly different from the control group (*n* = 1), $P < 0.001$ (X^2 analysis). Green columns (RA23- 30; C24-30), = male, blue = female.

Figures 6.5a and 6.4b display the levels of anti-mCRP IgM within the patient and control groups. Fig. 6.5a displays the level of anti-mCRP IgM auto-antibodies within the RA group and shows that 26 of the samples were found to have detectable levels of auto-antibodies (87%). The mean absorbance value across the group is 0.083 ± 0.016 SEM. The absorances ranged between 0.311 (RA01) and the lowest positive 0.008 (RA04). The number of high serum samples found above 48.39% (control group mean + 2SD), was less within this group than either the IgG or IgA mCRP directed antibody groups with 8 of 30 samples elevated. The mean absorbance of the patient group was slightly lower than that of the IgG assay result (0.083 vs 0.103), however the control group data differed greatly. Whilst a greater proportion of the control population were found to have the presence of anti-mCRP IgG when compared to anti-mCRP IgAs, this proportion was raised further to 73% of control samples with detectible IgM auto-antibody values, (22 of 30), as can be seen in Table 6.1.The mean absorbance of this group is much higher than either of the IgG or IgA assays at 0.053 ± 0.009 SEM. However, despite a higher proportion of normal samples found to contain IgM auto-antibodies, no control samples exceeded the determined upper normal level and there were therefore no elevated control samples. The number of elevated RA group samples was found to be significantly higher to those of the control ($P < 0.01$).

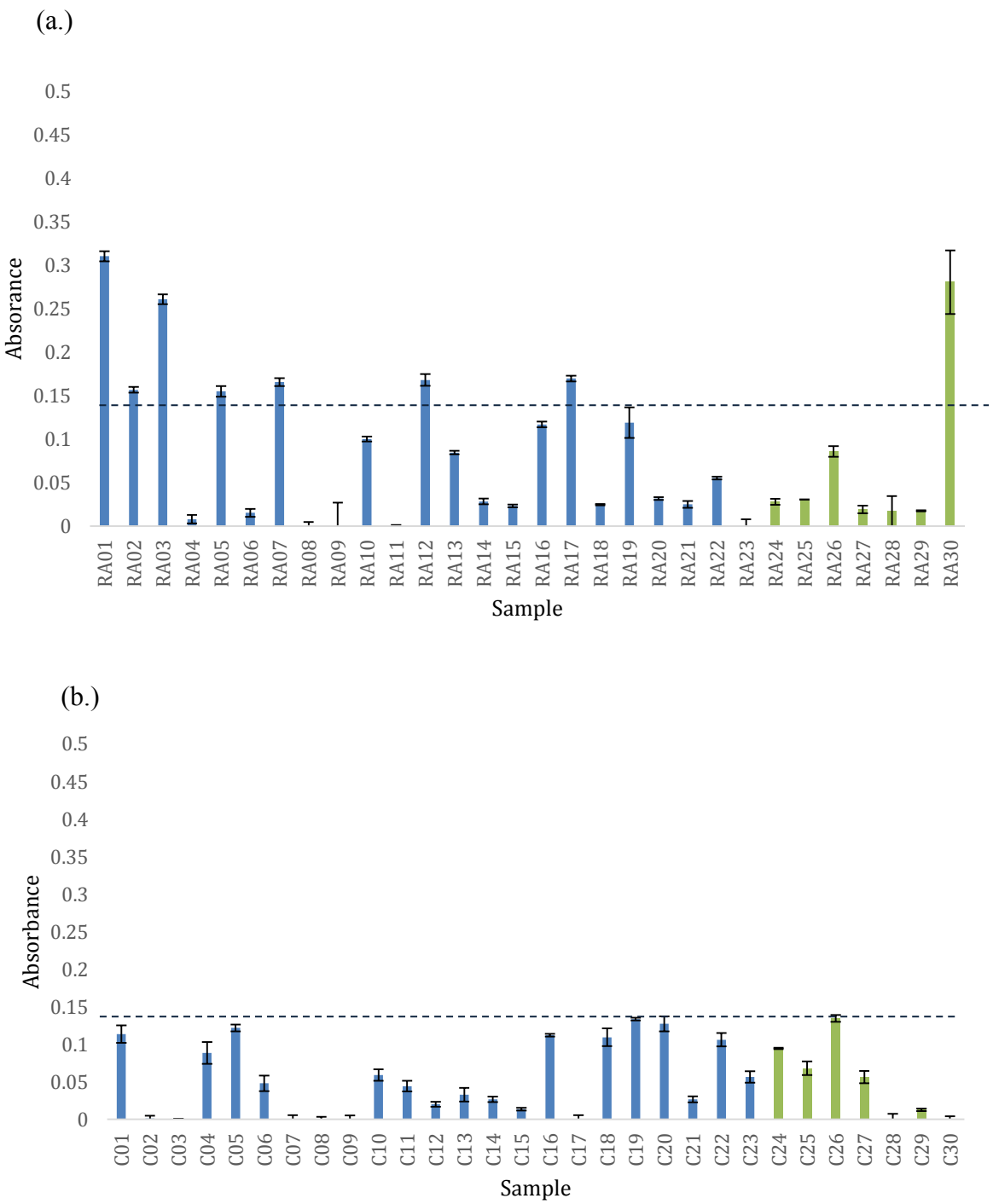


Figure 6-5 Bar graphs showing serum anti-mCRP IgM in both RA and control groups. (a.) RA group and (b.) control samples, auto-antibodies presented as Absorbance of sample. Data as determined by competitive ELISA (n=3), following sample dilution of 1:100 in triplicate. The dashed line represents the upper normal limit, those above are considered high serum, (≥ mean ± 2SD (0.149 OD)). The number of elevated RA group samples (n = 8) is significantly different from the control group (n = 0), $P < 0.01$ (X^2 analysis). Green columns = male, (C24-30; RA23- 30), blue = female.

Analysis of the pentameric form illustrate that auto-antibodies against the pCRP are also relevant within this area of study. Figure 6.6a and 6.6b display the level of IgG auto-antibodies directed towards pCRP within each of the 30 patient and control samples. Within the RA group (Figure 6.56), a high proportion, 28 of the 30 samples (93%), were found to have detectable levels of anti-pCRP IgG auto-antibodies, with a mean value across the samples of 0.129 ± 0.017 SEM. The absorbance of positive samples ranged between 0.297 (RA12) and the lowest positive of 0.006 (RA26), of the samples nine were elevated above 0.205 (control group mean + 2SD), indicating almost a third of patient samples had high levels of this auto-antibody within their serum. Figure 6.6b displays the control group auto-antibody levels represented as percentage OD of RA12, the highest sample within the RA group. Within this group 23 of the 30 (77%), were found to contain auto-antibodies with a mean value across the group of 0.072 ± 0.012 SEM. The recorded ODs of the group ranged between 0.257 (C08) and the lowest 0.0062 (C20). Of the samples, one was elevated. The number of elevated RA group samples was found to be significantly different to that of the control $P < 0.01$. The number of samples positive for IgG auto-antibodies directed against pCRP are similar to those of the mCRP group (Figure 6.3), with nine as opposed to ten in the RA group and one for both within the control groups. A significantly greater proportion of samples with pCRP directed antibodies present were observed within both the RA and control samples compared to those directed against mCRP, $P < 0.01$.

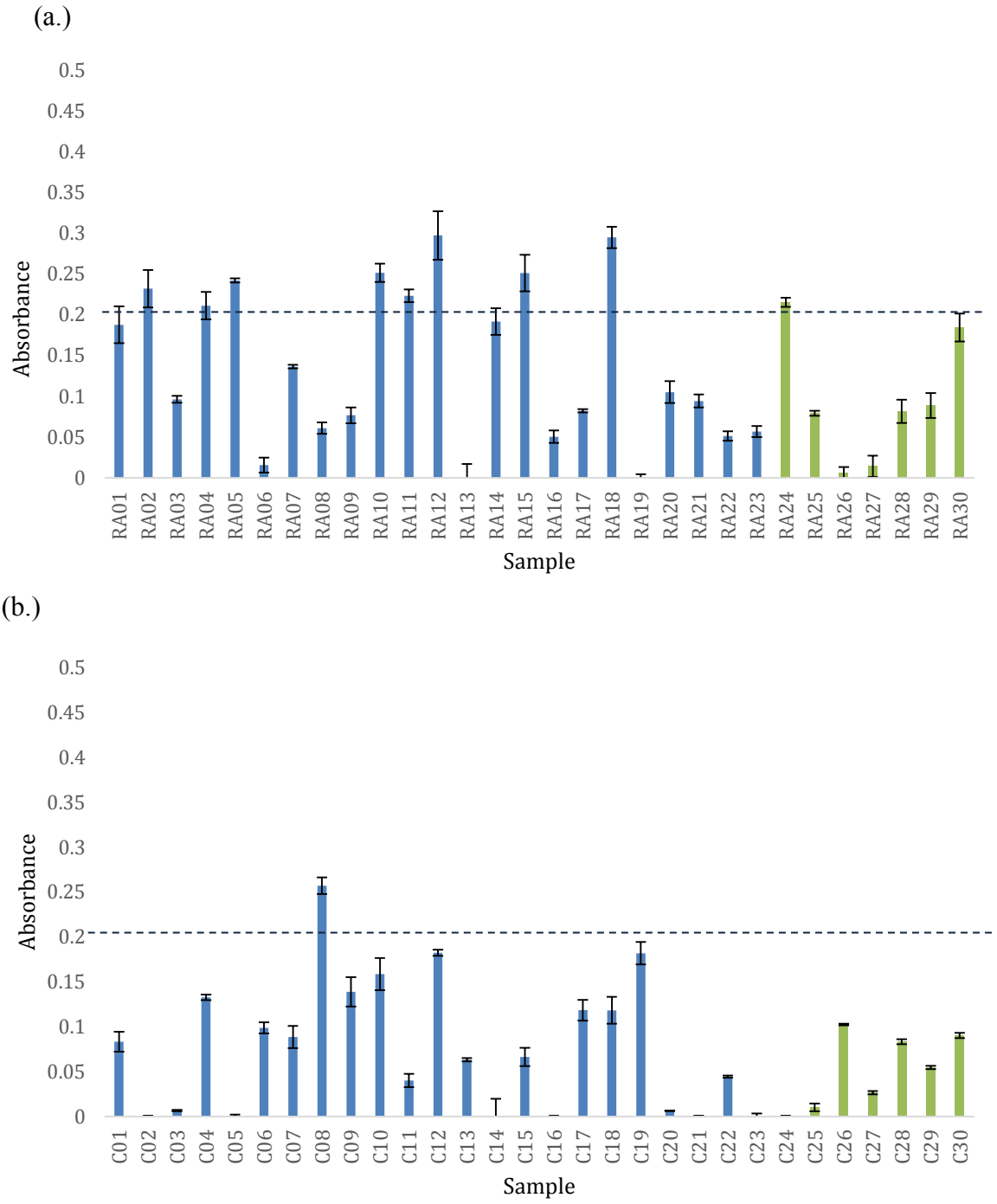
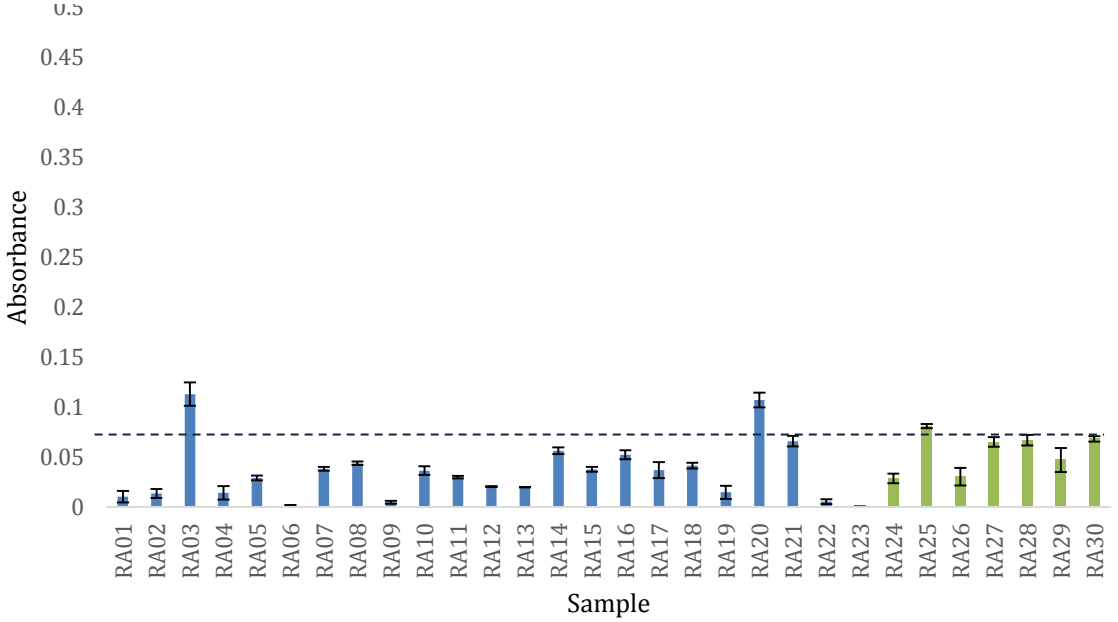


Figure 6-6 Bar graphs showing serum anti-pCRP IgG in both RA and control groups. (a.) RA group and (b.) control samples, auto-antibodies presented as Absorbance of sample. Data as determined by competitive ELISA (*n*=3), following sample dilution of 1:100 in triplicate. The dashed line represents the upper normal limit, those above are considered elevated, (≥ mean ± 2SD (0.205 OD)). The number of elevated RA group samples (n = 9) is significantly different from the control group (*n* = 1), $P < 0.01$ (X^2 analysis). Green columns = male, (C24-30; RA23- 30), blue = female.

Levels of IgA auto-antibodies directed towards pCRP (Figure 6.7) show the same number of patients with auto-antibodies detected within their serum, 28 of 30 (93%), as those seen in Figure 6.4a directed against mCRP. The mean absorbance value across the RA samples was 0.039 ±0.005 SEM. The recorded ODs of the RA group ranged between 0.113 (RA03) and the lowest positive 0.005 (RA09), and those of the control group between 0.08 (C13) and 0.001 (C26) with a mean of 0.019 ± SEM. There is no significant difference between the control and RA groups in respect of the number of high level samples (both having three), there is however a significant difference between the number of samples found to contain anti-pCRP IgA within the control group, 16 (53%), when compared to RA samples, $P < 0.01$ (X^2 analysis). There is also a significant difference between the numbers of positive control samples when compared to the number found within the anti-mCRP group, $P < 0.005$ (X^2 analysis), however there is no difference between the mCRP and pCRP RA groups which both have 28 of 30 samples containing anti-pCRP IgA.

(a.)



(b.)

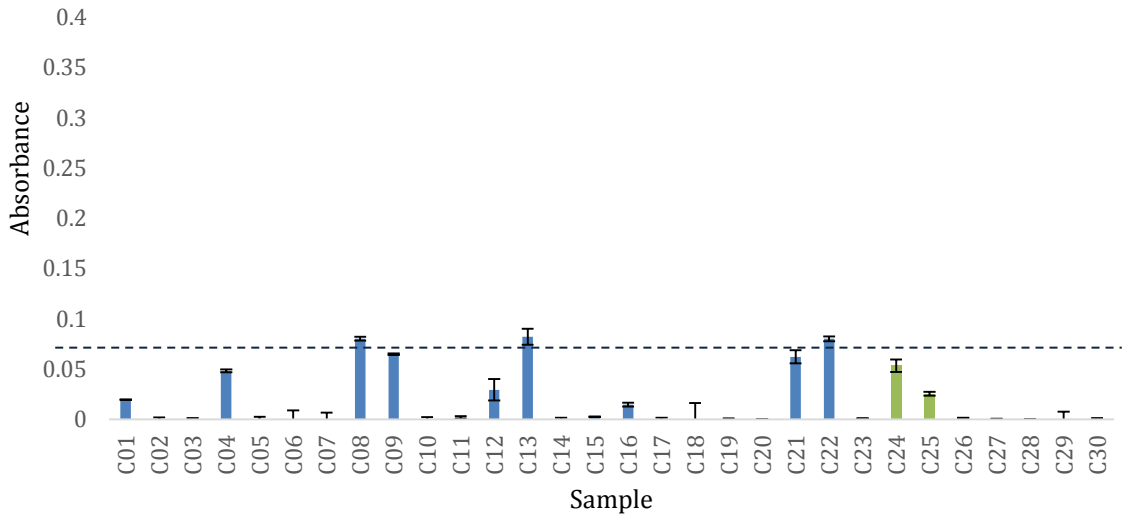
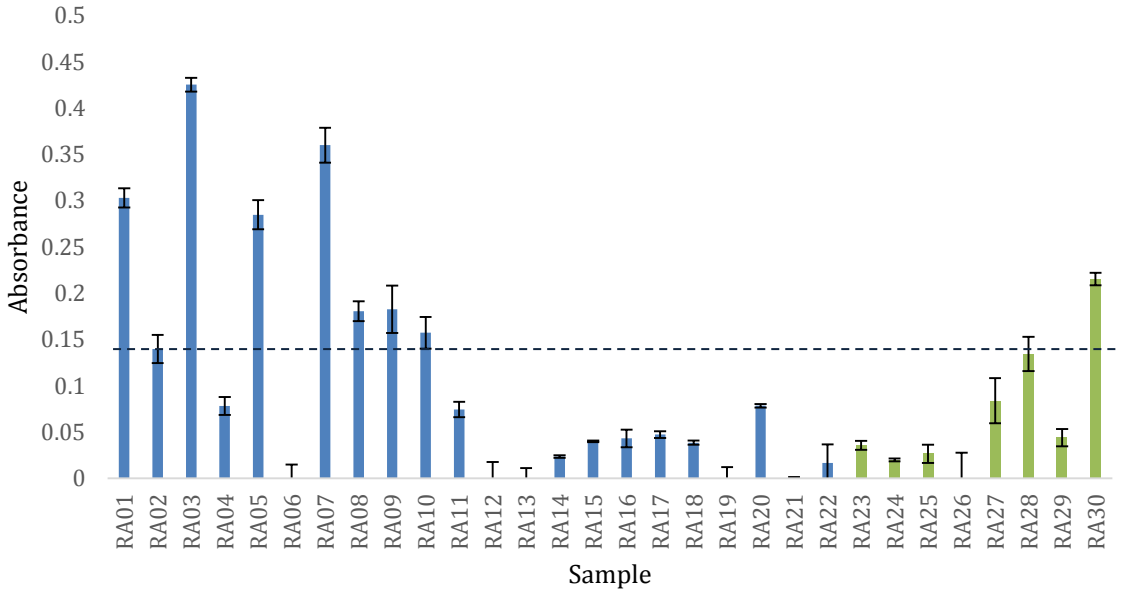


Figure 6-7 Bar graphs showing serum anti-pCRP IgA in both RA and control groups. (a.) RA group and (b.) control samples, auto-antibodies presented as absorbance of sample. Data as determined by competitive ELISA (n=3), following sample dilution of 1:100 in triplicate. The dashed line represents the upper normal limit, those above are considered elevated, (≥ mean ± 2SD (0.076 OD)). The number of RA group elevated auto-antibodies (n = 3) are not significantly different from the control group (n = 3), $P > 0.05$ (X^2 analysis). Green columns = male (C24-30; RA23- 30), blue = female.

Figures 6.8a and 6.8b illustrate the profile of IgM auto-antibodies directed against pentameric CRP. As can be seen in Figure 6.8a eight samples had elevated serum levels within the RA group above 0.137 (control mean +2SD), as opposed to three elevated within the control group (Figure 6.8b). Within the RA group a high proportion, 24 of the 30 samples (80%), were found to contain anti-pCRP IgM auto-antibodies, with a mean value across the samples of 0.101 ± 0.021 SEM. The absorbance values of positive samples ranged between the highest 0.425 (RA03) and the lowest positive of 0.016 (RA22). Figure 6.8b displays absorbance of control samples, within this group 16 of the 30 (53%), were found to contain detectable levels of auto-antibodies with a mean value across the group of 0.033 ± 0.01 SEM. The recorded absorbances of the group ranged between 0.1826 (C08) and the lowest 0.003 (C30), of the samples three were elevated with elevated levels exceeding the upper normal limit. There is no significant difference between the groups in respect of elevated samples ($P > 0.05$), however there is between the number of samples found to contain auto-antibodies within the control group when compared to RA samples, with 15 (50%) of control samples positive, $P < 0.01$ (X^2 analysis). There is no significant difference between the numbers of positive RA or control samples within the anti-pCRP IgM group when compared to those of the anti-mCRP group, $P > 0.05$ (X^2 analysis).

(a.)



(b.)

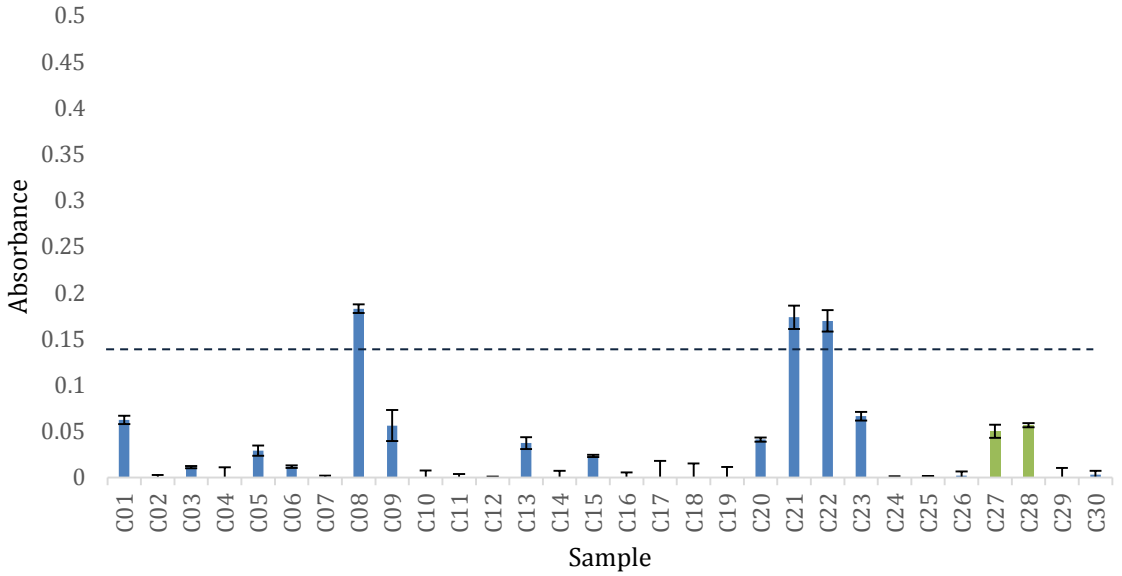


Figure 6-8 Bar graphs showing serum anti-pCRP IgM in both RA and control groups. (a.) RA group and (b.) control samples, auto-antibodies presented as % of known positive sample. Data as determined by competitive ELISA ($n = 3$), following sample dilution of 1:100 in triplicate. The dashed line represents the upper normal limit, those above are considered high, (≥ mean ± 2SD (0.137 OD)). The number of positive RA group samples ($n = 9$) is not significantly different from the control group ($n = 3$), $P > 0.05$ (X^2 analysis). Green columns = male, (C24-30; RA23- 30), blue = female.

6.3.3 Western Blot Analysis of Serum Samples

In order to determine the presence of auto-antibodies in RA patient samples that had previously been found positive by ELISA analysis, western blots were carried out. Native gels were run containing mCRP generated from 2M urea dissociation and also pCRP (Scripps). Patient samples were used as a primary antibody source in order to determine their capacity to recognise CRP isoforms. Anti-human IgA, IgM or IgG were applied as secondary antibodies. Control bands of mCRP and pCRP were also included on each gel for comparison and were blotted with both 3H12 anti-mCRP antibody and Clone 8. A representative set of samples chosen from the RA patient group are shown.

Figure 6.9 shows a typical western blot analysis of patient samples within the anti-pCRP IgG assays. Clear strong bands can be seen in each of the patient sample lanes. Lanes one and two contain duplicated control samples (mCRP and pCRP) with which the patients were compared against. The control image is separated from the patient samples as it was exposed for a shorter period of time (typically less than five minutes). The pentameric bands matched the 115kDa band position of the control samples. Figure 6.10 shows western blot analysis for patient anti-mCRP IgG analysis of serum auto-antibody content. Bands can be seen in lane one for the control samples at both 115kDa and 23kDa. Less well determined bands are apparent within the 23kDa monomer position, in lanes four, five and six, (RA22, RA15 and RA18).

Figure 6.11 shows a representative western blot of the anti-mCRP and anti-pCRP analysis combined on one gel, with nine samples and one control lane. Once again 23kDa and 115kDa bands can be seen in the control lane, with clear bands seen in the position of mCRP in lanes seven and nine (RA11 and RA17) with a further possible band in lane 4 (RA08). There is no visible band for anti-pCRP in any of the samples.

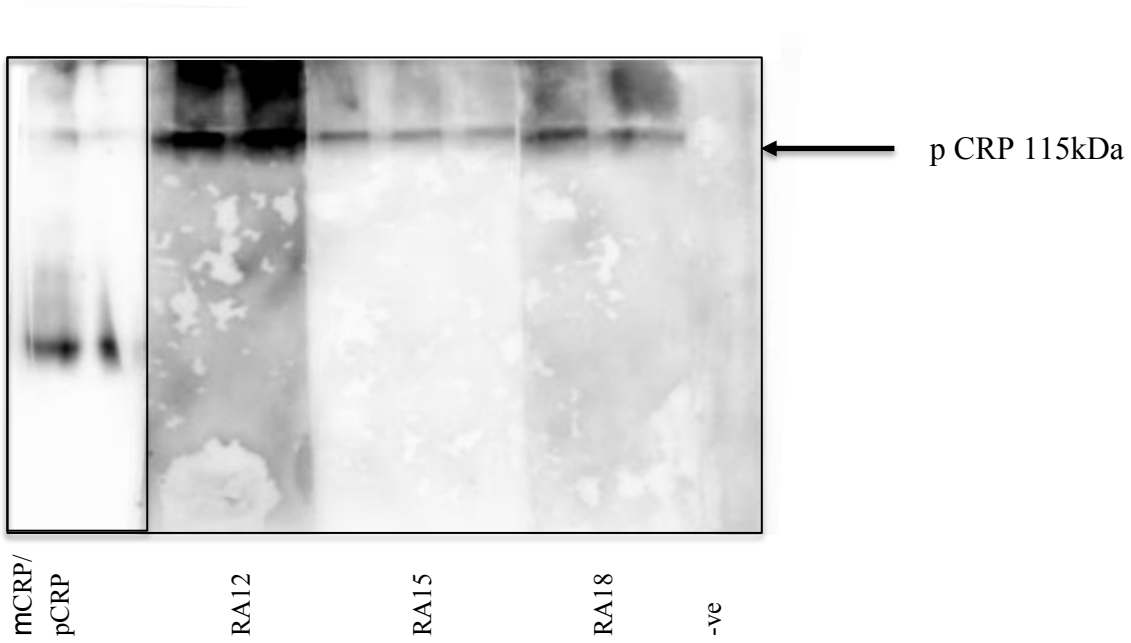


Figure 6-9 A representative western blot of anti-pCRP IgG auto-antibodies within patient samples, n=3. Lane 1 & 2: 1µg mCRP and pCRP, probed with mAb Clone 8 and 3H12; lanes 3-6 probed with patient sample serum RA12, RA15 and RA18, lane 10: negative control (no pCRP). Anti-pCRP detection is shown in all patient samples. 1µg pCRP was initially run within lanes 3-9 prior to probing with patient samples (diluted to 1:100).

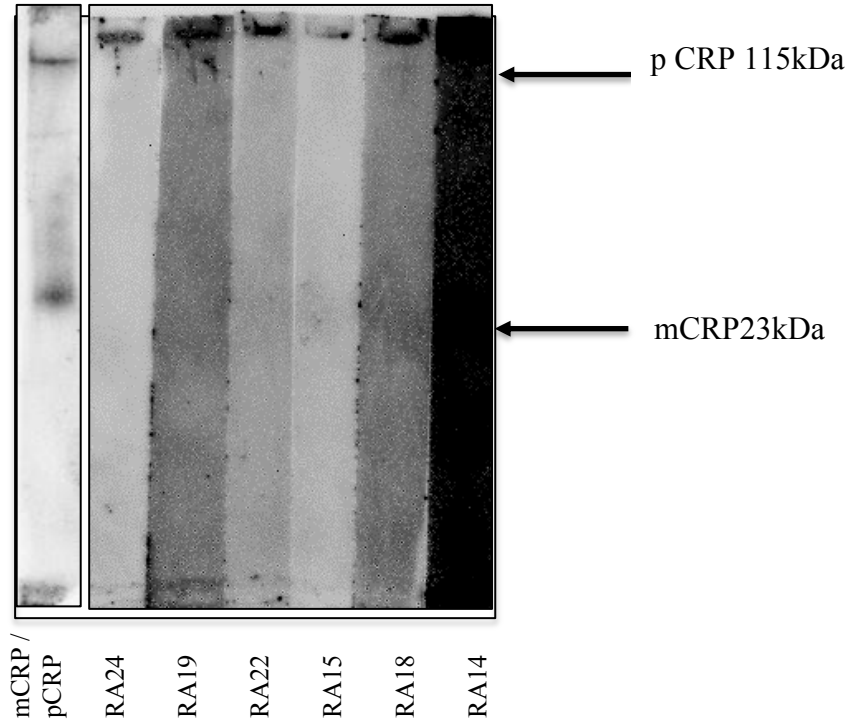


Figure 6-10 A representative western blot of anti-mCRP IgG auto-antibodies within patient samples, n=3. Lane 1: 1µg mCRP and pCRP, probed with mAb Clone 8 and 3H12, lane 2-7 probed with patient serum. Anti-mCRP detection is shown as a faint band in lanes 4, 5 & 6, samples RA22, RA15 and RA18. 1µg mCRP was initially run within lanes 2-7 prior to probing with patient samples (1:100 dilution).

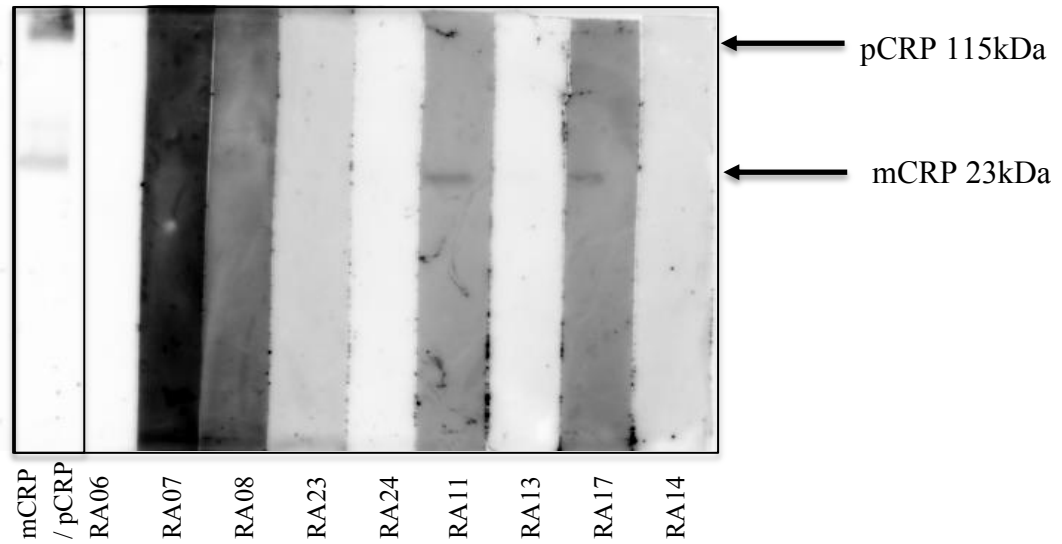


Figure 6-11 A representative western blot of anti-mCRP and anti-pCRP, IgA auto-antibodies within patient samples. Lane 1: 1µg mCRP and pCRP probed with mAb Clone 8 and 3H12, lane 2-10 probed with patient serum samples (1:100). Anti-mCRP detection is shown as a faint band in lanes 4, 7 & 9, samples RA08, RA11 and RA17, anti-pCRP is not visible in any lane. 1µg of mCRP and pCRP were initially run prior to probing with patient samples. *n*=3.

Lastly, Figure 6.12 which shows anti-mCRP and anti-pCRP IgM patient sample analysis. Lanes one to four contain patient samples with the control samples in lane 5 as describe previously. Bands can be seen located in the 23kDa mCRP position for each of the four patients, with band also visible in lanes two, three and four relative to pCRP. As is apparent with Figure 6.10, the bands within these two gels are not as clear as those within Figures 6.11 and 6.9; banding is not tightly confined or well resolved. It was not possible to develop them for longer as background interaction overcome the detection signal.

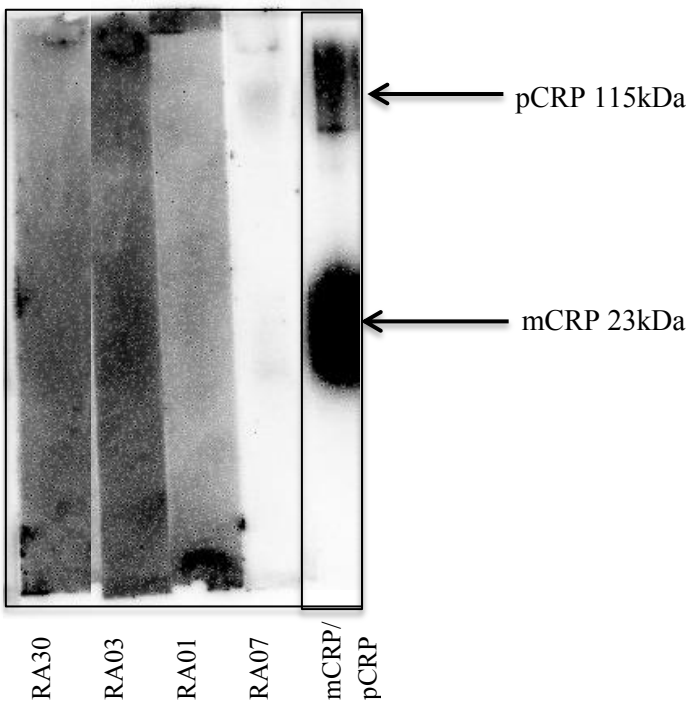


Figure 6-12 A representative western blot of anti-mCRP and anti-pCRP IgM auto-antibodies within patient samples. Lane 5: 1µg mCRP and pCRP, lane 1-4 patient samples RA30, RA03, RA01 and RA07 (1:100). mCRP detection is shown as a faint band in each lane, pCRP detection is shown by a faint band in RA3, RA01 and RA07. 1µg of mCRP and pCRP were intially run on the gel pre probing with patient samples, *n*=3.

6.3.4 Comparisons Between Anti-mCRP and Anti-pCRP Auto-antibody Levels

The difference between RA and control mean absorbance value for anti-IgA auto-antibodies directed towards mCRP is shown in Figure 6.13e and is more pronounced (0.103 ± 0.015 SEM vs 0.006 ± 0.003 SEM), than that found within all other groups. It is also apparent that the least difference between RA and control group mean absorbance can be observed within IgM auto-antibodies directed against mCRP (0.083 ± 0.016 SEM vs 0.053 ± 0.009 SEM) as seen in Figure 6.13f. Whilst Figures 6.3-6.8 illustrate individual auto-antibodies found within the sample of each subject, Figures 6.14 and 6.15 present the profile of all auto-antibodies detected per RA sample, directed against mCRP and pCRP respectively. These graphs illustrate the detectable level of antibody in each sample rather

than just those judged as elevated. No RA patients were identified as presenting negative for all three auto-antibodies, with at least half of samples found to have each of the three isotypes present (anti-pCRP 23 of 30, anti-mCRP 15 of 30).

When the profile of auto-antibodies is examined per subject within the control group samples, a different picture emerges. There were four samples that contained no detectable level of anti-mCRP auto-antibodies within their samples, C03, C07, C09 and C28 (Figure 6.16), and two samples within the anti-pCRP group samples C02 & C14 (Figure 6.17). A Venn diagram illustrating the distribution of auto-antibodies directed against either mCRP or pCRP in both control and RA groups is shown in Figure 6.18. Whilst the anti-mCRP detection assay showed eight RA samples contained the combination of IgM and IgA auto-antibodies (Figure 6.18b), the anti-pCRP detection assay revealed that no RA samples contained the same combination (Figure 6.18a), however the anti-pCRP assay included the most patient samples with all three isotypes present, 23 vs 15 within the anti-mCRP assay. The distribution of auto-antibodies across the control sample auto-antibody groups indicates there were more auto-antibodies present in the anti-pCRP group than that of the anti-mCRP group (Figures 6.18c and d), with a clear reduction in IgA auto-antibodies compared to the RA group. The anti-pCRP results showed a more balanced distribution across the classes. In each control assay group a greater number of samples contained a single antibody class (anti-pCRP = 8, anti-mCRP = 11) as seen in Figures 6.18c & d in comparison to RA sample group data (anti-pCRP = 4, anti-mCRP = 2) as seen in Figures 6.18a and b.

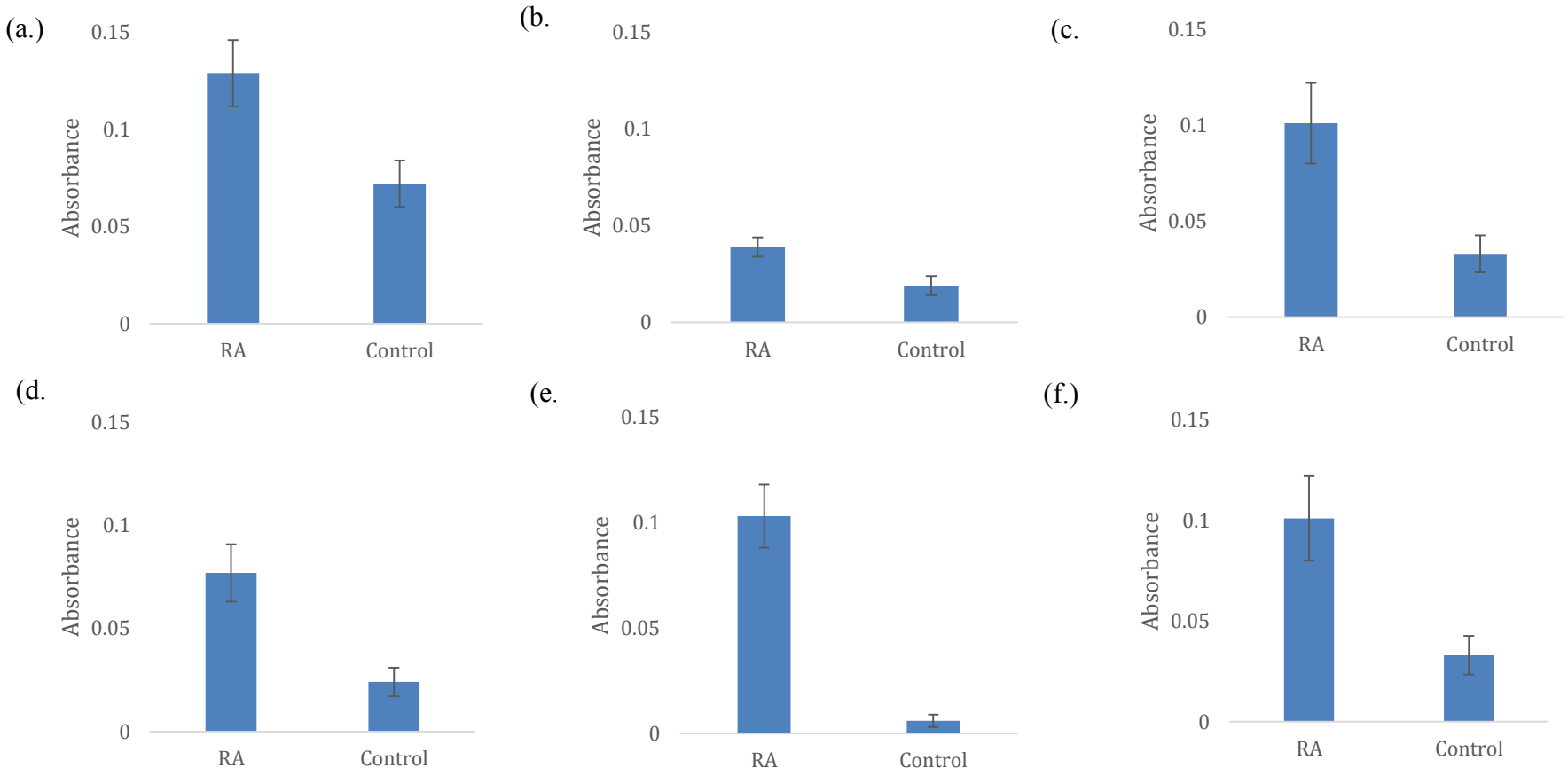


Figure 6-13 Bar graphs showing serum levels of anti-mCRP or pCRP in RA patient and control sample groups (± SEM) as determined by ELISA (*n*=3). 6.9 a-f, anti-pCRP IgG, anti-pCRP IgA, anti-pCRP IgM, anti-mCRP IgG, anti-mCRP IgA and anti-mCRP IgM respectively. A range of differences between the control and patient groups is evident with the greatest observed difference seen between the anti-IgA auto-antibodies directed against mCRP. Statistical analysis was not performed on the combined gender group mean data.

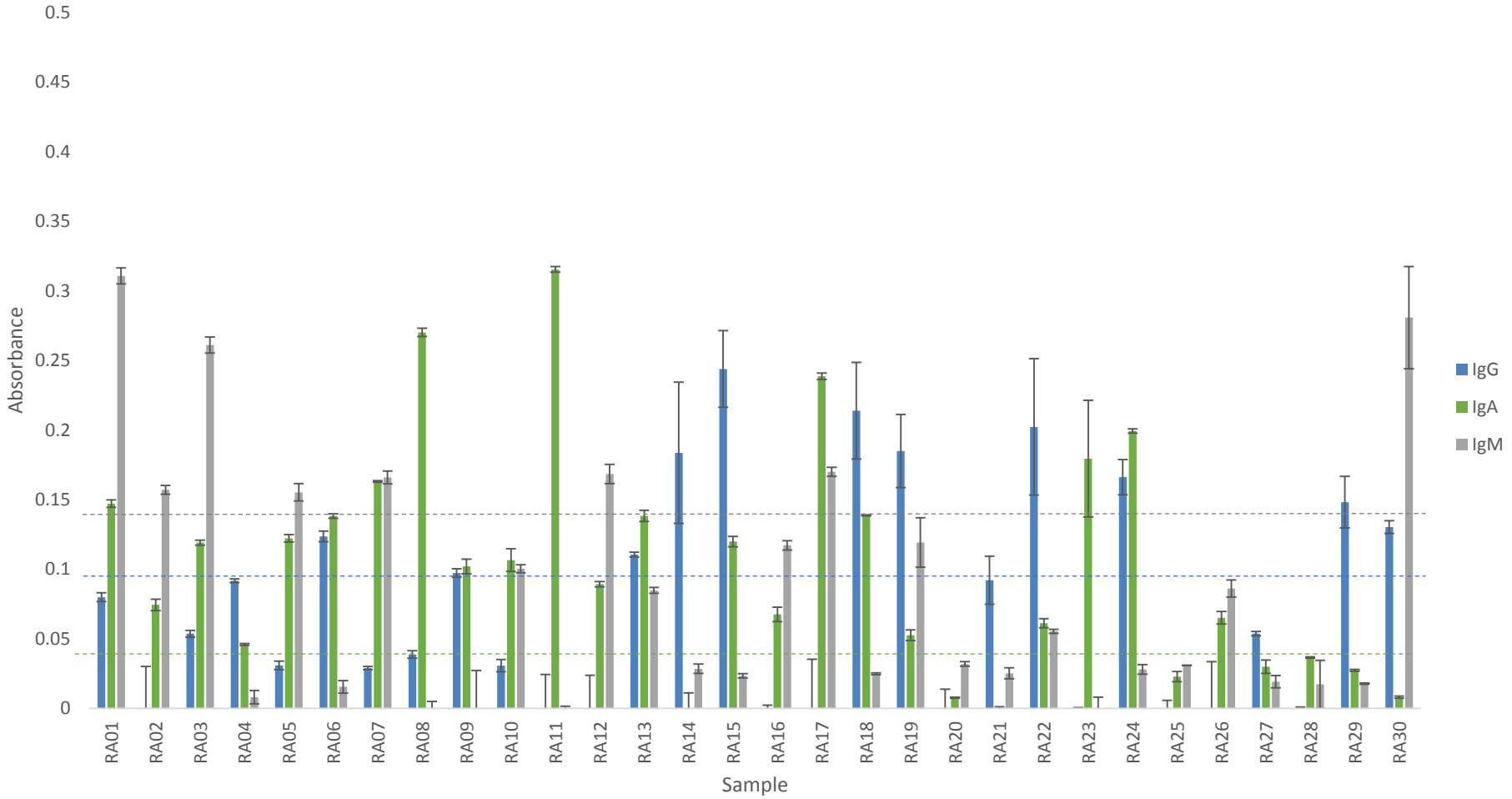


Figure 6-14 A bar graph illustrating levels of auto-antibody isotypes directed against mCRP present in individual RA patient samples, determined by competitive ELISA and expressed as absorbance ± SEM, (*n*=3). Dashed lines represent the upper threshold (mean ± 2SD normal control group), with samples above the line designated as elevated.

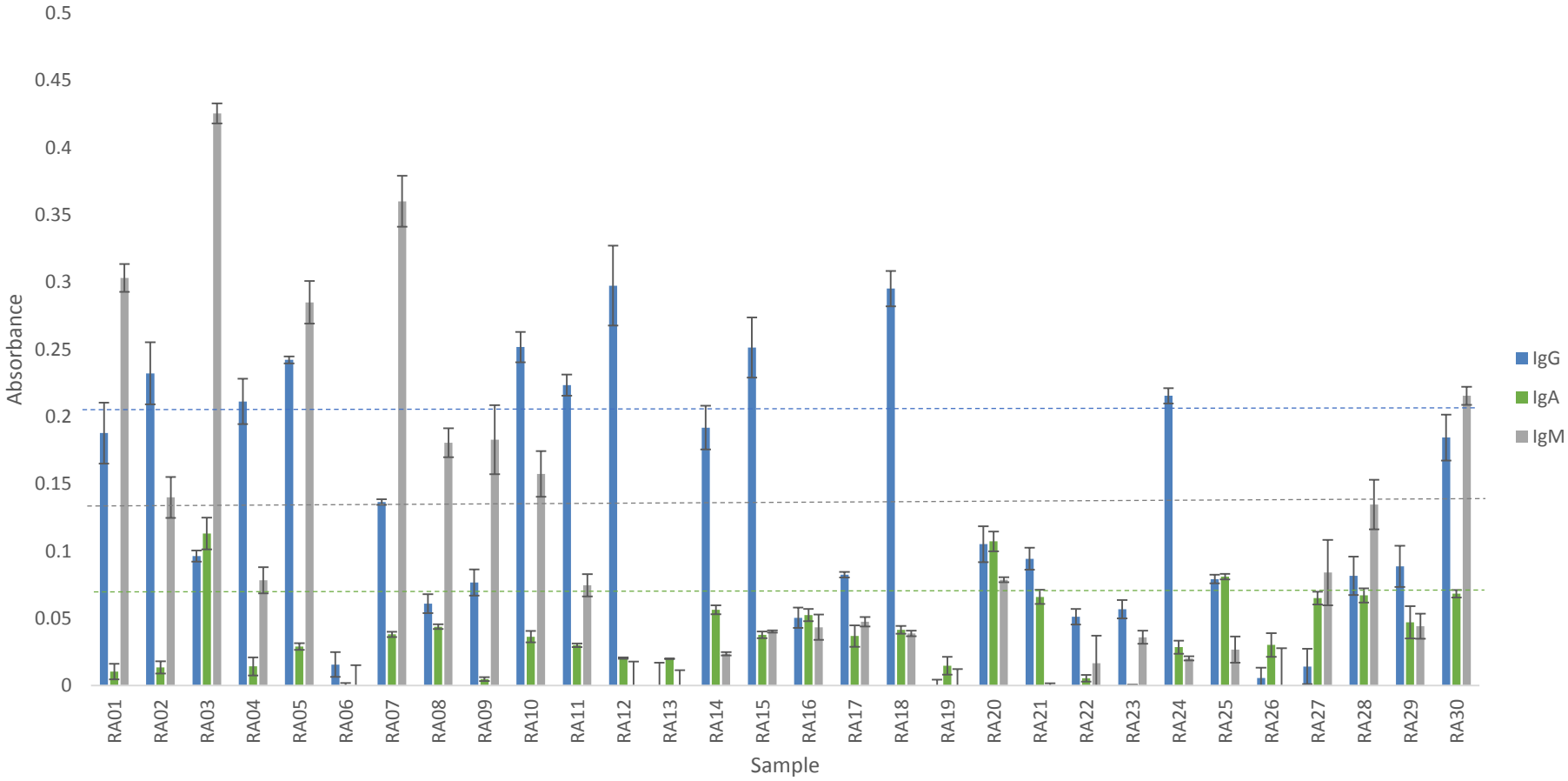


Figure 6-15 Bar graph illustrating normalised levels of auto-antibody isotypes directed against pCRP present in individual RA patient samples, determined by competitive ELISA and expressed as absorbance ± SEM, (*n*=3). Dashed lines represent the upper threshold (mean ± 2SD normal control group), with samples above the line designated as elevated.

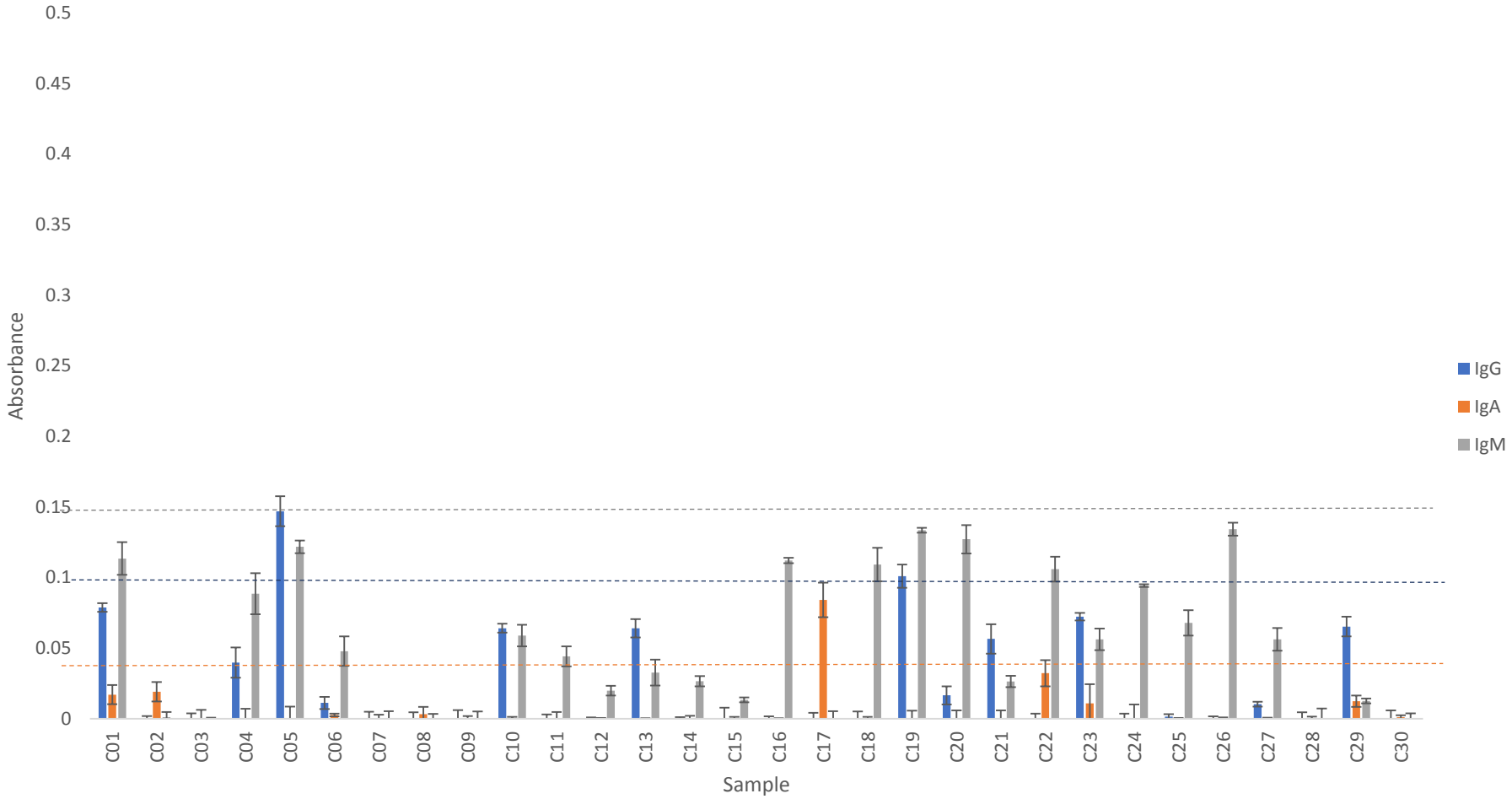


Figure 6-16 A bar graph illustrating normalised levels of auto-antibody isotypes directed against mCRP present in individual control patient samples, determined by competitive ELISA and expressed as absorbance ± SEM, (*n*=3). Dashed lines represent the upper threshold (mean ± 2SD normal control group), with samples above the line designated as elevated. There was no detectible level of any auto-antibody in four samples, C03, C07, C09 and C28.

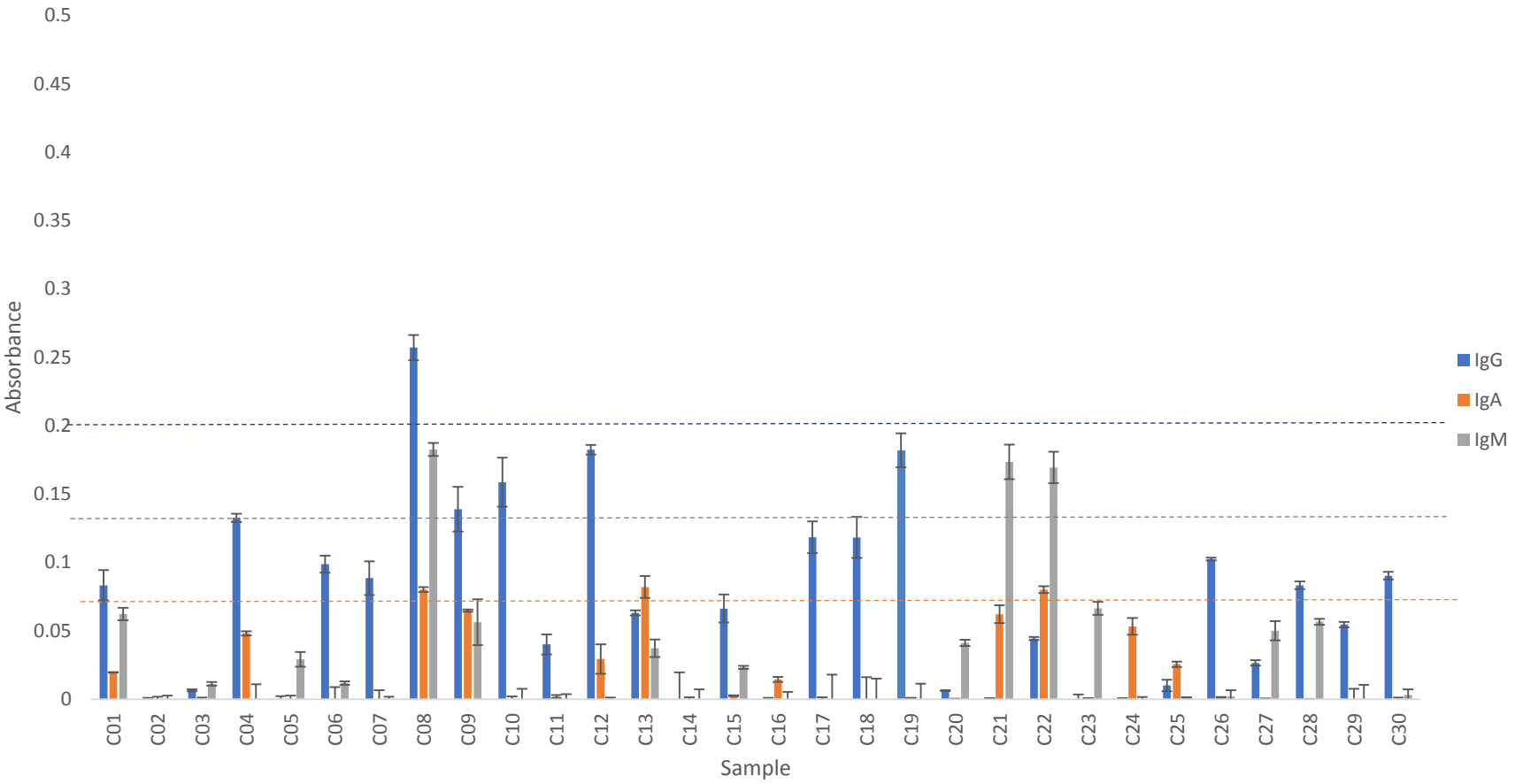


Figure 6-17 A bar graph illustrating normalised levels of auto-antibody isotypes directed against pCRP present in individual control patient samples determined by competitive ELISA and expressed as absorbance ± SEM, (*n*=3). Dashed lines represent the upper threshold (mean ± 2SD normal control group), with samples above the line designated as elevated. There was no detectible level of auto-antibodies in two samples, C02 and C14.

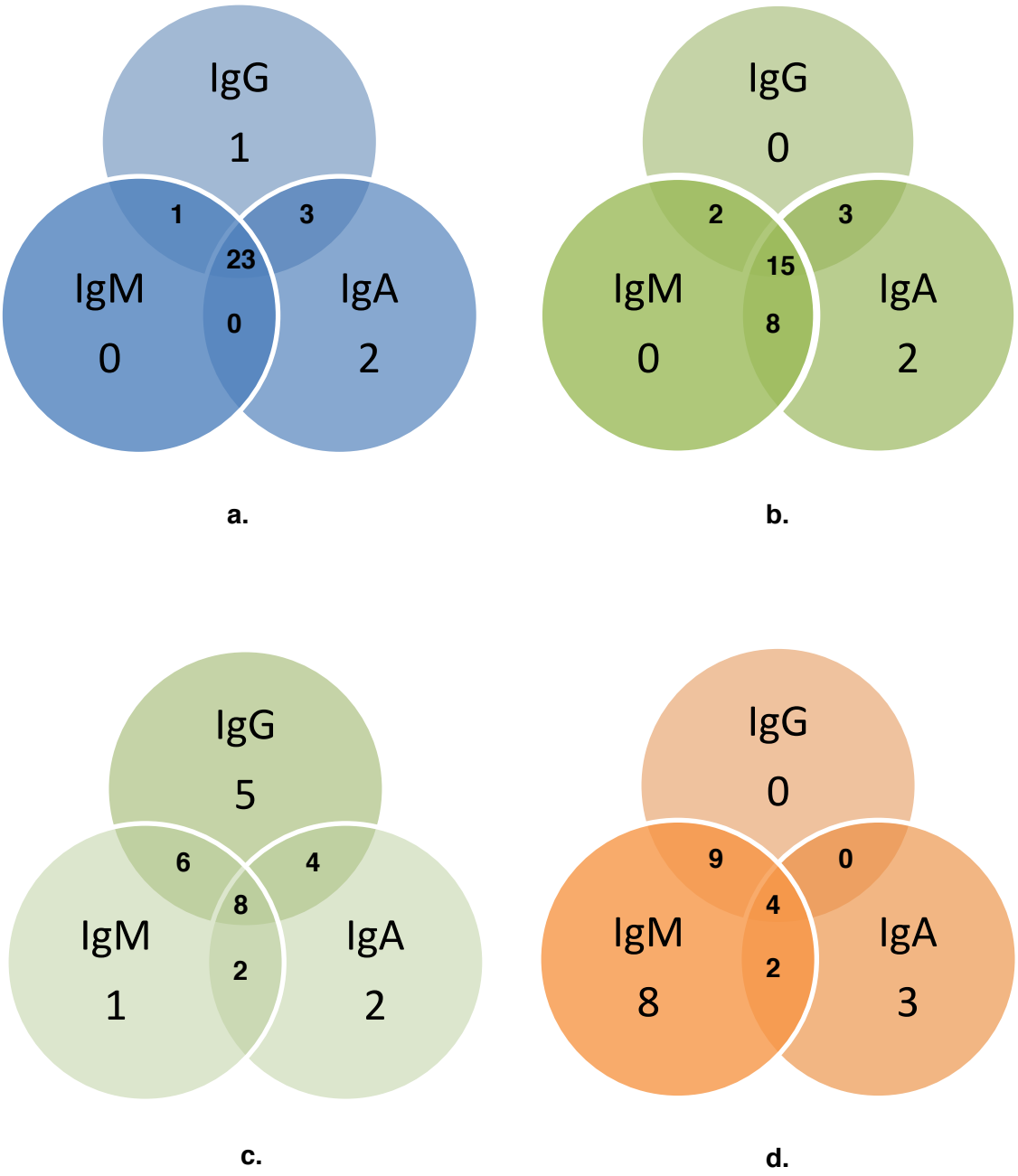
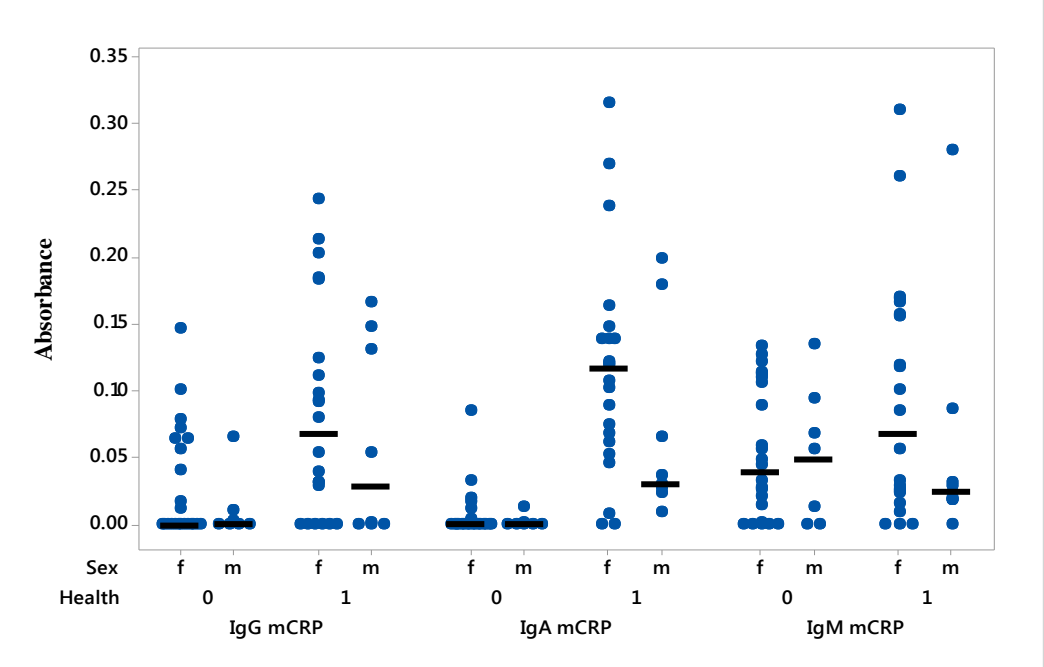


Figure 6-18 Venn diagram illustrating the isotype distribution of auto-antibodies within RA and control group samples as determined by competitive ELISA. Showing the frequency of samples with detectible auto-antibodies within the RA group a) anti-pCRP and b) anti-mCRP assays and the control group c) anti-pCRP, which included two samples with no detected auto-antibodies and d) anti-mCRP assays which included four samples with no detected auto-antibodies.

During initial statistical analysis it became apparent that the male cohort auto-antibody data was affecting analysis and in many cases the combination of male and female data sets produced bimodal data making detailed analysis based on comparison of mean or median group data unreliable, the serum level profiles can be seen in Figure 6.19 and Table 6.3. It was therefore necessary to subcategorise the data by gender into male and female groups before further detailed statistical analysis. The control group comprised of 7 males and 23 females and the patient group of 8 males and 22 females. Male groups were less likely to show statistically different patient values from those of their controls than the female groups due both to the low number of male samples and lower RA sample levels than those within the female cohort.

There was a significant difference between RA and control groups within the female subcategory in all but the anti-mCRP IgM assay, anti-pCRP IgG ($P < 0.01$), anti-pCRP IgA ($P < 0.01$), anti-pCRP IgM ($P < 0.001$), anti-mCRP IgG ($P < 0.01$) and anti-pCRP IgA ($P < 0.001$). Anti-mCRP IgM was not found to be significantly different from the control ($P < 0.05$). Within the male subgroup, anti-pCRP IgA and anti-mCRP IgA were observed to be significantly different from the control ($P < 0.002$, $P < 0.003$ respectively). Anti pCRP IgM, anti-pCRP IgG, anti-mCRP IgM and anti-mCRP IgG were not found to be significantly different from controls ($P > 0.05$). It is difficult to predict due to sample size whether these findings would be representative of a larger population size and no significance of these results can be assumed within the wider population.Table 6.3 illustrates the parameters of each group with data shown in terms of median (IQR) split by male and female group.

(a.)



(b)

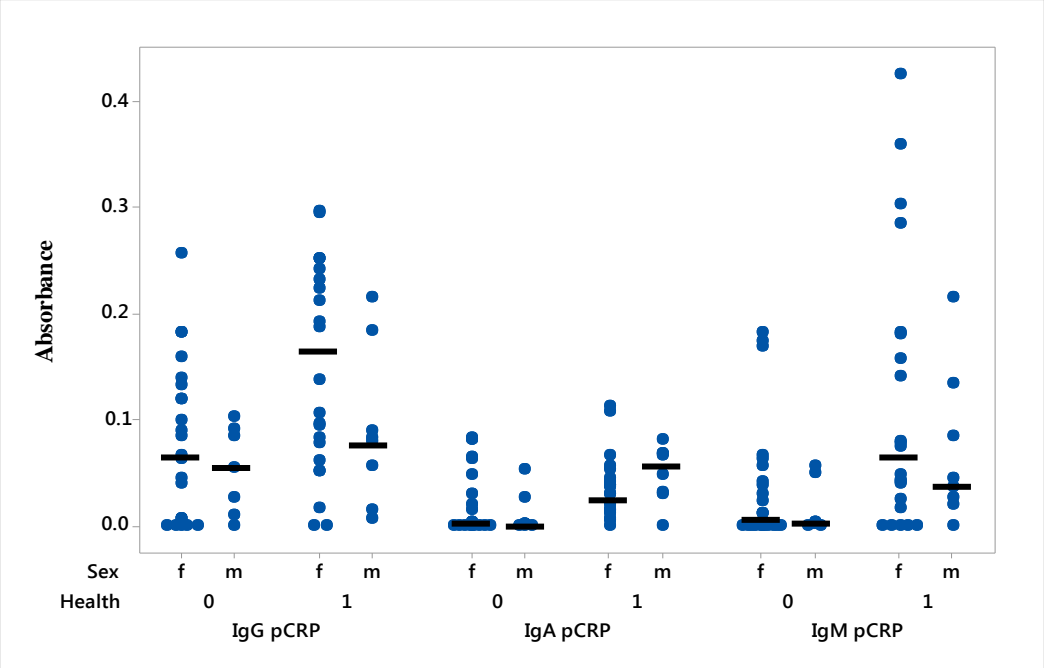


Figure 6-19 Graph illustrating the variation of median auto-antibody serum levels separated by gender. a) anti-mCRP and b) anti-pCRP auto-antibodies, separated by gender (m or f) and health (RA – 1, control – 0). The median is illustrated by horizontal bars and all data is expressed as Absorbance per auto-antibody isotype.

Table 6.3 Laboratory parameters (median IQR), for RA and control samples split by male (shaded) and female groups. *n* = 8 males, 22 females (RA group), 7 males, 23 females (control group).

Assay Group	RA	Control
Anti-mCRP IgG	0.067 (0.000-0.067)	0.000 (0.000-0.064)
Anti-mCRP IgM	0.070 (0.021-0.159)	0.044 (0.001-0.109)
Anti mCRP IgA	0.113 (0.059-0.141)	0.000 (0.000-0.003)
Anti-pCRP IgG	0.121 (0.058-0.235)	0.066 (0.000-0.133)
Anti-pCRP IgM	0.061 (0.012-0.181)	0.011 (0.000-0.056)
Anti-pCRP IgA	0.033 (0.014-0.046)	0.001 (0.000-0.048)
Anti-mCRP IgG	0.023 (0.00-0.144)	0.000 (0.000-0.010)
Anti-mCRP IgM	0.024 (0.017-0.072)	0.056 (0.000-0.090)
Anti mCRP IgA	0.033 (0.024-0.151)	0.000 (0.000-0.001)
Anti-pCRP IgG	0.080 (0.025-0.160)	0.055 (0.010-0.090)
Anti-pCRP IgM	0.040 (0.022-0.122)	0.000 (0.000-0.050)
Anti-pCRP IgA	0.056 (0.029-0.068)	0.000 (0.000-0.026)

There was little correlation between antibody isotype found within groups, with no interaction found between anti-mCRP IgG and either IgM or IgA in both male and female groups $p > 0.05$. Within the anti-pCRP auto-antibody groups there was also no intergroup correlation found $P > 0.05$, however a positive interaction was observed within the male group between anti-pCRP IgG and anti- mCRP IgG, $P < 0.022$, and negative correlations between both anti-pCRP IgM and anti mCRP IgM, $P < 0.027$ and anti-pCRP IgA and anti-mCRP IgA, $P < 0.007$.

No significant correlation was observed between either mCRP or pCRP and any auto-antibody with the exception of pCRP with both anti-mCRP IgA, $P < 0.021$ and pCRP IgA, $P < 0.021$ within the male group and mCRP against anti-pCRP IgG, $P < 0.02$ within the female group.

In order to address the question of whether age has an effect on health status and auto-antibody profile, the cohort of 30 was analysed by Spearman's Rank correlation as a combined gender group. An interesting correlation between age and auto-antibody concentration is evident within three of the 12 groups, those of anti-pCRP IgA and IgM within the RA group, both with negative correlations and also anti-pCRP IgM within the control group, a positive correlation ($P < 0.035$, $P < 0.002$ and $P < 0.02$ respectively). There were no other anti-pCRP vs age interactions within either RA or control group, nor were any anti-mCRP vs age groups considered to have a significant correlation. Although age and anti-mCRP IgA were not found to be significantly correlated in either the RA or control groups ($P > 0.05$), binary logistic regression indicated that there was a significant association between anti-mCRP IgA levels and disease. Tables 6.4 and 6.5 show details of Model 1, anti-mCRP IgA and health, and Model 2, anti-mCRP IgA, health and age ($P < 0.0001$), suggesting RA is significantly more likely when both age and anti-mCRP IgA levels increase.

Table 6.4 Estimated logistic regression model parameters for discriminating RA (n=22) from healthy female patients (n=23) based on (a) serum derived IgA-mCRP (model 1) and (b) serum derived IgA-CRP and age (model 2).

	Coefficient[a]	Odds ratio (95% CI)
a. Model 1		
Constant	-2.0370 ±0.6140	
IGA	0.1641 ±0.0495	1.1784 (1.0695, 1.2983)
b. Model 2		
Constant	-9.5800 ±3.2700	
IGA CRP	0.2086 ±0.0706	1.2319 (1.0727, 1.4148)
Age	0.1305 ±0.0500	1.1394 (1.0330, 1.2567)

[a] Mean of coefficient ± one standard error; sign indicates direction of relationship.

Table 6.5Assessment of the logistic regression model fit for health status (RA = 1, healthy = 0)

	Model 1 (IGA)	Model 2 (IGA + Age)
Significance of coefficients[a]		
G	34.57	45.65
df	1	2
p-value	0.001	0.001
Goodness of fit test		
Hosmer–Lemeshow[b]	4.66	3.47
df	6	8
p-value	0.588	0.902

[a] Test that all slopes (coefficients) are zero, significance level of 0.05, null hypothesis: slopes are equal

[b] Measure of how well health status is predicted by the model, significance level of 0.1, null hypothesis: model fits data.

6.4 Discussion

6.4.1 Overview

To our knowledge this study is the first that seeks to determine the presence of serum anti-mCRP and anti-pCRP IgA and IgM auto-antibodies in RA subjects, together with anti-pCRP IgG levels. The few studies that have considered anti-CRP auto-antibodies in RA have focussed on the detection of IgG antibodies directed towards mCRP and not pCRP (Minatani *et al*., 2001; Rosenau and Schur 2013). This chapter describes the presence of IgA, IgM and additionally IgG auto-antibodies directed against both mCRP and pCRP within RA serum. Our study successfully identified anti-mCRP and anti-pCRP auto-antibodies of all three isoforms within subjects of both the RA and control groups through development of a competitive ELISA. There were significantly higher levels of each auto-antibody present within RA patients compared to controls samples. It was established that anti-mCRP IgA auto-antibodies may play an important role in RA to the extent that anti-mCRP IgA was found to be a strong predictor of RA diseased state. Whilst the focus of the report considers the frequency of those samples found to be above the upper threshold for the normal control cohort, it is also interesting to consider the frequency of samples with a detectable level of auto-antibody present, as this also reveals some relevant findings between and within groups. Therefore, the discussion encompasses both levels of interpretation within its analysis.

6.4.2 Distribution of Auto-antibody Isotype within RA Patients

The first reported identification of auto-antibodies to CRP within serum detailed the finding within a single SLE patient (Robey *et al*., 1985). Ten years later a study on toxic oil syndrome, in which auto-antibodies directed towards CRP were again recorded,

highlighted the presence of auto-antibodies which only bound to CRP previously 'modified' by urea treatment or plastic bound, allowing inter-subunit epitopes to be exposed, the antibodies identified were class IgG (Bell *et al*., 1995). The same authors again identified anti-CRP auto-antibodies of the IgG class in 1998 (Bell *et al*., 1998), and once more identified the target of these antibodies as 'modified' CRP. These antibodies were found in 75.7% of SLE patients and 35.4% of healthy controls. Since these earlier studies many have sought to characterise anti-CRP auto-antibodies, of which all considered the IgG class immunoglobulin (Jakuszko *et al*., 2017, Li *et al*., 2017; Minatani *et al*., 2001; Sjowell & Wettero 2008; Sjowell *et al*., 2002,2003; Tan *et al*., 2008).

Our pilot study focused on a cohort of 30 RA patients consisting of 22 females and eight males, each confirmed sera positive for RA. All were under various ongoing drug regimens and many had lived with RA symptoms for many years. Data was analysed in two ways, either including all samples with a detectable presence of an auto-antibody whatever level that may have been, or those designated elevated (≥ mean +2SD normal control population). All RA patients were found to have the presence of at least one auto-antibody, with many showing the presence of all three, 23 of 30 patients within the anti-pCRP auto-antibody assays and 15 of 30 patients within the anti-mCRP auto-antibody assays contained detectable levels of IgG, IgA and IgM auto-antibodies. Within the anti-mCRP study 10 patients had elevated levels (≥ mean + 2SD normal controls) of IgG, 22 of IgA and eight of IgM. Within the anti-pCRP study nine patients were higher level for IgG, three for IgA and eight elevated for IgM. Analysis revealed significant differences between the frequency of patients with measurable presence of anti-mCRP and anti-pCRP auto-antibodies vs control subjects, with anti-mCRP IgA , anti-pCRP IgA and anti-pCRP IgG all having 28 of 30 samples with detectable antibody amounts. Perhaps surprisingly, in the anti-mCRP assay IgM and IgG isoforms were not the dominant class, with elevated

positive levels of IgA in 22 of 30 RA samples. This was found to be different within the anti-pCRP assays in which IgG had the greatest number of positive samples (nine), with IgM second with eight. Anti-pCRP IgA was the least likely auto-antibody to be positive within RA patient samples with just three samples elevated above the determined normal level.

With respect to anti-CRP IgG auto-antibodies, these were found to be present within subjects of both the RA and the control groups. Following separation of the initial group into gender subsets, analysis of female levels of anti-mCRP IgG revealed a significantly higher level of auto-antibodies directed against mCRP in the RA group when compared to the control group, $P < 0.018$. However this was not reflected within the male RA group samples which remained similar in value to that of the controls $P > 0.325$. A similar picture emerged within anti-pCRP IgG results in which the female group once again showed significantly higher levels of anti-pCRP IgG than the control group ($P < 0.02$), whilst the male group was again not significantly different ($P > 0.524$). This shows that significantly higher anti-mCRP and pCRP IgG auto-antibodies were found in RA rather than control subjects in the female group. When each auto-antibody class was directly compared between male and females, although female levels of auto-antibody were higher than their male counterpart in all auto-antibody groups within both RA and control subjects, with the exception of RA group anti-pCRP IgA, these differences were not significant. The trend apparent within these results supports the findings of Nagele *et al*., (2013) who also observed significantly higher levels of auto-antibodies within female subjects ($P = 0.004$), although these were only assessed within the control group, ($n = 18$ female, $n = 39$ male). The low number of male subjects within this study however suggests these results can not be regarded as relevant within a larger population unless further explored with a larger sample group.

Studies have indicated that the prevalence of RA is higher within females than males and a 2006 study by Kvien *et al* was also able to determine the incidence by age group. RA was four to five times more likely to occur within females than males below the age of 50, but this fell to a ratio of only two to one as the population age rose to between ages 60-70. The determination of higher levels of auto-antibodies within the female population may in part be responsible for the observed increased prevalence of RA in women. Smoking is also determined to be a predictor of RA in males but not consistently in females, suggesting further genetic influences on disease (Nagele *et al*., 2013) However disease severity was found to be significantly worse in female RA patients who had ever smoked than those who had not (Mattey *et al*., 2002a, 2002b).

Previous studies have focused detection methods on the 'modified' rather than native form of CRP as a target antigen, with identification of the CRP isoform determined by ELISA. Groups have bound CRP directly to a polystyrene plate, leading to conformational change and production of mCRP, or have used CRP that had previously been treated with urea in order to dissociate it (Jakuszko *et al*., 2017, Li *et al*., 2017; Minatani *et al*., 2001; Sjowall & Wettero 2007, Sjowall *et al*., 2002,2003; Tan *et al*., 2008). Therefore the presence of specifically anti-pCRP auto-antibodies, rather than those with dual antigenicity, may have been missed due to assay design. Auto-antibody studies within SLE patient groups (Jakuszko *et al*., 2017, Li *et al*., 2017; Minatani *et al*., 2001; Sjowall & Wettero 2007; Sjowall *et al*., 2002, 2003; Tan *et al*., 2008), have also focused upon IgG auto-antibodies directed towards monomeric and not pentameric CRP, as have those involving other diseases such as acute coronary syndrome (Wettero *et al*., 2009) and systemic autoimmune diseases including primary biliary cirrhosis, chronic graft-versus-host disease, systemic scleroderma and even toxic oil syndrome (Bell *et al*., 1995,1998). These assays have included inhibition studies eliminating the effect of pCRP auto-

antibodies, although they have only tested against immobilised mCRP rather than pCRP, suggesting any pCRP directed auto-antibodies would not have been identified. In these studies pentameric CRP was bound to the plate and left overnight to incubate and dissociate, serum was preincubated with urea treated mCRP and then incubated on half of the plate, with the other incubated with serum alone. The difference between the two values represented the inhibition by mCRP and therefore auto-antibodies present within the serum directed against it. However, as monomer only was used as a capture antibody, it was not possible to judge the presence or absence of anti-pCRP IgG. It was also not possible to tell how many of these IgG auto-antibodies may have contained dual recognition properties.

Our study showed 66% of RA patient serum samples contained anti-mCRP IgG auto-antibodies as did 43% of control subjects, this was higher than the study conducted by Rosenau and Schur, (2006), in which they found 22.3% of RA and 23% of SLE patients were positive for anti-CRP IgG. However these samples were judged positive when levels were equal or above mean OD ± 2SD for their healthy individual cohort; when our data is compared on a similar basis (≥mean + 2SD normal control population), 10 of 30 (33.33%) of RA samples were determined to be elevated above normal levels and one of 30 (3.33%) of control samples showing a significant difference between groups ($P < 0.003$ X^2 analysis). Further differences may be due to differences in methodology, a competitive ELISA was not used within their study and interactions between BSA and patient serum may have resulted in a lower frequency of positive patients. The differences within study designs may account for the differences found within the results of our study and those of previous. A second study by Minatani *et al*., (2000) showed 29% of RA patients were positive for anti-mCRP IgG, which is in closer alignment to results within our study. Levels of antibodies within healthy controls were unreported within Rosenau's study but

two of 49 healthy control samples (4%) were elevated within the work of Minatani and colleagues.

The number of RA high level samples for anti-pCRP IgG auto-antibodies is significantly higher than that of the control group ($P < 0.01$ X^2 analysis), illustrating a close association with disease. As existing studies have not sought to identify IgG auto-antibodies directed against pCRP, it is not possible to judge equivalent levels to ours, although 30% of our RA samples were elevated for anti-pCRP (≥ mean + 2SD normal controls), which is in broad alignment with the frequency of positive anti-mCRP IgG samples in ours and previous mentioned studies. Antibodies directed against pCRP were similarly lower in the control group with only one sample elevated (3.33%). Whilst our results indicated a significantly higher levels of auto-antibodies within RA patients than controls, Nagele *et al*., (2013) reported a general decrease in IgG auto-antibodies against their age matched controls within certain diseases; Alzheimer's disease, Parkinson's disease and multiple sclerosis all reporting a statistically significant decrease in the number of auto-antibodies against control. It is clear that the indication of high levels of IgG auto-antibodies may not always be considered detrimental to health. This is shown in the contrast between levels of anti-mCRP IgG within both RA and SLE, both are autoimmune diseases but SLE known for its high anti-CRP auto-antibodies and low CRP levels whereas RA is characterised by the opposite profile.

The significantly higher frequency of elevated IgG auto-antibody samples was quite different from the number of samples with detectable levels, of which little difference was observed between RA group 93%, and control group 77%. When mean levels of anti-mCRP IgG and anti-pCRP IgG auto-antibodies were compared there was no significant difference found ($P > 0.159$ female, $P > 0.318$ male). This suggests that although mean and median levels of anti-pCRP IgG were higher than anti-mCRP IgG in both male and female

groups, patients were not significantly more likely to have auto-antibodies directed towards one form of CRP at the expense of another. This may suggest that should an individual lose self-tolerance to one form of CRP it is likely to be a matched reaction to the other. If antigen presentation of mCRP leads to dual antigenicity and additional self-recognition of pCRP, it may be due to recognition of an epitope on the surface of the mCRP subunit, rather than one normally buried between subunits. If the epitope recognised by the immune system is located within the inter-subunit site, normally hidden, then recognition of the monomeric form only will occur. Specific antibodies to pCRP alone are suggested to recognise antigenic areas that bridge two subunits, thus not present in mCRP. Therefore should self-recognition occur with an epitope shared by both isoforms of CRP, then both would be vulnerable to immune system eradication. This may result in differences within disease profile dependent on the levels of mCRP and pCRP present within serum or joint, and also whether one or both isoforms are found to present pro-inflammatory rather than anti-inflammatory roles. It is entirely possible that one is protective and the other not, thus the ratio of each within serum may be an important consideration within disease diagnosis and progression. If mCRP is determined to be pro-inflammatory and pCRP anti-inflammatory as some studies suggest (Fujita, 2014; Khreiss, 2004; Molins, 2016; Thiele, 2015; Trial, 2016; Zouki, 2001), should mCRP be recognised and targeted by the immune system, pCRP could undergo the same fate. This would interrupt clearance of apoptotic cells and immune debris, possibly leading to the initiation of necrosis pathways and increased local and systemic autoreactivity. However our results showed no correlation between levels of mCPR and auto-antibody levels, nor between pCRP and either IgM or IgG, which suggests an alternative reason for similar levels between groups. Molecular mimicry may play a role, whereby a foreign antigen shares structural similarities or a sequence with a self-antigen (Cusick *et al*., 2012), therefore an as yet undetermined protein

may activate an immune response in which both mCRP and pCRP are caught up as innocent bystanders.

Where mean levels of detected anti-mCRP IgG differed in statistical significance across male and female groups, this was not found to be the case within anti-mCRP IgA. Once again females had significantly higher % auto-antibodies within RA group samples when compared to control samples ($P < 0.0001$), however the male group also exhibited a similar difference ($P < 0.002$). Upon analysis of anti-pCRP IgA mean detected levels, once more the number of samples with detectable levels in both male ($P < 0.033$) and female ($P < 0.02$) were significantly higher within the RA patient group than the control samples. When RA detected mean levels of anti-mCRP and anti-pCRP IgA were considered and compared, no significant difference between them was found ($P = 0.385$ female, $P = 0.123$ male), however there were significantly more elevated samples within the anti-mCRP than anti-pCRP group ($P > 0.001$ X^2 analysis). This implies that whilst the overall amount of samples found to have anti-mCRP or pCRP IgA were the same (28 or 30), the control groups mean levels varied greatly leading to a low normal threshold within the mCRP group. It is likely that as with IgG auto-antibodies, a possible level of dual antigenicity occurs as a similar number of patients appear to have antigenicity towards both CRP forms. Within RA, anti-citrullinated protein antibodies (ACPA) and rheumatoid factor (RF) are predominantly IgM or IgG isotype with little evidence to suggest IgA plays a diagnostic role. However studies have shown IgA auto-antibodies do seem to hold diagnostic value and our data agrees with this finding (Sieghart *et al*., 2016).Our data also suggests that IgA auto-antibodies may have a diagnostic value due not only to the significant difference between positive RA samples of anti-mCRP IgA when compared to controls, but also when associated with age, as a strong predictor of disease.

Analysis and comparison between RA and control group anti-mCRP IgM auto-antibodies revealed no significant difference between detected levels in either the male $P > 0.524$ or female $P > 0.204$ groups, although the male mean and median are actually higher within the control group than that of the RA. This was a surprise as IgM auto-antibodies are a feature of RA and therefore of the antibodies tested these were perhaps the class considered most likely to be present in RA serum. When anti-pCRP auto-antibodies are considered, the female RA group is significantly higher than the control ($P < 0.03$), whereas no significant difference was observed between male RA and control group levels of anti-pCRP IgM ($P > 0.118$). Again differences in gender are apparent; however the size of the male group prevents significant analysis of these findings.

Upon comparison of RA group anti-mCRP and anti-pCRP IgM auto-antibody levels, no significant difference was found ($P > 0.664$ female, $P > 0.270$ male), indicating that as with the IgA and IgG auto-antibody investigations, there was no apparent likelihood of auto-antibodies being more prevalent against mCRP or pCRP, or for one to influence the levels of the other. When the frequency of RA samples with elevated levels of anti-mCRP IgM in the combined gender group were compared to those within the control group, a significant difference was found, with a greater number of elevated RA patients (eight), than elevated control subjects (zero), $P < 0.01$, X^2 analysis. No significant difference was found between elevated patient and control samples in the anti-pCRP IgM detection assays, RA patients (eight), and control (three), $P > 0.05$.

IgM antibodies are not only the first class of antibody produced during a primary antibody response, but they predominantly produced by a subset of B cells, the long lived B1 cell, without necessary specific antigen stimulation. They also fulfil the role of ‘natural antibody’ present in the serum of healthy subjects prior to any infection (Boes, 2000). These natural antibodies are polyreactive to conserved structures such as heat shock

protein, carbohydrates, phospholipids and nucleic acids (Boes, 2000). It is possible that as a phylogenetically conserved protein, CRP may also be recognised by IgM natural antibodies. IgM plays a role in the enhancement of IgG response to foreign antigens and has been implicated in development of IgG autoreactivity, (Cornall *et al*., 1995). The binding of IgM to a self-antigen, such as mCRP or pCRP, could result in the activation of complement and formation of an immune complex (antigen-IgM-C) which may then go on to stimulate an IgG antibody response; however in this case the antibodies would be primed against a self-antigen, CRP, rather than a foreign one.

Our study also examined the presence of IgA anti-CRP auto-antibodies and results demonstrate that levels directed against both mCRP and pCRP are also significantly higher in RA patients than in normal control subjects. IgA class auto-antibodies are prevalent within RA and although they are not used within diagnosis in the same manner as IgG and IgM auto-antibodies are, a recent study by Sieghart *et al*., (2017) showed anti-RF IgA were found within 44.7% of tested patients with 31% also positive for anti-ACPA IgA auto-antibodies. Altogether 49% of 255 patients had at least one type of IgA auto-antibody (Sieghart *et al*., 2017). In their later study 50.68% of RA patients were identified as positive for IgA-RF (Sieghart *et al*., 2018). An early study showed elevated anti-cytokeratine-18 IgA was found (± mean plus 2SD of healthy controls) in 39.6% of RA patient sera compared to 1.4% of healthy controls. Levels of anti-epidermal keratin IgA were also raised in 20.75% of the RA patients and 4.5% of healthy controls (Borg *et al*., 1993). These levels although directed against different self-proteins, are considerably lower than the 73.33% of RA patients in our study who were found to have elevated levels of anti-mCRP IgA elevated above the normal upper limit (± mean plus 2SD of healthy controls), although in line with the frequency of positive elevated control samples (3.33%).

The results were quite different within the anti-pCRP IgA assay in which both RA and control groups had the same frequency of samples elevated above the normal range, three subjects (10%), considerably lower than both anti-mCRP IgA levels and the previously quoted studies. The reason this cohort contained a greater frequency of elevated anti-mCRP rather than pCRP IgA is not clear at present, it may be due to the very low levels observed within the anti-mCRP control group, resulting in a low upper threshold for samples to be considered high. The frequency of elevated samples differed between anti-mCRP and anti-pCRP assays, however the number of samples with detectable levels of IgA was the same with mean levels not found to be significantly different (Male $P > 0.128$; Female $P > 0.385$). Whilst some differences between IgG and IgA auto-antibodies are apparent, what was clear is that within the combined gender RA group anti-mCRP IgA levels show a significant positive correlation to mCRP levels ($P < 0.035$, Spearman's Rank correlation), whereas no such correlation was observed between anti-pCRP IgA levels and pCRP ($P > 0.087$). The origin of these antibodies is also not known (i.e. mucosal or circulatory), although it is possible they may be produced in response to mucosal compartment stimulation. Antigenic stimulation of the mucosal immune system can result in the appearance of IgA auto-antibodies within peripheral blood (Olas *et al*., 2005). Where secretory IgA is generally considered to be a non-inflammatory antibody due to the fact it does not trigger inflammatory processes upon antigen binding, serum IgA and also serum IgG have been shown to downregulate the release of the anti-inflammatory cytokines IL-10 and IL-12p40 from LPS-activated monocytes and peripheral blood monocytic cells (PBMC). This suggests that raised levels of serum IgA may lead to an enhanced inflammatory response (Olas *et al*., 2005). However in a study using LPS activated monocytes and PBMC, it was shown that IgA also had the effect of down regulating chemokine MCP1, MIP1α and MIP1β release in LPS-activated monocytes as well as

peripheral blood monocytic cells (PBMC). These chemokines are essential within an acute inflammatory and effective innate immune response, by stimulating cell activation and migration of neutrophils and basophils, therefore leading to induction of pro-inflammatory IL-6, TNF-α and IL-1 synthesis (Olas *et al*., 2005). This suggests that IgA auto-antibodies play a dual and possibly modulatory role within the normal immune response and therefore whilst elevated levels may provide key diagnostic information, the actual disease outcome of these auto-antibodies is unclear.

This interplay between immunoglobin classes may be responsible for CRP autoantigenicity within RA rather than control samples. It is evident from our results that RA is associated with elevated IgG, IgA and IgM antibody production. When considered individually the frequency of isolated auto-antibodies within samples is interesting, however the profile of multiple auto-antibodies within each patient and control subject is especially relevant. Figures 6.14 to 6.18 illustrate the combination of immunoglobulin classes evident within control and patient samples, with a far higher proportion of RA patients experiencing a combination of auto-antibodies. Within the RA group anti-pCPR investigations 23 patients had the presence of all three antibody classes within their serum, compared to only 15 of the anti-mCRP patients. Anti-mCRP patients had significantly higher individuals with a combination of with both IgM and IgA auto-antibodies present (eight subjects), whilst the anti-pCRP assay revealed no individuals with this combination, which was found to be significantly different ($P < 0.03$). The frequency of individuals with all three classes of auto-antibodies in the control group were lower in both mCRP (four compared to 15 RA, $P < 0.005$) and pCRP assays (15 compared to 23 RA).

It is possible that the diseased state and microenvironment of inflamed joints is in part responsible for changes to proteins leading to auto-antibody production. However it cannot be discounted that the combination and presence of these immunoglobulins may

possibly be responsible for some of the pathology surrounding an RA diagnosis. The presence of anti-mCPR and pCRP IgG auto-antibodies in serum may not have all resulted from a breakdown in self-tolerance, despite the evidence that diseases such as SLE and RA provide to the contrary. Auto-antibodies have been found in many non-autoimmune diseases including cancer and neurological diseases (Avramcas *et al*., 2007; Linnoila 2016; Nolan and Lokshin 2010). In their 2013 study Nagele *et al* showed that several disease groups including Alzheimer's disease, Parkinson's disease, multiple sclerosis and breast cancer showed profiles of high auto-antibody frequency. Within this study a group of 57 healthy controls were included and microarray analysis of 9,486 native human proteins revealed a mean of 1,996.9 different IgG auto-antibodies were present within each individual in all groups. Regardless of age, gender or health status 60% of samples contained a core of common and abundant auto-antibodies, with no individual having less than 301 auto-antibodies within their serum. This suggests that not only does the healthy population have a normal level of auto-antibodies, that they may each have core similarities and individual and identifiable profiles. These profiles may well correlate to specific diseases and provide future diagnostic help to distinguish between similar pathologies. Within our own study the profile of the three auto-antibodies is very clearly different between disease and non-disease and mCRP / pCRP assays. Within a larger population a clear blueprint may be ascertained allowing a predictive tool for disease.

The body may respond to elevated levels of pCRP and mCRP by raising an autoimmune response, ultimately bringing balance back to CRP concentrations. If however CRP, which has been considered a protective protein capable of clearing apoptotic cells and therefore avoiding necrosis, is cleared by these antibodies, further inflammation may be triggered or sustained. The mechanism of mCRP interaction with the immune system must be fully understood in order to elucidate whether auto-antibodies against it play a

positive or negative role. The levels of anti-mCRP detected within SLE patients suggest mCRP may play a protective role, with its loss via antibody clearance possibly enhancing the disease state (Tan *et a*l., 2008; .Minatani, 2001; Sjowall, 2009).

Previous studies indicate that levels of auto-antibody may increase in correlation with advancing age (Nagele *et al*., 2013). Our study results show that age was found to correlate with levels of anti-pCRP auto-antibodies present in two of the RA and one of the control group assays. Within the RA group anti-pCRP IgA and IgM both showed a negative correlations with age (Spearman correlation -0.387, $P < 0.035$; -0.540, $P < 0.002$ respectively). Of the control groups anti-pCRP IgM was positively correlated with age (Spearman correlation 0.431, $P < 0.018$), suggesting that as the RA population ages levels of anti-mCRP IgA and anti-pCRP IgM fall, whilst levels of anti-pCRP IgM rise within the control population. This finding was in part supported by Griffin *et al*., *(*2001) who observed a gradual decrease in natural IgM levels with age, however in contrast to Nagele *et al*., *(*2013), who found a significant correlation between rising age and rising auto-antibody concentrations. However Nagele *et al*. focused on IgG auto-antibodies alone.

Our study showed a trend of higher levels of IgG auto-antibodies with increasing age in both anti-pCRP and anti-mCRP assays, although this was not found to be significant. Rising levels of auto-antibodies in the control population may in general provide an element of protection or moderation to the immune system which is not present in the diseased population. Findings within anti-pCRP assays were however not universally repeated within those of the mCRP investigations in which age bore no significant impact on the levels of anti-mCRP auto-antibodies in either RA or control groups, although the trend suggestes that as age increased in the RA group, so did levels of IgG and IgM auto-antibodies whereas IgA auto-antibody levels appeared to fall.

It is interesting to note that the negative correlation between age and antibody level for anti-pCRP IgA is reflected in the albeit non-significant negative trend between age and anti-mCRP IgA. Anti-mCRP and anti-pCRP IgG both show positive associations with age, but this concordance between anti-mCRP and pCRP is not seen between IgM auto-antibodies, where a positive anti-mCRP vs age trend is seen, but a significantly negative anti-pCRP vs age correlation is also apparent. Whilst incidence of RA is often associated with age due to a greater frequency of diagnosis with increasing age, it is interesting to see that whilst anti-mCRP IgA was not only found to be a strong positive indicator of RA ($P < 0.001$, R^2 51.54%), this indication was found to increase in significance when age was also taken into account ($P < 0.0001$, R2 69.74%). It is unknown at this stage whether this indication of disease is due to cause or effect. As age increases within the anti-mCRP group, the level of anti-mCRP IgA does not appear to rise. These results appear to contradict the logistic regression showing age and anti-mCRP IgA levels correlate with disease, however the regression takes into account the much higher RA auto-antibody levels in older RA individuals (mean age 62.73 years), than those found within the youngest of an already low level control group (mean age 46 years). The reflected increase in the likelihood of RA occurring may possibly result from either a reduction in the level of mCRP due to increased likelihood of anti-mCPR IgA; or alternatively, the increase in RA risk in conjunction with ageing leads to an increase in anti-mCRP IgA levels due to the systemic inflammatory state created by the disease, therefore anti-mCRP IgA is not actually complicit RA development. The results of this study therefore have not been able to clarify fully whether the significant difference in IgA levels are implicated in pathogenesis of RA or are caused by it.

When an antigen is detected within the body an immune response where necessary includes generation of antibodies, supporting the presumption that higher levels of either

mCRP or pCRP detected within serum would correlate with higher levels of auto-antibody present. This was the case within the combined male/female group, in which a significant positive correlation was found between levels of anti-mCRP IgA and mCRP ($P < 0.035$), illustrating that as mCRP levels rise so do associated IgA auto-antibodies. However when split by gender, RA mean levels of mCRP did not correlated against anti-mCRP IgG, IgA and IgM in either male or female groups $P > 0.05$, a finding reflected within the control cohort.

As discussed previously there were strong gender differences between male and female groups, making some direct comparisons unreliable. A positive correlation was found in the male but not female group when pCRP and anti-pCRP IgA levels were investigated ($P < 0.021$), however it is difficult to draw conclusions from these results due to the small sample size. It is unknown as to whether this would be reflected within a larger population and no such indication was apparent within the larger female group. No significant correlation was observed between pCRP and either anti-pCRP IgG and IgM. Previous studies have also indicated that there was no correlation between CRP levels and those of auto-antibodies directed against it, but these have focused on IgG rather than IgM or IgA auto-antibodies (Jakuszko *et al*., 2017; Rosenau and Schur 2006; Sjowell *et al*., 2003, Tan *et al*., 2003).

The RA patients within this study whilst confirmed RA seropositive and all under clinical management at a routine hospital drug monitoring clinic, were not assessed for interaction with their drug regime as this was not an agreed aspect of the ethical review and permission. Therefore some results may have been impacted by unknown interactions, including certain classes of drug which modify immune pathways and influence the level of CRP within serum. Correlations between mCRP, pCRP and auto-antibodies directed against them may have been masked by the class of drug individuals were taking and the

length of time it had been prescribed. The degree of disease activity and length of time from diagnosis, if the individual was experiencing an RA flare, together with comorbidity factors were also unknown and may have impacted upon analysis made within this study.

6.4.3 Overall Conclusion

To our knowledge this is the first study to address multiple auto-antibodies directed against both mCRP and pCRP within rheumatoid arthritis. The ELISA design was optimised to ensure interactions found within previous research studies, such as those between human serum and BSA, did not affect the results found within our study. RA patients were found to have significantly higher levels of auto-antibodies than those of normal control subjects, with the exception of the IgM isotype. This may be due to the level of natural antibodies present within both control and RA patient serum which exist as part of normal physiological and immunomodulatory roles, IgM may not be pro-actively produced but be the result of low level constant secretion. Gender differences were evident with significantly higher RA to control level auto-antibodies within the female group. Auto-antibodies were not found to differ significantly in detection between anti-mCRP or pCRP suggesting possible dual antigenicity with both CRP conformations. Auto-antibodies of each isotype were present within control samples, with only four subjects negative for IgM, IgG and IgA anti-mCRP auto-antibodies and just two negative for anti-pCRP antibodies. This indicates that not only is there a ‘normal level’ of serum pCRP and possibly mCRP, but also a retained level of self-directed antibodies against both. There was a strong correlation found between disease and anti-mCRP IgA, further enhanced when age was considered. It is possible that anti-mCRP IgA could prove to be an effective diagnostic tool which may aid diagnosis. Levels of auto-antibodies vary across the groups, however it is clear that although not all correlated, subtle interactions existed. The

diagnostic power of autoimmune diagnosis may be further enhanced by increasing the number of auto-antibodies detected within routine testing and analysis of the auto-antibody profile, rather than relying predominantly upon IgG and IgM auto-antibodies.

Our research highlights the advantage of assessing whether additional tests focused upon both mCRP and pCRP auto-antibodies, which together with auto-antibody profiling, may further support physicians determining diagnosis.

6.4.4 Future Work

Future work should continue to determine whether auto-antibody levels detected within this pilot study are linked to RA development, prognosis or drug prescribed. A significant correlation between anti-mCRP IgA levels, age and RA has been established, this should be further investigated to determine how this relates to disease activity and if the predictive capabilities suggested in this study are a possible predictive tool of future disease, or severity of disease. This information may help determine the type of RA drug intervention chosen. Investigation between gender differences are a key area of research and a larger study population with greater proportion of male subjects would enable powerful statistical analysis of the trends suggested within this study. RA established risk factors include smoking, determined to be a predictor of RA in males but not consistently in females (Nagele *et al*., 2013). Disease severity is suggested to be significantly worse in female RA patients who had ever smoked than those who had not (Mattey *et al*., 2002a, 2002b). Unfortunately our study did not collect smoking history and therefore we are unable to assess whether the levels of auto-antibodies determined in our study may have been affected by the influence of smoking history. Future work should also consider this

variable within experimental planning to ensure the influence of life style is also taken into account.

Finally research should focus on establishing comparable methodologies in which simple and standardised assays reliably determine which form of CRP is the target of auto-antibodies, including commercially available mAb with specificity against the monomeric form of CRP. Within both clinical and research settings variations in CRP and anti-CRP detection methods may be adding to an already confused and contradictory body of research, preventing clear determination of the role played by monomeric CRP and its auto-antibodies within inflammatory disease.

Chapter 7 – Discussion, Conclusion and Future Work

7.1 Overview

Since it was first observed by Oswald T. Avery in 1930 (Tillet & Francis, 1930), CRP has become a key biological marker for the presence of inflammation, infection or tissue damage within the body (Bell *et al*., 1998; Ford, 2003; Pepys & Hirschfield, 2003; Sowall & Wettero, 2007; Zhang *et al*., 2012). However previous research has reported conflicting roles, with CRP suggested to be both pro and anti-inflammatory (Hanriot *et al*., 2008; Pasceri *et al*., 2000; Pepys & Hirschfield, 2003; Raaz-Schrauder *et al*., 2014; Thiele *et al*., 2015). The acute phase protein is widely accepted to be a strong independent risk factor for, and predictor of, future disease; for instance, cardiovascular disease (Chen *et al*., 2018; Pepys & Hirschfield *et al*., 2003; Ridker *et al*., 1998; Shrivastava & Singh 2015); chronic obstructive pulmonary disease (Dahl *et al*., 2007) and type two diabetes (Pradhan *et al*., 2001) and is a component of the DAS28 testing and biomarker within RA. Alternative evidence has suggested that CRP may play a regulatory role within the immune system, moderating inflammation and other components within the immune system (Gershov *et al*., 2000). CRP is also correlated with SLE risk and severity due to a polymorphism associated with both development of SLE and decreased levels of CRP. This perhaps suggesting a protective role within SLE, possibly due to the clearance of nuclear antigen by CRP, a target of autoimmunity in SLE (Cunninghame *et al*., 2004). Whilst elevated concentrations of CRP are a feature of RA, it is unclear whether the raised levels found in RA patients result from their inflammatory state, or are directly implicated within pathology of this disease.

The two main aims of this pilot research study were designed to clarify whether molecular variations of human CRP were present, detectable and quantifiable within RA

patient serum, and to determine the existence of auto-antibodies directed against both mCRP and pCRP within the same serum. Although the material within this study is limited to 30 rheumatoid arthritis patient and 30 control samples, many interesting and novel observations were made. The research shows experimental evidence contributing towards a greater understanding of CRP conformations and auto-antibodies within RA. Through optimised ELISA and western blot analysis our research identified a monomeric form of CRP within RA and control serum, together with the presence of IgG, IgA and IgM class antibodies directed against both monomeric and pentameric CRP.

7.1.1 Production of monomeric CRP

The native pentameric form of CRP has been studied and characterised extensively, however the physiological conformation of mCRP, its function and creation mechanism are still under consideration. The experimentation within Chapter 3 sought to produce and purify mCRP *in vitro,* evaluating methods of dissociation in order to determine optimum conditions for the creation of mCRP for further studies. Initial investigations considered the effect of pH on CRP. Whilst pH modification has previously been used to dissociate CRP *in vitro*, it was not considered an appropriate method of mCRP generation within this study, as the effects upon the dissociated protein could not be determined and the amount generated without heating the sample was limited. Localised physiological drops to pH are known to occur within certain diseases, however they are not found to fall as low as pH 4.0. (Helmlinger et *al*., 1953; Kimmoun *et al*., 2015) It is therefore unlikely pH levels within the body would provide an environment supportive of the dissociation found within this *in vitro* testing system.

Whilst an existing body of research indicates a potentially pro-inflammatory role for mCRP, the nature and origin of mCRP used within these studies has varied, for instance

8M urea was used within studies by Motie *et al*. *(*1988) and Slevin *et al*. (2015) whilst the groups of Chirco *et al*.*(* 2016) and Fujita *et al*. (2014) produced and used recombinant mCRP. However a greater proportion of previous studies generating mCRP *in vitro* used similar dissociation methods to the urea induced dissociation of subunits found within our study. However, many of these studies have used the harsher method of 8M urea rather than the 2M conditions developed within our research. Experimental evidence suggests 3M urea dissociated mCRP retains an ability to bind PC in a calcium dependent manner (Williams, 2016), however further functional clarity has not been determined within our research. Potential modifications to CRP by urea could not be confirmed, however it is postulated that urea may act either via its ability to interact with polar and non-polar components, allowing disruption of non-covalent bonds or weaken hydrophobic interactions between subunits (Caballero-Herrera *et al*., 2005; Hua *et al*., 2008; Rossky, 2008; Zou *et al* 1998). It is also suggested it acts by directly interacting with the backbone of CRP (Caballero-Herrera *et al*., 2005; Hua *et al*., 2008; Stumpe & Grubmuller, 2007), or disrupts intersubunit salt bridges (Sun *et al*., 2014). The true structural effects may only be revealed following determination by crystallographic studies, however the functional phosphocholine binding properties of urea dissociated CRP are retained (Williams, 2017), suggesting minimal disruption of tertiary structure. Despite this it remains to be determined whether *in vitro* produced mCRP proves to be physiologically relevant.

Dissociation investigations also revealed the importance of calcium to the stability of CRP. It was not possible to fully dissociate pCRP with either extremes of pH or urea alone, with dissociation achieved only after the addition of calcium chelator EDTA. Calcium is shown to maintain structural integrity of the subunits via interactions protecting key sites from proteolysis (Ramadan *et al*., 2002; Shrive *et al*., 1996) and this was evident within our study.

Perhaps surprisingly the reintroduction of calcium to mCRP in solution did not result in re-association of the protein, due possibly to the necessity of a final stage chaperone protein within physiological assembly of pCRP, or a required involvement of the 19 residue N-terminal signal peptide present pre cleavage. It is also possible that intersubunit salt bridges may become carbamylated by urea, preventing reassociation of subunits.

Several theories have been previously suggested as to how mCRP may arise *in vivo* and what mechanisms may be involved, including mechanical dissociation initiated following membrane binding, or possible local expression of CRP as a monomer rather than a pentamer. It is also theoretically possible that within the inflammatory confines of an RA joint, reactive oxygen species and oxidative stress thought to be involved within the pathogenesis of RA (Biemond *et al*., 1984; Mateen *et al*., 2016; Ozturk *et al*., 1999), result in oxidation and damage to CRP. Key protein oxidation markers were found by Mateen *et al*. (2016) within RA patient plasma, alongside significantly low levels of non-enzymatic antioxidants (reduced glutathione and vitamin C). It is possible the oxidative environment found within RA joints may destabilise the pentameric structure of CRP, making it more vulnerable to previously mentioned dissociation factors. Physiological mCRP may however be a combination of several of these factors dependent on disease or inflammatory states within the body. Despite doubts as to the functional relevance of the monomer created within our study, it proved to be recognised not only by commercially available anti-mCRP antibody Clone 8, but by patient and control serum. This indicates that key structural recognition domains remain following urea treatment.

The techniques used within this study provided a reliable method to reproducibly generate stable mCRP for further investigations within patient serum and antibody studies. Our results confirmed those previously reported, with the creation of a stable subunit

isoform of CRP. However our methods have been adapted to reduce the harsh techniques used within much existing research. The monomer created has been confirmed as 23kDa via calibrated size exclusion elutions and travels on native PAGE to a position estimated to be that of a monomeric subunit, in a distinctly different way to the pentamer. Western blotting further confirms the identity of the created subunit through the use of specific antibodies designed to recognise the intersubunit epitopes.

7.1.2 Optimisation of Detection Assays

CRP presents many challenges when attempting to carry out quite routine molecular biology or immunoassays involving it. It does not run 'normally' within a native gel in its monomeric form, due to a change in pI from that of its original pentamer formation. The pentamer itself does not all travel into the resolving section of a gel, with a quantity remaining within the stacking gel. It is not possible to run a standard direct or indirect ELISA to determine patient CRP isoform content as pCRP is unstable and dissociates on contact with the ELISA plate (Sjowell *et al*., 2002; 2003). In addition to this, components of both the RA and control serum interacted with BSA used within the blocking stages of both western blotting and ELISA and potentially any capture antibodies used. CRP within our study was also found to interact directly with both goat anti-human IgG and rabbit anti-mouse IgG, although not significantly at the dilutions used within this study.

Our assay design carefully took into account the various factors required in order to avoid non-specific binding or high noise to signal ratio. Possible interaction with other pentraxins was countered by pre-testing antibodies with both SAP and PTX3 for specificity. A competitive ELISA was developed which minimised confounding variables at risk of creating false positive or negative results. Following ELISA, western blots were

carried out, and serum incubated with the milk blocking solution overnight in an attempt to reduce interaction with the blocking stage. Antibodies directed against mCRP are also known to bind Hsp60, with the epitope region of Hsp60 matching 26.6% amino acid sequence to residue region 77 to 90 of CRP (Udvarnok *et al*., 2007). This 58-65kDa protein was not pre tested within our assays, however the mouse anti-mCRP 3H12 used within our detection of mCRP was tested by Udvarnok *et al*. (2007) and found not to recognise Hsp60. Additionally it is known that 3H12 antibody recognises CRP residues 199-206 rather than 77-90.

Positive samples were also confirmed by western blotting, although only two initially determined positive for mCRP by ELISA were later identified as positive by western blot. Western blotting is less sensitive than ELISA and the concentration of mCRP found in both patient and control samples low, therefore only the highest samples provided visible bands. However it is important to ensure samples are positive within both western blot and ELISA, removing the risk of false positive results due to interference of system components. Within the study by Williams (2016), CRP was first isolated by affinity chromatography before size determination by both western blotting and size exclusion chromatography. It is possible that future studies could adopt this system to ensure mCRP antibody recognition and ELISA results are accurate.

Possible interactions between assay components and serum antibodies were avoided by use of a competitive ELISA system and western blotting confirmed the ELISA results in all but anti-pCRP IgA detection assays. The absorbance of ELISA results ranged between 0-0.113 in the anti-pCRP IgA RA group and potential bands present may not be visualised over the background interaction between serum and blocking solution. Although all interactions may not have been fully eradicated, our study underlines the importance of thorough study design and optimisation before initiating assays.

7.1.3 Detection of CRP within Human Serum

The serum of 30 RA patients and 30 control samples was tested for the presence of mCRP. Patients were recruited at a routine hospital drug monitoring clinic and were all confirmed seropositive for RA before informed consent was gained. Although a small sample size (study limitations discussed in section 7.7), our study did show that mCRP could be detected within patient serum samples. We identified both monomeric and pentameric CRP within ELISA assays.

Statistical analysis, prior to splitting the cohorts into gender subsets, found a significant difference between concentration levels of pCRP detected between the RA and control groups ($P < 0.001$), (RA group pCRP mean 22.85mg/l, control group pCRP mean 0.742mg/l). Detectable levels of mCRP were found in 29 of the 30 RA samples and 24 of the 30 control samples, with significantly higher levels within the RA group ($P < 0.01$), (RA group mCRP mean 0.092mg/l, control group mean 0.069mg/l). However, once groups were split by gender no significant difference remained.

To our knowledge this is one of the few studies to identify the presence of mCRP within patient serum samples. In 1987 Potempa *et al*. identified molecules expressing neo-CRP antigenicity in the plasma of two RA patients (Potempa *et al*., 1987). A more recent study by Wang *et al*., *(*2015), also provided evidence suggesting the existence of soluble circulating mCRP (0.021 ± 0.002 mg/l). The study by Wang *et al* considered plasma of patients who recently had suffered acute myocardial infarction (AMI), however both of these studies considered plasma rather than serum. Plasma may contain microparticles, therefore presenting the possibility that mCRP found within the samples was microparticle bound, rather than soluble within serum. A recent study that did consider serum was that of Williams (2017), who isolated mCRP from serum, concentrating it before identifying and

quantifying it by ELISA and western blotting (Williams, 2017). The highest levels reported within Williams' study were approximately 2.5 mg/l which is in the region of 100 times higher, although this study selected patients with in excess of 100mg/l serum CRP levels initially therefore potentially higher mCRP content than Wang *et al*., *(*2015). The results from our study are more comparable to Wang *et al* with 0.132mg/l in the highest patient sample.

Concentration levels of pCRP were universally higher than mCRP across all samples within our study, with an increase observed within the RA group. There is less difference within the mean mCRP concentration between an RA state and non RA control state, with no samples elevated above normal threshold, suggesting that a low level of mCRP may be present in the serum of a normal healthy population. It is not possible to determine if a rise in the level of mCRP would be linked to inflammation or infection in the same way pCRP is, however, six of 30 (combined gender) control participants were negative for mCRP, whereas only one was negative within the RA group ($P < 0.05$). This suggests that like pCRP, mCRP may also be more prevalent within inflammatory or autoimmune disease and our research shows a small but significant increase within the RA cohort. It is also the case that once separated by gender there is no significant difference between RA and control serum levels, suggesting mCRP may circulate at a 'normal' albeit low level across the general population.

Elements of the mCRP identified within both patient and control samples may have originated from circulating pentameric CRP through local dissociation, which has been well described within recent literature (Ji *et al* ., 2009, Thiele *et al*., 2014, Eisenhardt *et al*., 2009). A recent study by Braig *et al*., (2017) provides evidence for a mechanism allowing pCRP to become 'activated' by binding to cell membranes resulting in a relaxed pentameric form, allowing C1q binding. The engagement of C1q within the central cavity

is suggested to further allow dissociation in mCRP (Braig *et al*., 2017). Autoimmune disorders such as RA can involve impairment to the clearance of apoptotic cells, linked to the development and progression of chronic inflammatory dieseases (Kruse *et al*., 2010; Mũoz *et al*., 2010; Szondy *et al*., 2014). When pCPR recognises and binds apoptotic cells, normal clearance procedures may not allow time for dissociation of the pentamer on the cell surface; however in situations of impaired clearance, dissociation may be enabled. This could ultimately lead to the clearance of apoptotic cells more rapidly, following secretion of pro-inflammatory cytokines by monocytes and macrophages induced by the newly formed mCRP. In this way mCRP may facilitate the recruitment of further immune cells to the locality, enhancing the clearance of cellular debris. The normal function of mCRP may therefore be the amplification of signal allowing a rapid recruitment of immune cells when apoptosis is inefficient. However within a diseased state this amplification may in part be responsible for perpetuating inflammation and auto-antibody generation, eventually resulting in auto-antibodies generated against mCRP itself as demostrated by the detection of IgG, IgA and IgM autoantibodies in patient serum within our study.

Scenarios suggesting that mCRP originates purely from pCRP imply that its levels would correlate with reduced but corresponding concentrations of mCRP to those of pCRP. Such a correlation may have suggested the soluble mCRP detected within our serum samples was of pentameric origin, resulting from degradation or dissociation. However, no correlation was found between levels of pCRP and mCRP in either the RA or control group, RA ($P > 0.708$), control ($P > 0.646$), demonstrating perhaps that mCRP is not entirely resultant of physiological pCRP dissociation. Local pCRP expression at sites of inflammation or tissue damage have been identified (Calabro *et al*., 2005; Gould *et al*., 2001; Jabs *et al*., 2003; Thiele *et al*., 2014; Yasojima *et al*., 2001). It is possible that local expression of mCRP may also take place, although by an as yet to be determined pathway.

It has been demostrated within U937 macrophage studies that the form of CRP expressed by these cells is mCRP rather than pCRP (Ciubotaru *et al*., 2005), suggesting that a pathway exists allowing this event, perhaps during chronic disease state or during periods of high oxidation at the site of inflammation. It is possible that local disociation of pCRP upon a cellular surface would result in an activated intermediate form of bound CRP predisposed to complete dissociation. This dissociation may stimulate surrounding macrophage and other immune cells to secrete a combination of cytokines leading to cellular expression of mCRP in preference to pCRP and thus amplifying complement activation via C1q activation. Further exploration of an mCRP mechanism independent of pCRP dissociation must be sought in order to fully explain the levels of mCRP/pCRP found within our study and the impact it has, if any, on disease states.

When age and gender were considered as variables, interesting differences between the groups emerge. Within the pCRP detection assay, control group female levels were higher than males (mean 0.80mg/l, mean 0.54mg/l respectively, $P > 0.05$), however within the pCRP RA group, male levels were higher than females (male 24.70mg/l, female 21.79mg/l, $P > 0.05$), this finding is supported by research by Siemons *et al,* (2014) who reported that in early RA men had 1.2 times higher CRP levels than women (Siemons *et al*., 2014). There was no significant difference between levels of male and female mCRP in either control or RA groups. Whilst both male and female mCRP levels within the RA group increase slightly as they grow older and both show declining pCRP values with age, these figures are based on a small sample size and are therefore affected by two particular male samples showing a greater decline in pCRP with age.

It is evident that although standard clinical tests such as ELISA are able to quantify CRP levels, they may not be able to distinguish between the two isoforms, for instance the DuoSet used within our study is widely used within research and unable to detect mCRP.

Should further studies confirm additional forms of CRP within human serum or tissue, the question of accuracy and implied meaning of previous testing could be called into question, both within clinical and research settings (Crawford *et al*., 2006). Automated systems used within clinical settings rely upon the formation of an antigen-antibody complex and therefore results are subject to the choice of antibody. Findings generated over the last 20 years may have worked on the assumption of native pentameric CRP detection, when in fact samples could possibly have contained monomeric CRP or a combination of the two forms together. It is plausible to suggest the complex and contradictory results showing both the pro-inflammatory and protective effects assigned to CRP, may result from a of lack of determination of the isoform of CRP detected, with mCRP and pCRP playing potentially different roles within the inflammatory environment.

7.5 Detection of Anti-CRP Auto-antibodies within Human Serum

Anti-human IgG, IgM and IgA auto-antibodies directed against mCRP and pCRP were found in both control and RA serum. RA is a well categorised autoimmune disease, with serum auto-antibodies playing a key role not only within the development of RA but within its diagnosis. Where the presence of auto-antibodies within RA is well established, to our knowledge this is the first study to report the profile of IgG, IgA and IgM auto-antibodies directed against both mCRP and pCRP.

Whilst anti-mCRP auto-antibodies are a feature of SLE (Meyer, 2010; Sjowell *et al*., 2002), they are less extensively studied within RA, with few studies considering anti-pCRP auto-antibodies. Two previous studies have identified anti-mCRP antibodies in RA, with between 22-29% of patients positive for anti-mCRP IgG auto-antibodies within their serum (Minitani *et al*., 2001; Rosenau & Schur, 2006). Our study showed 66% of RA

samples contained detectable anti-mCRP IgG as did 43% of controls, although when adjusted for those above the normal threshold, levels dropped to 33% and 3.33% respectively, close to the top range of Minitani *et al*, 2001 study. Of interest was the profile of serum auto-antibody content within our study. All RA samples contained detectable levels of at least one auto-antibody class, with many determined to contain multiple isotypes. Table 7.1 provides a summary of all results including mCRP and pCRP levels with the equivalent auto-antibody level and western blot confirmation where available. Detectable levels of all three IgG, IgM and IgA auto-antibodies directed against pCRP were found within each of 23 of 30 RA patients and 15 of 30 control samples. This was in contrast to the lower frequency of samples with auto-antibodies directed against mCRP, of which 15 of 30 RA and 4 of 30 control samples possessed all three. Whilst IgG, IgM and IgA auto-antibodies were found to be directed towards both mCRP and pCRP in RA patients, they are also shown to be present within non-RA control samples, indicating a background level present within the healthy population. The nature of these auto-antibodies is unknown; however they may play a role in the clearance of cellular debris at low levels. However the higher number of RA samples with detectable levels present within serum suggests that within RA patients their role may be linked to disease. Cross reactivity between antibodies directed against Hsp60 may account for a proportion of these auto-antibodies. It is known that anti-Hsp60 auto-antibodies are prevalent within RA (van Halm *et al*., 2006; Watanabe *et al*., 2003) and it is possible antibodies directed against mCRP found within serum in our study originated against Hsp60.

Once again the whole cohort was split into male and female groups, with the focus of attention on the female group due to its larger size. Data taken from the female cohort shows significantly higher levels of auto-antibodies in RA samples directed against both mCRP and pCRP of each antibody class than that of controls ($P < 0.05$), with the exception

of anti-mCRP IgM, which showed no significant difference. Binary logistic regression showed a significant association between anti-mCRP IgA levels and disease ($P < 0.0001$), showing increased anti-mCRP IgA associated with an increased likelihood of RA. When age is also taken into account this becomes a stronger indicator disease ($P < 0.0001$), suggesting RA is significantly more likely to occur with an increase in both age and anti-mCRP IgA levels. However, the age profile of the female control group is lower than that of the RA group. The nature of control samples, with lower IgA auto-antibody levels and a younger age profile, suggests that this finding may not be repeated within an age matched control group.

7.1.4 Interactions between CRP and Anti-IgG Antibodies

Our study found interesting interactions between anti-IgG and CRP, which were stronger between the antibody and mCRP than pCRP. Existing research has suggested the risk of cross reactivity is elevated in proteins with a high level of homology amongst species (Delgado *et al*. 2003), which may also include CRP. It has also previously been reported that mCRP binds immunoglobulins non-specifically. Our research indicated an interaction with mCRP as low as 0.5µg/ml, that increased with protein concentration and resulted in considerable binding at both 2.5 and 5µg/ml. It was thought possible that key similarities between Fcγ binding of both CRP and IgG may result in an area of homology leading to molecular mimicry; however no interaction was found when Fc specific anti-IgG antibodies were used to test this theory. The exact nature of CRP and anti-IgG interaction therefore remains unknown, however the location of interaction on CRP may be an area close to the interface between subunits as there appears to be higher level of interaction between mCRP than pCRP. Steric hinderance between IgG and pCRP may reduce the capacity of interaction.

Table 7.1 Summary table containing pCRP, mCRP (mg/l) and auto-antibodies (absorbance) quantification as determined by ELISA (*n*=3), for each patient or control sample. Additionally western blot (*n*=3) results are indicated. Quantification of mCRP / pCRP - green highlighted boxes represent samples with mg/l below the calculated normal threshold (SEM ± 2SD), orange indicates those mg/l above the threshold. Western blot results are indicated by green for those samples tested but without a visable band and orange for those with a positive band, blue represents those samples which were not tested.

ID	mCRP (mg/l)		pCRP (mg/l)		WB result pCRP	WB result mCRP	AAB mCRP IgG		AAb mCRP IgA		AAb mCRP IgM		AAb pCRP IgG		AAb pCRP IgA		AAb pCRP IgM	
	Mean	± SEM	Mean	± SEM			Mean	WB	Mean	WB	Mean	WB	Mean	WB	Mean	WB	Mean	WB
RA01	0.099	0.0010	1.985	0.0013	+	-	0.080		0.147		0.311	+	0.188		0.010		0.303	+
RA02	0.079	0.0023	55.905	0.2141	+	-	0.000		0.074		0.157	-	0.232	+	0.013		0.140	
RA03	0.085	0.0012	43.742	0.0748	+		0.054		0.119		0.261	+	0.096		0.113		0.425	+
RA04	0.077	0.0025	7.943	0.0262	+		0.092		0.046		0.008		0.211	+	0.014		0.078	
RA05	0.073	0.0022	18.701	0.0066	+		0.031		0.122		0.155	-	0.242	+	0.029		0.285	+
RA06	0.101	0.0017	22.501	0.0515	+	-	0.124	-	0.138		0.015		0.016		0.000	-	0.000	
RA07	0.101	0.0008	83.688	0.0101	+	-	0.029		0.163	-	0.166	+	0.136		0.038	-	0.360	-
RA08	0.118	0.0065	5.289	0.3151	+	-	0.039		0.270	+	0.000		0.061		0.044	-	0.181	-
RA09	0.083	0.0002	2.621	0.0181	+		0.097		0.102		0.000		0.077		0.005		0.183	-
RA10	0.105	0.0005	4.464	0.0621	+	-	0.031		0.106		0.100		0.252	+	0.036		0.157	-
RA11	0.096	0.0004	11.495	0.0561	+		0.000		0.315	+	0.000		0.223	+	0.030	-	0.075	
RA12	0.099	0.0013	5.587	0.1052	+		0.000		0.089		0.168	-	0.297	+	0.020		0.000	
RA13	0.102	0.0011	9.528	0.0570	+	-	0.110	-	0.138	-	0.085		0.000		0.020	-	0.000	
RA14	0.073	0.0002	1.079	0.0128	+		0.184	-	0.000		0.028		0.192		0.056	-	0.024	
RA15	0.105	0.0003	8.963	0.0952	+	-	0.244	+	0.120		0.023		0.251	+	0.038		0.040	
RA16	0.131	0.0018	55.353	0.0161	+	+	0.000		0.067		0.117	-	0.050		0.052		0.043	
RA17	0.119	0.0007	24.151	0.0913	+	-	0.000		0.239	+	0.170	-	0.082		0.037	-	0.047	
RA18	0.054	0.0011	22.839	0.2109	+		0.214	+	0.139		0.025		0.295	+	0.041		0.039	
RA19	0.128	0.0022	29.258	0.2664	+	+	0.185	-	0.053		0.119		0.000		0.015		0.000	
RA20	0.053	0.0010	21.542	0.2528	+		0.000		0.008		0.032		0.105		0.107		0.079	
RA21	0.083	0.0015	21.188	0.1281	+		0.092		0.000		0.025		0.094		0.066		0.000	
RA22	0.106	0.0007	29.968	0.7266	+	-	0.202	+	0.061		0.055		0.051		0.005		0.017	

RA23	0.133	0.0020	13.466	0.1931	+	-	0.000		0.179		0.000		0.057		0.000	-	0.036	
RA24	0.102	0.0006	5.274	0.0750	+	-	0.166	-	0.199		0.028		0.215	+	0.028		0.020	
RA25	0.098	0.0016	37.871	0.0515	+		0.000		0.023		0.031		0.079		0.081		0.027	
RA26	0.000	0.0000	28.321	0.1781	+		0.000		0.065		0.086		0.005		0.030		0.000	
RA27	0.089	0.0015	23.300	0.4198	+		0.054		0.030		0.019		0.014		0.065		0.084	
RA28	0.095	0.0002	24.632	0.5216	+		0.000		0.036		0.017		0.082		0.067		0.135	
RA29	0.055	0.0038	23.726	0.1204	+		0.148	-	0.027		0.018		0.089		0.047		0.044	
RA30	0.109	0.0035	41.014	0.2854	+	-	0.130		0.008		0.281	+	0.184	-	0.068		0.215	-
C01	0.092	0.0007	0.236	0.0078	-		0.079		0.017		0.113		0.083		0.020		0.062	
C02	0.086	0.0010	0.236	0.0089	-		0.000		0.019		0.001		0.000		0.000		0.000	
C03	0.087	0.0009	1.995	0.0666	+		0.000		0.000		0.000		0.007		0.000		0.011	
C04	0.084	0.0011	0.951	0.0230	+		0.040		0.000		0.088		0.133		0.048		0.000	
C05	0.062	0.0003	0.056	0.0012	-		0.147	-	0.000		0.122		0.000		0.000		0.029	
C06	0.077	0.0007	0.165	0.0010	-		0.011		0.003		0.048		0.099		0.000		0.012	
C07	0.098	0.0011	0.420	0.0041	-		0.000		0.000		0.000		0.089		0.000		0.000	
C08	0.087	0.0014	0.147	0.0018	-		0.000		0.003		0.000		0.257	+	0.080		0.183	-
C09	0.078	0.0007	0.321	0.0028	-		0.000		0.000		0.000		0.139		0.065		0.056	
C10	0.096	0.0011	0.078	0.0009	-		0.064		0.000		0.059		0.159		0.000		0.000	
C11	0.075	0.0011	1.995	0.0057	+		0.000		0.000		0.044		0.040		0.002		0.000	
C12	0.104	0.0017	0.946	0.0010	+	-	0.000		0.000		0.020		0.182		0.029		0.000	
C13	0.074	0.0009	0.946	0.0246	+		0.064		0.000		0.033		0.063		0.082		0.037	
C14	0.066	0.0007	5.406	0.0018	+		0.000		0.000		0.027		0.000		0.000		0.000	
C15	0.093	0.0020	2.470	0.0257	+		0.000		0.000		0.013		0.066		0.003		0.023	
C16	0.102	0.0003	0.190	0.0250	-	-	0.000		0.000		0.112		0.000		0.014		0.000	
C17	0.000	0.0002	0.310	0.0015	+		0.000		0.084		0.000		0.118		0.000		0.000	
C18	0.115	0.0009	0.184	0.0097	-	--	0.000		0.000		0.109		0.118		0.000		0.000	
C19	0.086	0.0001	0.259	0.0026	-		0.101		0.000		0.133		0.182		0.001		0.000	
C20	0.075	0.0004	0.086	0.0002	-		0.016		0.000		0.127		0.006		0.000		0.041	
C21	0.000	0.0000	0.065	0.0012	-		0.056		0.000		0.026		0.000		0.062		0.173	
C22	0.090	0.0012	0.119	0.0008	-		0.000		0.032		0.106		0.044		0.080		0.169	
C23	0.085	0.0087	0.928	0.0286	+		0.072		0.011		0.056		0.000		0.001		0.066	

C24	0.094	0.0010	0.081	0.0014	-		0.000		0.000		0.094		0.000		0.053		0.000	
C25	0.000	0.0000	0.117	0.0007	-		0.002		0.000		0.068		0.010		0.026		0.001	
C26	0.058	0.0001	1.297	0.0073	+		0.000		0.000		0.134		0.102		0.001		0.002	
C27	0.000	0.0012	0.000	0.0001	-		0.010		0.000		0.056		0.027		0.000		0.050	
C28	0.000	0.0000	0.724	0.0014	+		0.000		0.000		0.000		0.083		0.000		0.057	
C29	0.000	0.0011	0.592	0.0061	+		0.065		0.012		0.012		0.055		0.000		0.000	
C30	0.104	0.0007	0.951	0.0218	+	-	0.000		0.001		0.000		0.090		0.000		0.003	

7.1.5 Study Limitations

The pilot study design did not incorporate ethical approval for the correlation of CRP to disease or drug treatment history, nor were lifestyle indicators such as smoking history collected. Future studies should address these limitations to study analysis as levels of CRP may have been impacted by either prescribed RA drugs, severity of disease or secondary conditions. Control samples were not age matched as they were collected from an existing bank of healthy volunteers within the NHS setting. Once again this proved to be a limitation as the age of the control volunteers was generally younger than those with RA. The result of this was a correlation between age and disease when perhaps one would not exist within an age and gender matched population.

7.1.6 Conclusion and Future Work

Our research provides evidence for a physiological monomeric form of CRP through the use of ELISA and western blotting analysis. Our ELISA and western blot protocols were carefully optimised with the presence of monomer confirmed by western blot in two patients who were among three showing highest level of mCRP within the ELISA. Monomeric CRP was generated with results confirmed by size exclusion chromatography. The study has shown detectable levels of mCRP within both RA patient and control samples, although once split for gender no significant difference was found. There is a suggestion that just as pCRP exists below a designated normal level within a healthy population, that mCRP may also be present at a normal baseline level. It is not known if, as is the case with pCRP, levels of mCRP increase in response to inflammation or infection, although results indicate higher levels of mCRP within the RA group than that of the control. There was no apparent correlation between mCRP and pCRP serum

concentrations indicating that dissociation of the pentameric form is not solely responsible for mCRP found within serum.

Serum auto-antibodies against both mCRP and pCRP were found, again in both RA and control populations. However the frequency of samples found with elevated levels was higher within the RA group than the control. The profile of auto-antibodies was different between the RA and control groups, and between assays investigating a different target antigen, mCRP or pCRP as visible within. This suggests that the profile of auto-antibodies within RA serum may have value as a future diagnostic tool. It is possible that the auto-antibody profile may change as disease progresses, or could be apparent before symptoms manifest.

The significance of anti-IgA auto-antibodies is interesting. Serum IgA interacts with FcαRI (CD89) expressed on the surface of immune cells. When complexed with an antigen, IgA binds to FcαRI promoting degranulation of eosinophils and basophils, phagocytosis and antibody-dependent cell-mediated cytotoxicity. It also results in respiratory burst, leading to localised oxidation conditions (Morton *et al*., 1996; Snoeck *et al*., 2006) creating an environmental with potential for pCRP dissociation. Should the local RA environment contain a protein such as Hsp60 with part homology to CRP, which is recognised and bound by the higher levels of IgA auto-antibodies present in RA, this may result in dissociation of pCRP to mCRP, leading to mCRP recognition by IgA originally directed against Hsp60. Excessive production of reactive oxygen species (ROS) can damage proteins (Hitchon and El-Gabalawy, 2004), it is therefore important to replicate these conditions in order to address the potential of oxidation within the generation of physiological mCRP.

Further areas of future research include the requirement to repeat this pilot study within a larger sample group, with age and sex matched control groups. Key differences

between disease and drug treatment profile, together with lifestyle history may further enhance the value of our results. Auto-antibody studies may be further improved by the purification of antibodies from serum prior to testing, therefore avoiding problems with interactions between assay and serum components. The interesting interaction between anti-IgA and mCRP is an area requiring further investigation. RA is characterised as an autoimmune disease and many autoantigens have been profiled, however the interaction found within our studies may provide further useful information with regard to auto antigenicity.

Lastly, the structural changes of *in vitro* dissociation methods upon CRP must be resolved through X-ray crystallographic studies. Although urea dissociated CRP appears to retain PC binding capabilities in a $CaCl_2$ dependent manner, it is unknown whether this form of the protein retains the same structural fold as a subunit of pCRP. It is demonstrated from this research that consistent methodology for the detection of both mCRP and pCRP within clinical and research studies is required. Although the clinical significance of our research is yet to be revealed, it is clear from this study that there is a necessity for further research involving mCRP and auto-antibodies directed against it and against pCRP. Only then can we come to an understanding of the full role of mCRP and pCRP within the human immune system.

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NHS
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06 November 2012

Mrs Jennifer A Moran
PhD Student
Huxley Building
Keele University
Keele, Staffordshire
ST5 5BG

Dear Mrs Moran

Study title:	**Investigations into the possible involvement of C-Reactive Protein (CRP) in the progression of rheumatoid arthritis.**
REC reference:	**12/NW/0818**
IRAS reference:	**100079**

The Proportionate Review Sub-committee of the NRES Committee North West - Lancaster reviewed the above application on 07 November 2012.

Ethical opinion

On behalf of the Committee, the sub-committee gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

You should notify the REC in writing once all conditions have been met (except for site approvals from host organisations) and provide copies of any revised documentation with updated version numbers. Confirmation should also be provided to host organisations together with relevant documentation.

Approved documents

The documents reviewed and approved were:

Document	*Version*	*Date*
Covering Letter		31 October 2012
Evidence of insurance or indemnity		26 July 2012
Investigator CV	Jennifer Moran	26 October 2012
Investigator CV	Derek Mattey	
Other: Email		02 November 2012
Participant Consent Form	1.0	01 May 2012
Participant Information Sheet	1.0	01 May 2012
Protocol	1.1	01 August 2012
REC application	3.4	29 October 2012
Referees or other scientific critique report		13 August 2012

Membership of the Proportionate Review Sub-Committee

The members of the Sub-Committee who took part in the review are listed on the attached sheet.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

12/NW/0818 **Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

pp.

Dr Lisa Booth
Chair

Email: nrescommittee.northwest-preston@nhs.net

Enclosures: *List of names and professions of members who took part in the review*

"After ethical review – guidance for researchers"

Copy to: *Nicola Leighton, Keele University*
Mrs Christine Woolven, West Midlands (North) CLRN)

A Research Ethics Committee established by the Health Research Authority

After ethical review

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With the Committee's best wishes for the success of this project

Yours sincerely

pp.

Dr Lisa Booth
Chair

Email: nrescommittee.northwest-preston@nhs.net

Enclosures: *List of names and professions of members who took part in the review*

"After ethical review – guidance for researchers"

Copy to: *Nicola Leighton, Keele University*
Mrs Christine Woolven, West Midlands (North) CLRN)

A Research Ethics Committee established by the Health Research Authority

Non Portfolio

Staffordshire and NHS
Stoke on Trent Partnership
NHS Trust

Any queries to:
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17 April 2013

Mrs Jennifer A Moran
PhD Student
Keele University
Keele,
Staffordshire
ST5 5BG

Dear Mrs Moran

Confirmation of NHS permission for Research

Full Study Title	**Investigations into the possible involvement of C-Reactive Protein (CRP) in the progression of rheumatoid arthritis.**
Short Title	**C-Reactive Protein**
REC reference:	**12/NW/0818**
Local Investigator:	**Mr Derek Mattey**
Sponsor:	**Keele University**
Proposed local study end date:	**01/12/2015**

I can confirm that the above project has been given NHS Permission for Research by the Research & Development Department for Staffordshire and Stoke on Trent Partnership NHS Trust and the details entered on to the R&D database.

I note that this research project has been approved by NRES Committee North West - Lancaster

NHS permission for the above research has been granted on the basis described in the REC application form, protocol and supporting documentation. The local documents reviewed and approved were:

Document	Version Number	Date Ethics approved
Protocol	1.1	01 August 2012
Participant Consent Form	1.0	01 May 2012
Participant Information Sheet	1.0	01 May 2012

The research sponsor or the Chief Investigator, or the local Principal Investigator at a research site, may take appropriate urgent safety measures in order to protect research participants against any immediate hazard to their health or safety. The R&D office should be notified that such measures have been taken. The notification should also include the reasons why the measures were taken and the plan for further action. The R&D office should be notified within the same time frame of notifying the REC and any other regulatory bodies.

Staffordshire and NHS
Stoke on Trent Partnership
NHS Trust

Approval by the R&D Dept therefore assumes that you have read, understand and agree to comply with the:

- Research Governance Framework (www.doh.gov.uk/research)
- Data Protection Act
- Health and Safety Act
- ICH Guidelines on good clinical practice
- All applicable Trust policies & procedures

In line with these requirements may I draw your attention to the need for you to provide the following documentation/notifications to the R&D Department throughout the course of the study and that all amendments (including changes to the local research team) need to be submitted to R&D in accordance with guidance in IRAS:-

- Annual Progress Report Form
- End of Study Declaration Form (available on IRAS website)
- Changes to study start and end dates
- Changes in study personnel

Please note that the NHS organisation is required to monitor research to ensure compliance with the Research Governance Framework and other legal and regulatory requirements. This will be achieved by random audit by our department.

I would like to take this opportunity to wish you well with your research. If you need any further advice or guidance please do not hesitate to contact us.

Yours sincerely

Dr Douglas Wulff
Medical Director
Staffordshire and Stoke on Trent Partnership NHS Trust

Copies by email to:
Julie Gray, Clinical Trials Manager, Haywood Hospital
Janet Turner - Research Assistant
Dr Derek Mattey – Local investigator

Staffordshire and NHS
Stoke on Trent Partnership
NHS Trust
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Fax: 01782 673912

PATIENT INFORMATION SHEET

We would like to invite you to take part in a new research study. Before you decide whether to take part, it is important that you understand why the research is being done and what it would involve for you. One of the research team will go through the information sheet with you and will answer any questions you may have. We expect this should take no more than 15 minutes. If you are happy to participate we will ask you to sign a written consent form to acknowledge this.

Study title: *Investigations into the possible involvement of C-Reactive Protein (CRP) in the progression of rheumatoid arthritis.*

What is the purpose of the study?
The purpose of the study is to understand whether C-reactive protein (CRP) plays a role in the progression of auto-immune, inflammatory diseases such as rheumatoid arthritis. We will use cells isolated from blood and/or joint fluid to identify factors contributing to joint destruction in patients with arthritis. It is hoped to improve our understanding of inflammatory disease progression and joint destruction, leading to possible treatments.

Why have I been chosen?
You have been chosen because you have a rheumatic disease, such as rheumatoid arthritis or systemic lupus erythematosus (SLE). Your blood may contain higher than normal levels of either C-reactive protein or antibodies to it.

Do I have to take part?
No, taking part in the study is voluntary. If you do decide to take part you will be asked to sign a consent form and you may keep this information sheet for future reference. You will retain the right to withdraw from the study at any time.

What will I have to do if I take part?
Your role in the study will not require you to take any additional medication, involve a change in your treatment or initially to undergo any additional procedures. In taking part you will:

(i) Agree for us to use the joint fluid that is drained from your knee effusion during the routine procedure performed by the Doctor (if a rheumatoid arthritis patient).
(ii) Agree to provide an extra tube of blood (approx 1 tablespoon) when having a blood sample taken by the phlebotomist, as requested by the Doctor.
(iii) Agree to provide an additional sample (approx 2 tablespoons) of blood to the initial tube at a later date if necessary.

Version 1.0, May 2012

Staffordshire and NHS
Stoke on Trent Partnership
NHS Trust

For the purpose of the study, collection of joint fluid will only take place once. We may request an additional volume of blood should tests on the initial sample indicate it necessary. This will complete your role in the study.

What is the benefit of taking part?
Although you will not benefit directly from the study, the data we collect will help us understand the process of joint destruction. This may help in the development of new treatments and better care.

Will taking part change my treatment and care?
Taking part in the study will not influence your treatment and care. This will continue as directed by your Doctor. There will be no further risk to you outside that already present in the routine collection of joint fluid and blood.

Will my information be kept confidential?
All study information and data will be kept confidential, anonymous and securely. You will never be identified by name in any communications or publications arising from the research.

What will happen to the samples I donate?
The samples will be used in a series of laboratory experiments to determine levels of immune proteins present within the blood and/or synovial fluid, and how they may be responsible for joint destruction. Samples will also be stored for possible use in future studies investigating rheumatic diseases. The consent form will ask for your consent for us to do this.

Who is conducting the research?
The research is lead by Dr. Derek Mattey (Haywood Hospital). His co-investigators are Mrs Jennifer Moran and Dr Annette Shrive, both of Keele University. The study will be conducted at the Haywood Hospital. It is being funded by Keele University and The Medical Research Council.

Who should I contact for more information?
Please contact Mrs Jennifer Moran or Dr. Derek Mattey for further information about the study and its outcome, or if you have any problems relating to taking part.

Mrs Jennifer Moran
Postgraduate researcher
Huxley Building, Keele University
Keele
ST5 5BG.
Tel: 01782 734441

Dr. Derek Mattey
Senior Scientist
University Hospital of North Staffordshire
Haywood Hospital
Stoke-on-Trent, ST4 7QB
Tel: 01782 673813

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Staffordshire and NHS
Stoke on Trent Partnership
NHS Trust
Haywood Rheumatology Centre
Haywood Hospital
High Lane, Burslem
Stoke on Trent, ST6 7AG
Fax: 01782 673912

PATIENT CONSENT FORM

Title of Project: Investigations into the possible involvement of C-Reactive Protein (CRP) in the progression of rheumatoid arthritis.

Name of Researcher: *Dr Derek Mattey*

Study Centre: *Haywood Hospital.*
Study Number: CRP1
Patient ID Number for this study: *E001.*

Please initial box

1. I confirm that I have been guided through and understand the information sheet dated May 2012, version 1, for the above study, and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason and without my medical care or legal rights being affected. ☐
3. I understand that sections of my medical notes may be looked at by responsible individuals from regulatory authorities, the NHS trust or from Keele University, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my data. ☐
4. I agree to take part in the above study. ☐

Name of Patient	Date	Signature
Name of Person taking consent	Date	Signature
Researcher	Date	Signature

[1 for patient; 1 for researcher; 1 to be kept with hospital notes]

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