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**C-reactive protein and
enhancement of tissue damage:
a potential therapeutic target**

**Thesis submitted in fulfilment for the Ph.D. degree of
the University of London**

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1 Declaration

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27/9/2005

**To my mother: I continue to rely on you for wisdom, support
and love. No day passes without a thought for you.**

2 Abstract

Human C-reactive protein (CRP), the classical acute phase reactant, is a member of the pentraxin family of calcium dependent ligand binding proteins. When ligand bound, particularly to phosphocholine residues found in cell membranes, bacterial cell walls and lipoprotein particles, CRP activates the complement cascade through engagement of C1q. Prior studies have suggested an important role for CRP both epidemiologically and biologically in atherothrombosis and its consequences. In the largest study to date of the epidemiological association between CRP and future cardiac events in healthy individuals, we demonstrate in contrast to previous studies, that the additive value of this inflammatory marker is less than previously thought. Further study of the biology of this ancient molecule in a number of animal models of disease has failed to confirm a protective role for CRP in endotoxaemia, and despite evidence of upregulated production in a transgenic mouse strain prone to atherosclerosis, we have demonstrated no apparent effect of human CRP on atherogenesis. Finally we have clearly and robustly shown that decamerisation of pentameric CRP by a novel low molecular weight palindromic inhibitor effectively prevents the ligand binding properties of this protein. This has established a platform for pharmacological inhibition of CRP in myocardial infarction and stroke where the complement activation initiated by CRP binding to necrotic cells has been suggested to be pro-inflammatory and deleterious.

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5 Frequently used abbreviations

apoE	Apolipoprotein E
BMI	Body mass index
BP	Blood pressure
BSA	Bovine serum albumin
C1q	Complement component 1q
C3	Complement component 3
C4	Complement component 4
CFU	Colony forming unit
CHD	Coronary heart disease
CIA	Collagen induced arthritis
CRP	C-reactive protein
CoF	Cobra venom factor
CPHPC	R-1-[6-[R-2-carboxy-pyrrolidin-1-yl]-6-oxo-hexanoyl]pyrrolidine-2-carboxylic acid
CPS	C-polysaccharide
CVD	Cardiovascular disease
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELDL	Enzyme modified LDL
ESR	Erythrocyte sedimentation rate
FEV1	Forced expiratory volume in one second
hCRPTg	human CRP Transgenic mice
HDL	High density lipoprotein
hsCRP	High sensitivity CRP
ICAM	Intercellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL-1R	Interleukin-1 receptor
IL-1ra	Interleukin-1 receptor antagonist
i/p	Intra-peritoneal
i/v	Intra-venous
LBP	Lipopolysaccharide binding protein

LD	Lethal dose
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
MBL	Mannose binding lectin
MI	Myocardial infarction
MIRA	Roche MIRA Autoanalyser
NF-κB	Nuclear factor- κ B
NHS	Normal human serum
NMS	Normal mouse serum
OD	Optical density
OxLDL	Oxidised LDL
PC	Phosphocholine
PE	Phosphoethanolamine
PPARα	Peroxisome proliferator-activated receptor alpha
PtC	Phosphatidylcholine
RA	Rheumatoid arthritis
RMM	1,6-Bis[{{(trimethylammonium)ethoxy}phophinyl}-oxy]hexane
s/c	Sub-cutaneous
SAA	Serum amyloid A protein
SAP	Serum amyloid P component
SLE	Systemic lupus erythematosus
TLR	Toll-like receptor
TNF	Tumour necrosis factor
WBC	White blood cell count
VLDL	Very low density lipoprotein
Wt	Wild type
w/v	weight/volume

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8 Introduction

8.2 Introduction

C-reactive protein (CRP), named for its capacity to precipitate the somatic C-polysaccharide (CPS) of *Streptococcus pneumoniae*, was the first acute phase protein to be described, and is an exquisitely sensitive systemic marker of inflammation and tissue damage with broad clinical utility for monitoring and differential diagnosis. As a member of the pentraxin family of plasma proteins, the other major member being serum amyloid P component (SAP), it is part of the lectin fold superfamily of calcium-dependent ligand binding and lectin (carbohydrate binding) proteins [1-3].

In 1930 Tillet and Francis reported the mixing of acute phase sera (i.e. sera obtained from an individual during an acute inflammatory illness) with varying dilutions of *Fraction C*, a non-protein somatic fraction of *Streptococcus pneumoniae*; this fraction was chemically distinct from both the type-specific capsular polysaccharide and the non-type-specific “*somatic nucleoprotein*”. The presence or absence of precipitation was noted and Figure 1 shows the original findings in a subject with pneumococcal pneumonia.

SEROLOGICAL REACTIONS IN PNEUMONIA WITH A NON-
PROTEIN SOMATIC FRACTION OF PNEUMOCOCCUS*

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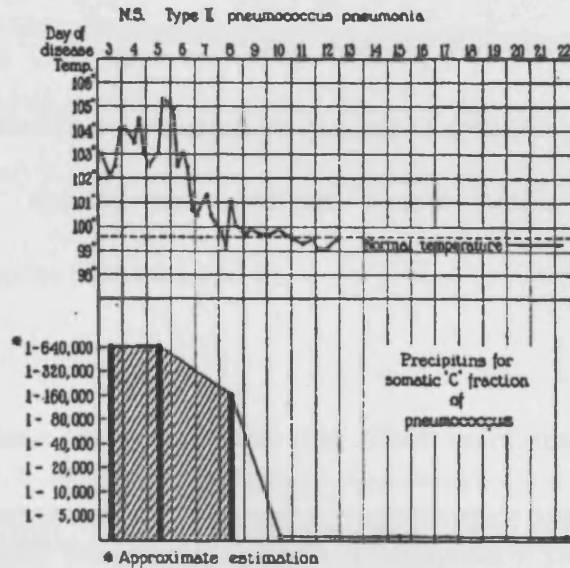


Figure 1 Reproduction of the first demonstration of CRP

- after Tillet and Francis [4]

Their report described how serum obtained on admission to the hospital reacted with high dilutions of Fraction C. This reactive capacity completely disappeared over the next two days, as the disease resolved, mirroring the body temperature. Abernethy, Macleod and Avery in two further papers in the Journal of Experimental Medicine in 1941, showed that “C precipitin” was a protein which was separate from antibodies and required calcium for precipitation [5, 6]. Maclyn Mcarty continued the pivotal work of Avery at the Rockefeller Institute crystallising the protein and raising specific antibodies that were used for detection of CRP in patients [7].

8.3 The Acute Phase Response

The acute phase response is the immediate set of host inflammatory reactions that occur after such challenges as tissue damage, infection, inflammation, and malignant neoplasia [8, 9]. A complex cascade of cytokine production occurs in all endothermic species and mediates a non-specific physiological and biochemical response (Tables 1 and 2).

Table 1 Acute phase phenomena other than effects on plasma proteins

Acute phase phenomena	Description
Neuroendocrine	Fever Increased vasopressin secretion Increased catecholamine secretion
Haematological	Leukocytosis Thrombocytosis Anaemia of chronic disease
Metabolic	Decreased gluconeogenesis Increased lipogenesis and lipolysis Cachexia
Non-protein	Hypozaemia Hypoferraemia Increased retinol and glutathione

The synthesis of a number of proteins is in particular rapidly up-regulated, principally in hepatocytes [10], under the control of cytokines originating at the site of pathology (notably interleukin-1 [IL-1], tumour necrosis factor- α [TNF- α] and interleukin-6 [IL-6]). An acute-phase protein has been defined as one whose plasma or serum concentration increases (positive acute phase proteins) or

decreases (negative acute phase proteins) by at least 25% during inflammatory disorders (Figure 2) [9]. The responses of serum amyloid A protein (SAA) and of the pentraxins, CRP, and, in the mouse, SAP are exquisitely sensitive, even to subclinical processes. Other acute phase proteins include proteinase inhibitors, coagulation, complement, and transport proteins, but the only molecule that displays sensitivity, response speed, and dynamic range comparable to CRP is SAA [11-14].

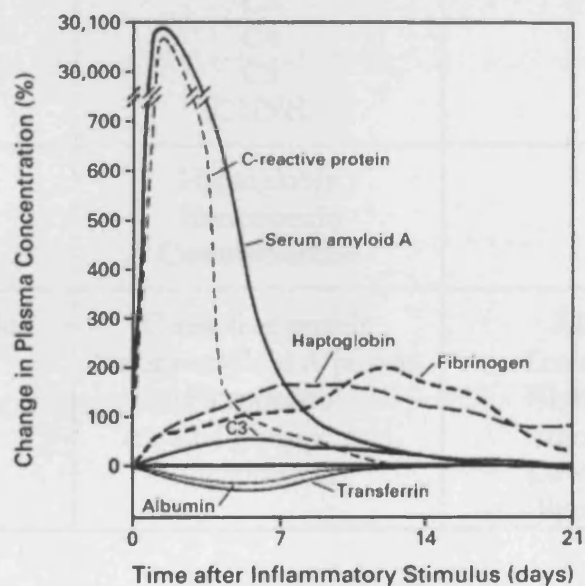


Figure 2 Changes in Plasma Concentrations of Acute Phase Proteins

- after Gabay and Kushner [9]

Table 2 The acute phase response and changes in plasma protein concentration

	Positive acute phase reactants (increased)	Negative acute phase reactants (decreased)
Proteinase inhibitors	α 1-antitrypsin α 1-antichymotrypsin α 2-macroglobulin Antithrombin	Inter α -antitrypsin
Coagulation/fibrinolysis proteins	Fibrinogen Prothrombin Factor VIII Plasminogen	
Complement cascade proteins	C1s C2 B C3 C4 C5 C1INH	Properdin
Transport proteins	Haptoglobin Haemopexin Caeruloplasmin	
Miscellaneous proteins	C-reactive protein Serum amyloid A protein Fibronectin α 1-acid glycoprotein Gc globulin	Albumin Transthyretin High density lipoprotein Low density lipoprotein

The pertinent features of the most widely used direct laboratory markers of the acute phase response are summarised in Table 3. The erythrocyte sedimentation rate (ESR) is an indirect marker of inflammation and is a measure of red blood cell aggregation. Factors influencing the ESR include the haematocrit, plasma viscosity, and the concentration of plasma proteins such as fibrinogen and albumin. It therefore lacks the precision of CRP or SAA.

No clearly defined role for this broad innate response is held, but it probably arose as a means to isolate and neutralise pathogens and prevent further pathogen entry while minimising tissue damage and promoting repair processes, thereby permitting host homeostatic mechanisms to rapidly restore normal physiological function [14]. Although the acute phase response has most likely evolved as a survival mechanism for the short term, its maintenance over the longer term in cases of chronic inflammation may have negative consequences. A good example of this is AA amyloidosis, which has a lifetime incidence of 1-5% among patients with chronic inflammatory disorders such as juvenile and adult rheumatoid arthritis; chronic inflammation results in amyloid deposits containing fragments of the acute phase reactant SAA, with subsequent end organ damage, particularly in the kidney [15].

Table 3 Summary characteristics for CRP, SAA, Albumin and Leukocyte count

Characteristic	CRP [1]	SAA [13]	Albumin [16, 17]	Leukocyte count [17, 18]
Description	Classical pentraxin acute phase protein	Apolipoprotein of high density lipoprotein particles (polymorphic)	Negatively charged protein; major protein component of serum	White blood cells
Biological properties	Calcium dependent ligand binding protein Binds damaged cells and certain micro-organisms Activates complement when ligand bound	Displaces apo-A-I from HDL particles; Involved in cholesterol transport and metabolism, and in modulating immunological responses	Contributes to maintenance of osmotic pressure; Transport protein and assists in the distribution, metabolism, or regulation of a number of marginally soluble substances	Cellular and humoral defence (e.g. antibody and cytokine production)
Main site of synthesis	Hepatic	Hepatic	Hepatic	Bone marrow
Main regulators of synthesis	Interleukins 1 and 6 Tumour necrosis factor alpha	Interleukins 1 and 6 Tumour necrosis factor alpha	Negative acute phase reactant (values fall as cytokines stimulate hepatic synthesis of positive acute phase proteins)	Various cytokines/growth factors
Important associations	Smoking BMI Other acute phase reactants e.g. fibrinogen	Cholesterol BMI Other acute phase reactants e.g. fibrinogen	Smoking BMI Blood pressure	Smoking BMI Blood pressure
Baseline values	Median 1.41mg/l 90th centile 5.82mg/l	Median 3mg/l 90 th centile 10mg/l	40 +/-3.0 g/l	7.0 +/-1.8 x 10 ⁹ /l
Self-correlation (r)	~0.6	Not reported	~0.5	~0.7
Inflammatory response	~10000 fold rise	~1000 fold rise	20% fall	3 fold rise

8.4 C-reactive protein in health and disease

In healthy volunteer blood donors the median concentration of CRP is 0.8 mg/l [19], but following an acute phase stimulus, values may increase by the order of 10000 fold, with *de novo* hepatic synthesis starting very rapidly, serum concentrations beginning to rise by about 6 hours, and peaking around 48 hours after a single stimulus [20]. In the general, ostensibly healthy, population the median baseline value is slightly higher and tends to increase with age; females are often reported as having slightly higher circulating concentrations, perhaps secondary to oestrogen effects on CRP synthesis; however the precise characteristics of the population studied is important and not all studies show a gender difference [21-24]. In most, but not all diseases (Table 4) the circulating value of CRP much more accurately reflects on-going inflammation than do other biochemical parameters of inflammation, such as plasma viscosity or the erythrocyte sedimentation rate. This is because the plasma half-life of CRP is the same, at about 19 hours, under all conditions, and the sole determinant of the plasma concentration is therefore the synthesis rate, which, in turn, reflects the intensity of the pathological process(es) stimulating CRP production [25]. *In vivo* turnover studies of human CRP in man did not demonstrate any detectable tissue deposition of CRP, even in inflamed or infected foci [25], and in animal studies the only significant cellular site of CRP clearance and catabolism was the hepatocyte [26].

Table 4 Clinical disease and the CRP response

<i>Major CRP response</i>	
Infections	Bacterial Fungal Mycobacterial Viral (severe or systemic)
Hypersensitivity complications of infection	Rheumatic fever Erythema nodosum
Acquired and inherited inflammatory diseases	Rheumatoid arthritis Juvenile chronic arthritis Ankylosing spondylitis Psoriatic arthritis Systemic vasculitis Polymyalgia rheumatica Reiter's disease Crohn's disease Familial Mediterranean fever
Tissue necrosis	Myocardial infarction Tumour embolisation Acute pancreatitis
Trauma	Surgery Burns Fractures
Neoplasia	Lymphoma Carcinoma Sarcoma
<i>Modest/absent CRP response</i>	
Systemic lupus erythematosus Scleroderma Dermatomyositis Ulcerative colitis Leukaemia Graft-versus-host disease	

Liver failure impairs CRP production [27], but no other intercurrent pathologies and very few drugs reduce CRP values unless they also affect the underlying acute phase stimulus. The CRP value is thus a very useful non-specific biochemical marker of inflammation, measurement of which contributes importantly to (Table 5):

- Screening for organic disease;
- Monitoring the response to treatment of inflammation and infection, and
- Detecting intercurrent infection in the few specific diseases characterised by modest or absent acute phase responses to those diseases themselves.

It is not known why systemic lupus erythematosus (SLE) and the other conditions listed in Table 4 fail to elicit major CRP production, despite evident inflammation and tissue damage [28], nor why the CRP responses to intercurrent infection are apparently intact in such patients [29]. Animal models of lupus mirror this observation, with levels of murine SAP (the equivalent acute phase protein in mice) not rising in response to progression of autoimmune lupus-like disease in (NZB x NZW) F1 mice [30]. Furthermore just as in human lupus, where the occurrence of intercurrent microbial infection can stimulate an acute-phase response, so injection of bacterial lipopolysaccharide or casein also stimulated "normal" acute phase SAP production [30].

Table 5 Routine and speculated uses for CRP measurement

Clinical uses of CRP
<p><i>Screening assay for organic disease</i></p>
<p><i>Assessing disease activity in inflammatory conditions</i></p> <p style="text-align: center;"> Juvenile chronic arthritis Rheumatoid arthritis Ankylosing spondylitis Reiter's syndrome Psoriatic arthropathy Vasculitides Crohn's disease Rheumatic fever Familial fevers including familial Mediterranean fever Acute pancreatitis </p>
<p><i>Diagnosis and management of infections</i></p> <p style="text-align: center;"> Bacterial endocarditis Neonatal septicaemia and meningitis Intercurrent infection in systemic lupus erythematosus Intercurrent infection in leukaemia and its treatment Postoperative complications including infection and thromboembolism </p>
<p><i>Differential diagnosis / classification of inflammatory disease</i></p> <p style="text-align: center;"> Systemic lupus erythematosus versus rheumatoid arthritis Crohn's disease versus ulcerative colitis </p>
<p><i>Risk prediction in cardiovascular disease (Speculative)</i></p> <p style="text-align: center;"> Long term future predictive value of CRP in healthy populations Outcome of patients with acute coronary syndromes and following invasive coronary procedures Instigation of statin therapy for the primary prevention of cardiovascular disease </p>

8.5 Structure and function

Human CRP is a calcium dependent ligand binding protein, which binds with highest affinity to phosphocholine (PC) residues, as well as a variety of other autologous and extrinsic ligands, and aggregates or precipitates the cellular, particulate or molecular structures bearing these ligands [31-35]. The known ligands can be divided into three major groups: 1) compounds that contain phosphocholine or related structures [36-41], 2) polycationic compounds such as poly-L-lysine and protamine sulphate [42, 43], and 3) carbohydrates that contain D-galactose-related structures [44]. Autologous ligands include native and modified plasma lipoproteins, damaged cell membranes, a number of different phospholipids and related compounds, small nuclear ribonucleoprotein particles, and apoptotic cells. Extrinsic ligands include many glycan, phospholipid and other components of micro-organisms, such as capsular and somatic components of bacteria, fungi and parasites, as well as plant products [1, 45, 46]. When human CRP is ligand bound, it is recognized by C1q and it potently activates the classical complement pathway, engaging C3, the main adhesion molecule of the complement system, and the terminal membrane attack complex, C5-C9 [47-52]. Bound CRP may also provide secondary binding sites for factor H, and thereby regulate alternative pathway amplification and C5 convertases [53-56] (see Figure 88, Chapter 13). The secondary effects of CRP that follow ligand binding resemble some of the key properties of antibodies, suggesting that under various circumstances CRP may contribute to host defence against infection, function as a pro-inflammatory mediator, and participate in physiological and pathophysiological handling of autologous constituents [32, 57-60] (Figure 3).

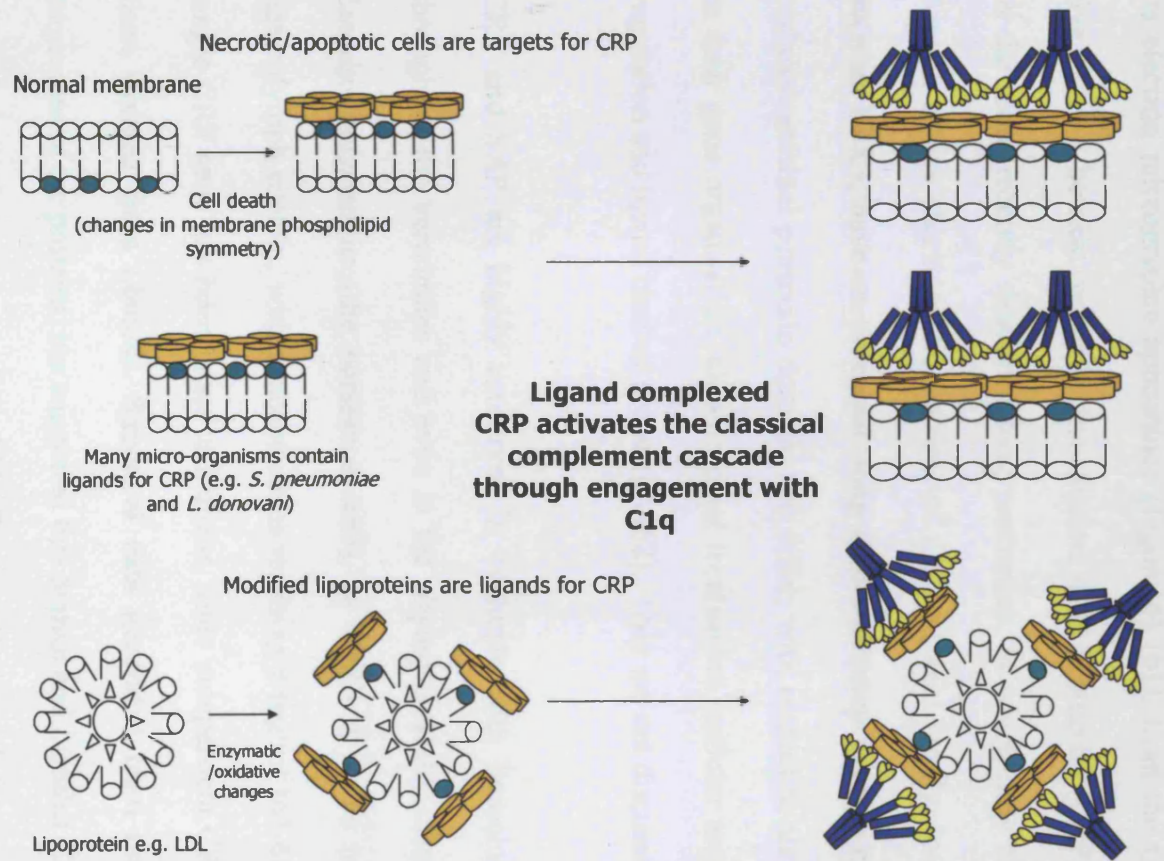


Figure 3 Representation of suggested functions of CRP

8.5.1 Phylogeny

The pentraxins are a superfamily of conserved proteins that are characterised by a cyclic multimeric structure [12]. This family of proteins, named originally for its electron micrographic appearance (Figure 4) [61], from the Greek *penta* – five, *ragos* – berries, comprises the classical short pentraxins (CRP/SAP) as well as the more recently described long pentraxins, in which only the C-terminal domain shows characteristic features of the pentraxin family; long pentraxins, such as PTX3, have an unrelated, long amino-terminal domain coupled to the carboxy-terminal pentraxin domain, and differ, with respect to short pentraxins, in their gene organisation, chromosomal localisation, cellular source, synthetic regulation and ligand binding properties [62]. They are not discussed further.

CRP and SAP are highly conserved in evolution with homologous proteins throughout the vertebrates and even in the phylogenetically distant arthropod, *Limulus polyphemus* (the horseshoe crab), in which it is the most abundant haemolymph protein, with concentrations reaching 6 mg/ml [63-68]. Whereas a single CRP gene has been found in humans, three independent CRP genes have been isolated from *Limulus*. Structural data generated from the amino acid sequences of the proteins, has suggested that human and *Limulus* CRPs' diverged about 500 million years ago [69, 70]. Structurally the calcium-mediated ligand binding by CRP, human SAP and *Limulus polyphemus* CRP is similar, but sequence differences in the hydrophobic pocket explain the differential ligand specificities exhibited by the homologous proteins. Insertions and deletions

elsewhere most probably account for the different hexameric quaternary structure of *Limulus polyphemus* CRP.

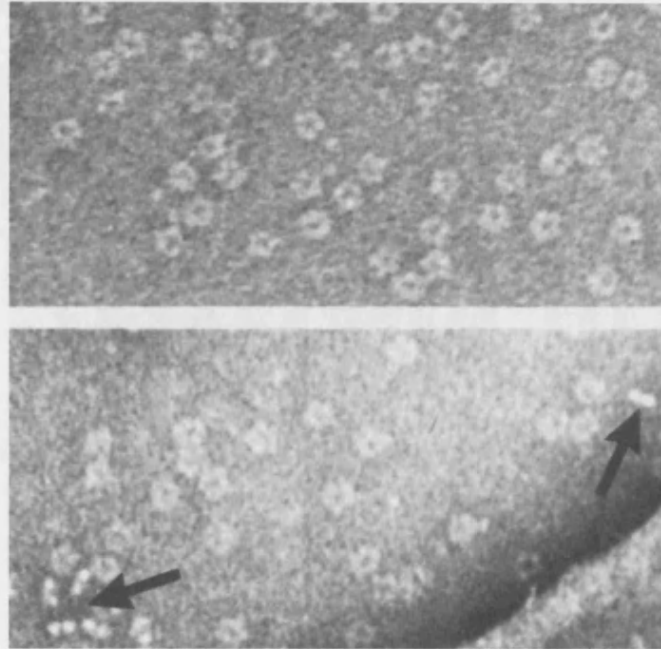


Figure 4 Negatively stained electron micrograph of human CRP

- this shows the typical pentameric disc like structure face on and side on (arrows) [3].

The cyclic pentameric or hexameric arrangements with several equivalent binding sites, one on each subunit, is important for the biological functions of pentraxins, which involve binding or linking several cells or molecules together to form a stable multicomponent system [71].

In contrast to man, mouse CRP is a trace protein the concentration of which increases only modestly in the acute phase response to a maximum of about

2 mg/l [72]. No mouse CRP knockout has yet been made, and *in vivo* work on CRP function has largely been confined to mice transgenic for rabbit [73] or human CRP [74]. It is important to be aware that artefactual heterologous systems may not provide physiologically relevant information. In spite of the evolutionary conservation of sequence, subunit organisation and protein fold, there are major structural and functional differences between CRP's of different species, including glycosylation, capacity to activate autologous complement, and regulation of basal and acute phase synthesis. For example rat CRP closely resembles human CRP in its amino acid composition, in having five subunits per molecule and in its electron microscopic appearance as a pentameric annular disc. It differs, however, from all other mammalian CRP's in being a glycoprotein bearing a single complex oligosaccharide on each polypeptide subunit. Furthermore one pair of its subunits per molecule is linked by an interchain disulphide bridge whereas in other animals the subunits of both CRP and SAP are all non-covalently associated [75]. Another species difference is seen with rabbit CRP, which in acute phase hyperlipidaemic serum, circulates in association with very low density lipoproteins [76, 77], and is therefore unlike human CRP which is always uncomplexed [78]. Rabbit CRP however, unlike rat CRP, behaves more like human CRP in terms of its dynamic changes during an acute phase response [79, 80]. These and other such differences therefore command extreme caution in extrapolating from animal models to man, and generate difficulties in designing robust experiments to investigate the properties of pentraxins.

8.5.2 Serum amyloid P component

SAP, named for its universal presence in amyloid deposits [81], is a constitutive, non-acute phase plasma glycoprotein in man and all other species studied, except the mouse in which it is a major acute phase protein [11]. SAP binds to DNA in chromatin exposed to or released in the extracellular environment by apoptosis or necrosis, stabilises it, and protects it from degradation [82-84]. SAP deficient mice, created by gene targeting, degrade chromatin more rapidly than normal, have enhanced antibody responses to exogenous chromatin, and spontaneously develop marked anti-chromatin autoimmunity [85]. However it has become clear latterly that the autoimmunity seems to be independent of SAP deficiency, and reflects murine strain effects, namely expression of 129/Sv chromosome 1 genes on a C57BL/6 background [86-88]. This point reiterates the extreme caution needed in interpretation of models in which manipulation of protein expression occurs in artefactual and heterologous systems, including the possibility of unexpected effects of interstrain genetic interactions or ectopic protein expression.

Other properties of SAP relate to its binding affinity for bacteria such as *Streptococcus pyogenes*, as well as *Neisseria meningitidis* and rough variants of *Escherichia coli*. This binding has powerful antiopsonic effects, reducing phagocytosis and killing of bacteria and thus enhances their virulence [89].

Finally SAP binds to all types of amyloid fibrils, the abnormal protein aggregates responsible for the amyloidosis diseases [90, 91]. Binding of SAP stabilises

amyloid fibrils *in vitro* [92] and in mice appears to contribute to the pathogenesis of amyloidosis (as shown by delayed and reduced amyloid deposition in SAP deficient mice) [93]. These observations taken together generated the impetus to design a low molecular weight molecule capable of pharmacological depletion of SAP, with the aim of developing a novel treatment for human amyloidosis. This has now entered clinical studies and will robustly test the hypothesis in patients [94].

8.5.3 Innate immunity

The conservation of the structure of CRP and of its calcium-dependent specific binding of ligands containing PC and related substances, together with the lack of any known deficiency or protein polymorphism, suggest that this protein must have had survival value. Microbial infection is a major driving force of change during evolution, and CRP has many features compatible with an important role in innate immunity.

The innate immune system discriminates self from non-self using a restricted number of pattern recognition receptors that recognise pathogen associated molecular patterns, and most micro-organisms that penetrate the body's external barriers are recognised and cleared by cells and molecules which exhibit these broad specificities [95-97]. If infectious organisms evade these mechanisms, the specific antigen receptor-bearing lymphocytes of the adaptive immune system come into play, and significant subsets of lymphocytes, even in previously

unexposed animals, express germ line encoded specificity for immunogenic epitopes of pathogens. The T-cell independent natural IgM antibodies produced by the progeny of these cells comprise a significant proportion of serum immunoglobulins at birth, and can both provide protection against some viral and bacterial infections [98], and play a role, via complement activation in enhancing specific adaptive T cell dependent antibody production [99]. The antigen-specific receptors of the natural antibody producing lymphocytes often contain highly conserved sequences, and represent an “evolutionary memory” bridging pattern recognition molecules and the phylogenetically more recent and sophisticated adaptive responses. Importantly, many of the natural antibodies encoded by nonmutated germline V_H or V_L genes react with a variety of self-determinants, including carbohydrates and glycolipids, as well as cross-reacting with bacterial or oncofoetal antigens. They may thus have a significant role in handling autologous ligands as well as extrinsic antigens.

One important class of highly conserved natural antibodies expresses the so-called T15 idiotype, binds to PC as does CRP, and like CRP protects against *Streptococcus pneumoniae* infection [100-103]. PC is a component of many prokaryotes and is universally present in eukaryotes [104]. In *Streptococcus pneumoniae*, PC is present in the somatic ribitol teichoic acid component [37] and is associated with different sugar residues in a variety of other organisms, including other *Streptococci*, *Clostridium*, and *Bacillus* species, and *H. influenzae* [105, 106]. PC is also found in the external components of a variety of pathogenic protozoa, fungi, nematodes, and other intestinal parasites [107]. PC is ubiquitous in the phospholipids of cellular membranes of higher

animals, and importantly also in the circulating plasma lipoproteins that are intimately involved in the pathogenesis of atherosclerosis [108]. The specific binding of CRP to low density and very low density lipoproteins (LDL and VLDL), [109-111] and to the membranes of damaged cells has long been recognised, and more recently it has been observed that CRP also recognises apoptotic cells in a Ca^{2+} dependent manner, enhancing their opsonisation and phagocytosis by macrophages [59]. The original finding that native, non-aggregated, human CRP does not bind to native LDL has lately been confirmed and extended with the observation that native CRP does bind to oxidised LDL [112]. This, as well as the binding of CRP to apoptotic cells and to oxidised but not to non-oxidised phosphatidyl choline (PtC), is mediated by recognition of exposed PC residues. The interaction between CRP and cholesterol in lipoprotein particles in particular is reportedly mediated by the PC binding site of CRP and the 3-beta-hydroxyl group of cholesterol [113]. The spectrum of ligand recognition by CRP closely resembles that of EO6, an autoantibody to oxidised LDL, which is a T15 clono-specific anti-PC antibody with specific binding to PC on oxidised but not on native PtC [114, 115].

An important physiological role of CRP may thus be in handling of autologous materials including oxidised PC-bearing phospholipids within oxidised LDL and on the plasma membranes of apoptotic cells. Similarly, pathophysiological effects of CRP may also be mediated via these interactions with autologous ligands [116]. Intriguingly, the spectrum of autologous ligand recognition by CRP overlaps that of pathogenic anti-phospholipid autoantibodies [117].

8.5.4 Structure

The human CRP molecule (M_r 115,135) is composed of 5 identical non-glycosylated polypeptide subunits (M_r 23,027) each containing 206 amino acid residues. The protomers are non-covalently associated in an annular configuration with cyclic pentameric symmetry. The CRP protomer has the characteristic “lectin fold” composed of a two layered β sheet with flattened jellyroll topology. Two calcium ions are bound 4Å apart by protein side-chains coming from loops at the concave face, termed the *B* face as this is the site of ligand binding. The other, *A*, face carries a single α helix (Figure 5) [32, 118].

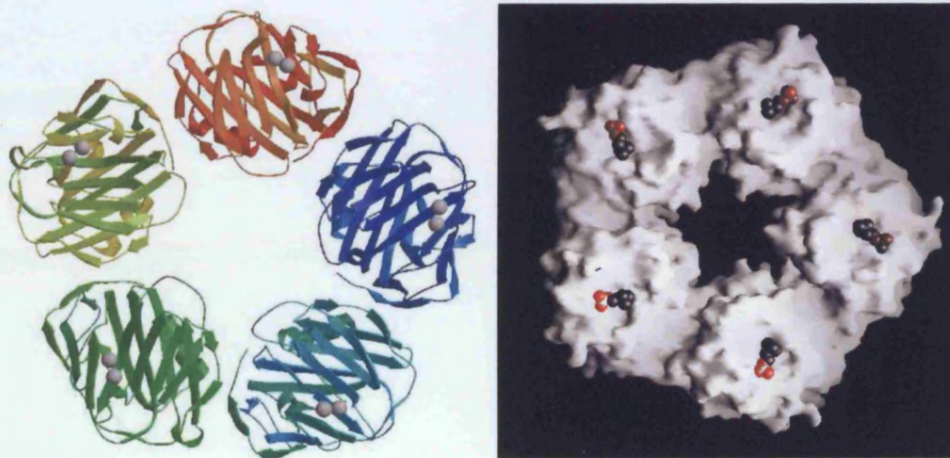


Figure 5 Ribbon diagram and space filling model of CRP

- The ribbon diagram on the left shows the lectin fold with two calcium atoms (spheres) in the ligand binding site of each protomer; the space filling model (right) shows a single phosphocholine molecule located in the ligand binding site of each protomer [3].

The crystallographic structure of the CRP-PC complex, and indirect evidence from mutagenesis studies, show that key residues in the ligand binding pocket responsible for recognition of PC are Phe66 and Glu88 [119, 120]. C1q, the recognition protein of the classical pathway, probably binds to complexed or aggregated CRP in a pocket at the open end of a cleft on the A faces of the protomers in the intact pentamer. Mutagenesis studies of CRP suggest that Asp112 and Tyr175 are important contact residues for C1q binding, that Glu88 influences the conformational change in C1q necessary for complement activation, and that Asn158 and His38 probably contribute to the correct geometry of the binding site [121, 122].

In vitro studies have suggested that some bioactivities of CRP are expressed on loss of the pentameric symmetry, resulting in formation of modified or monomeric CRP (that has distinct antigenic and physicochemical characteristics) [123-127]. The lack of any *in vivo* evidence for the presence of modified CRP, along with the knowledge of the robustness of the non-covalent association of the monomers (6-8M urea chelation or acid treatment being required to generate monomers), suggests that great caution should be exercised before accepting a biological function for modified CRP.

8.5.5 Genes and gene expression

The genes for both CRP and SAP are on chromosome 1, and the CRP gene consists of a coding sequence for a signal peptide of 18 residues and the first 2

amino acids of the native protein, followed by an intron of 278 base pairs and then the coding sequence for the remaining 204 residues [128-130]. A single non functional pseudogene [131] with 50-80% region-specific homology is found close to the authentic CRP gene and typical promoter sequences are located upstream of the cap region (104 nucleotides from the start of the signal peptide). Plasma CRP is produced by hepatocytes [10], although other sites for CRP synthesis and possibly secretion have been suggested [132-136]. CRP may thus have local roles in particular micro-environments as well as its functions effected via the systemic circulation.

Increased CRP production is induced predominantly by the cytokine IL-6, but either IL-1 or TNF- α may also contribute [137, 138]. Control of CRP expression is principally at the level of transcription and *in vitro* studies in hepatocyte cell lines have identified some of the intra-cellular signalling pathways [139-141] and also shown that secretion is more efficient during an acute phase response [142]. Thus cell culture studies investigating the transcription of CRP in Hep 3B cells suggest expression is induced by IL-6, acting through C/EBP isoforms (CCAAT-enhancer-binding proteins) and STAT3. STAT (signal transducers and activators of transcription) family members, induce their effects by binding to palindromic sequences with TT(N)5AA motifs and an IL-6 responsive element in the proximal region of the human CRP 5'-flanking region that bears a TT(N)4AA motif, has been demonstrated and termed the CRP acute phase response element. IL-1 β , which alone has no effect, greatly enhances IL-6-induced transcription and IL-1 β activates the NF- κ B system. This is suggested to involve IL-1 β induced nuclear translocation of p50-containing dimers; p50 interacts with

C/EBP β activated by both IL-6 and IL-1 β to induce CRP expression. Binding of Rel p50 to the nonconsensus κ B site enhances and stabilises binding of C/EBP β to the CRP promoter and binding of both C/EBP β and p50 to their overlapping cognate sites is required for induction of CRP expression by IL-6. More recently a protein kinase C dependent transcriptional regulation of CRP gene expression has also been demonstrated in cell culture [143]. Such knowledge of the cell biology may of course be used to identify inhibitors of CRP synthesis.

Healthy subjects tend to have rather stable, individual, baseline CRP concentrations [144], that in family studies are significantly 35-40% heritable [145]. Predictably there are significantly higher correlations of CRP (and SAA) values among monozygotic than among dizygotic twins [146], where the estimated heritability reaches ~50% for CRP [146]. Nevertheless CRP values remain strongly related to other factors such as total and central abdominal obesity, blood pressure, and lipid levels, independent even of the genetic influences seen in monozygotic twins [147].

Although human CRP is notable for the lack of reported deficiency states or protein polymorphisms [148], associations between CRP production and genetic polymorphisms in IL-1 [149], IL-6 [150], tumour necrosis factor [151] and apoE [152-155] have been reported. Increasing information is now also available on non-coding genetic polymorphisms within the CRP gene itself. In one large prospective cohort of apparently healthy men, plasma CRP concentrations were shown to be significantly reduced among carriers of a 1059G/C polymorphism in the human CRP gene (GC or CC) as compared with non-carriers (GG).

However, there was no significant association with risk of arterial thrombosis [156]. When the genotypes of two common polymorphisms within the CRP gene, an exonic 1059G/C and an intronic T/A base substitution were assessed in a moderately sized community-based study, there were effects, albeit small, but persistent even after adjustment for age, sex, body mass index, ethnicity, hypertension, smoking, diabetes, hyperlipidaemia, and aspirin use, on baseline CRP values: GG versus GC or CC genotypes, median CRP levels 0.22 vs 0.15 mg/l and TT versus AT versus AA genotypes, median CRP levels 0.19 vs. 0.23 vs. 0.24 mg/l [157]. A +1444C/T polymorphism in the CRP gene has also been associated with differences in CRP concentration and one study suggests an effect of this polymorphism on the CRP response to an inflammatory stimulus. Individuals homozygous for the +1444T allele showed higher CRP concentrations compared with C-allele carriers after periodontal therapy [158]. A polymorphic GT repeat in the intron of the CRP gene is reportedly also associated with differences in baseline CRP concentrations in both normal individuals and in patients with SLE [159], and with susceptibility to invasive pneumococcal disease [160]. Similarly a polymorphism at the CRP gene locus (in man the genes for CRP and SAP map to 1q23.2 within an interval linked with SLE) influenced gene expression and predisposed patients to SLE [161].

Specific genetic polymorphisms that reliably correlate both with baseline CRP concentrations and/or CRP production in the acute phase response will increasingly be used to test, through genetic association studies, the case for a pathogenetic role of CRP in inflammatory disease [162-167].

8.6 C-reactive protein and coronary heart disease

Inflammation has a pivotal role in atherothrombotic cardiovascular disease and individuals with coronary artery disease have elevated markers of inflammation such as CRP [168, 169] (Figure 6). Earlier work suggested a prognostic association between increased CRP production and outcome after acute myocardial infarction [170] and in acute coronary syndromes [171]. In the mid 1990's immunoassays for CRP, with greater sensitivity than those previously in routine use, revealed a powerful predictive relationship between increased CRP production, even within the range previously considered to be normal, and atherothrombotic events [172-186]. Circulating CRP values correlate closely with other markers of inflammation, some of which show similar, albeit generally less significant, predictive associations, and baseline CRP values also reflect metabolic states associated with atherothrombotic events [16, 17, 179].

Hence there is a powerful predictive association between raised serum CRP values and the outcome of acute coronary syndromes, and, remarkably, between even modestly increased CRP production and future atherothrombotic events in otherwise healthy individuals. Meta-analysis of all published studies up to the year 2000, comprising a total of 1,953 coronary events, showed a relative risk of approximately 2.0 for a future coronary event in subjects with a single initial base-line CRP value in the upper third compared with those in the lower third of the distribution in the general population. (tertile cut offs, >2.4 vs. <0.9 mg/l) [179].

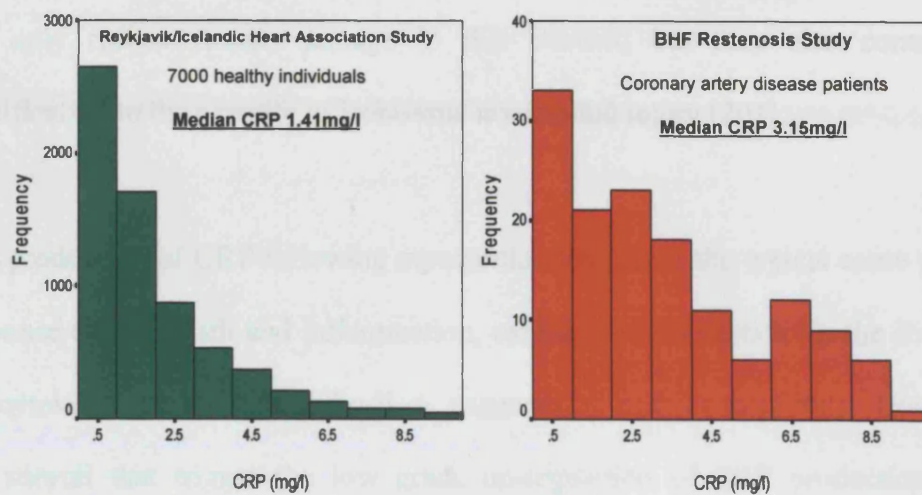


Figure 6 Higher baseline CRP values in those with established coronary disease

- In two studies where I measured CRP values, when comparing the population distribution of CRP in healthy individuals [168], with those known to have coronary artery disease [169], a raised median CRP concentration is evident.

CRP itself is particularly interesting with respect to cardiovascular biology and pathology because not only does it bind selectively to LDL [111], especially oxidised and enzyme modified LDL as found in atheromatous plaques [187], but it is deposited in the majority of such plaques [188-190] and it has a range of pro-inflammatory properties that could potentially contribute to the pathogenesis, progression and complications of atheroma [3, 191]. Tissue necrosis is a potent acute phase stimulus, and following myocardial infarction, there is a major CRP response the magnitude of which reflects the extent of myocardial necrosis [20, 170]. Furthermore the peak CRP values at around 48 hours after the onset, powerfully predict outcome after myocardial infarction [170, 192-195].

Importantly, CRP is deposited, with complement, within all acute myocardial infarcts [196-200], and experimental evidence suggests that the CRP response not only reflects tissue damage in this context, but may also contribute significantly to the severity of ischaemic myocardial injury [201].

The production of CRP following myocardial necrosis is the typical acute phase response to cell death and inflammation, mediated by the action on the liver of the cytokine cascade, especially IL-6, triggered by such events [202]. However the stimuli that trigger the low grade up-regulation of CRP production that predicts coronary events in general populations [168, 179, 203, 204], or the more substantial CRP values associated with poor prognosis in severe unstable angina [172, 205-208] or after angioplasty [209, 210], are not clear (Figure 7). The association with future stroke as well as the outcome following stroke [211-214], along with the ability of CRP to powerfully predict outcome in chronic renal disease also require explanation [215-217]. Potential acute phase stimuli may arise from inflammation within atheromatous lesions themselves, and thus reflect their extent and/or severity or instability [218]. However atherosclerotic burden *per se* as determined by coronary artery calcification, for example, does not necessarily always correlate with CRP concentrations [219-221], although one analysis from the Framingham Heart Study did find an association between CRP and coronary calcification [222].

The distinction between so called “disease markers” and “process markers” is important particularly with respect to inflammation, not least because our concepts of the underlying disease processes continue to evolve. Measures of

inflammation, such as CRP, and indices of atherosclerosis, such as coronary artery calcification may provide quite distinct information regarding cardiovascular risk. Hence, for example, the evidence that there is widespread activation of neutrophils across the coronary vascular beds of unstable angina patients, challenges the concept of a single vulnerable plaque in unstable coronary syndromes [223]. Similarly in the CAPTURE study (Chimeric c7E3 AntiPlatelet Therapy in Unstable angina REfractory to standard treatment trial) [224] troponin T, but not CRP, was predictive of cardiac risk during the initial 72 hour period, whereas CRP was an independent predictor of both cardiac risk and repeated coronary revascularisation (coronary artery bypass graft surgery and percutaneous transluminal coronary angioplasty) during the six months of follow-up. At four year review [225], both elevated troponin T and CRP were associated with impaired outcome, independently of other established risk factors, but with a different time course. Elevated troponin was associated with increased procedure related risk, and elevated CRP with increased risk for subsequent events.

Alternatively increased production of CRP may reflect inflammation elsewhere in the body, although there is no strong correlation with serological evidence of the various chronic microbial infections, such as *C. pneumoniae* and *H. pylori*, that have been putatively linked with coronary heart disease [179]. Indeed, within what was until recently accepted as the reference range for circulating CRP concentration, up to 5 or 10 mg/l [19], higher values have now been found to be strongly associated with increased body mass index [226, 227] and, also with many features of the insulin resistance or metabolic syndrome [228-232], up

to and including frank diabetes mellitus [233-235]. This may reflect in part the fact that adipocytes are the source of a substantial portion of baseline IL-6 production [236], but in general it suggests that some or even most of the inflammatory marker profile associated with increased atherothrombotic risk in the population at large, may not be triggered by inflammation or tissue damage in the classical sense. Rather it may be a sign of a particular metabolic state which happens also to be pro-atherogenic, or at least predisposing to atherothrombotic events [237]. Indeed, CRP production predicts the development of type 2 diabetes independently of traditional risk factors [238]. In insulin resistant obese individuals, elevated CRP values fall in parallel with improvements in insulin resistance associated with weight loss, but the association between CRP and insulin resistance is independent of body mass [239]. Oral contraceptive use [229] and systemic but not transdermal post-menopausal hormone replacement therapy [240-244] are also associated with significantly raised baseline CRP concentrations without any sign of tissue-damaging inflammation. Other reported associations with elevated baseline CRP values include periodontal disease, smoking, consumption of coffee, and stress [16, 245-250]. Also, lately, other conditions not classically viewed as involving inflammation have been associated with elevations in CRP concentration, for example, atrial fibrillation [251-253]. Weight loss predictably leads to lower baseline CRP concentrations [254-256], as does moderate alcohol intake [257-259] and there is an association between exercise and reductions in CRP production [260-262].

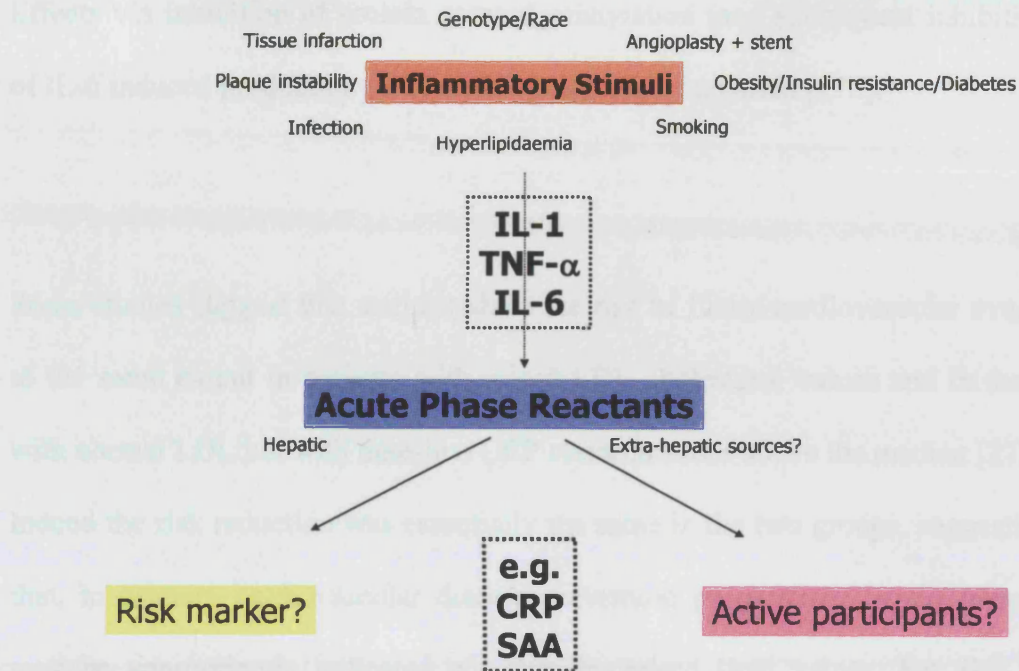


Figure 7 Sources and roles of acute phase reactants in cardiovascular disease

Of note, is the class effect of HMG CoA-reductase inhibitors (statins), which reproducibly reduce CRP values, independent of their effects on lipid values [263-267]. A similar effect seems to occur with the fibrates [268, 269]. As well as possible reductions in atherosclerotic plaque inflammation [218] direct hepatocyte effects seem important. PPAR α -activators (e.g. fenofibrate) notably directly suppress IL-1-induced but not IL-6-induced expression of CRP in cell culture [270] and it appears that the anti-inflammatory action of statins and fibrates on human CRP expression in hepatocytes is based on up-regulation of the cytosolic inhibitor of NF κ B, I κ B α , resulting in reduced NF κ B activity. Administration of statins or PPAR α activators to human CRP transgenic mice results in lowered basal and IL-1 β -induced human CRP gene expression [271].

Effects via inhibition of protein geranylgeranylation (and subsequent inhibition of IL-6 induced production of CRP) have also been suggested [272].

Some studies suggest that statins reduce the risk of future cardiovascular events to the same extent in patients with raised LDL cholesterol values and in those with normal LDL but with base-line CRP concentrations above the median [273]. Indeed the risk reduction was essentially the same in the two groups, suggesting that, in primary cardiovascular disease prevention programmes, statin therapy may be appropriately indicated not only by raised lipid values, but also by increased CRP concentrations [274]. Ridker *et al.* have now published findings they suggest demonstrate that patients with acute coronary syndromes in whom low CRP levels are achieved after statin therapy have a decreased risk of recurrent myocardial infarction or death, regardless of the levels of LDL cholesterol attained. The observed benefit was largely attributed to the anti-inflammatory effects of statins, as assessed by CRP values. Hence the controversial suggestion that strategies to lower cardiovascular risk with statins should include monitoring CRP as well as cholesterol [275].

Ridker has also extensively propagated the idea that increased CRP values identify individuals at risk who are not detected, for example, by the Framingham risk score [276, 277]. Such a suggestion, and the proposed change in clinical practice, however requires further extensive work. It is critically important to recognise that the CRP response is non-specific and is triggered by

many disorders unrelated to cardiovascular disease. In using CRP for coronary heart disease risk assessment it is therefore essential to clearly establish true baseline CRP values, not distorted by either trivial or serious intercurrent pathologies. At the individual level, as opposed to epidemiological studies, this requires at least a proper history and physical examination of the patient, and possibly also appropriate relevant investigations, together with 2 or 3 serial CRP measurements if the first result is in the higher risk range (>2.5 mg/l) (Table 6). If the CRP value persistently remains above 10 mg/l, indicating the presence of a significant acute-phase response, a full history and physical examination of the patient is indicated, ideally together with relevant investigations, to determine the cause and alleviate it if possible [3]. Interestingly though, chronic inflammatory conditions, such as rheumatoid arthritis and haemodialysis for end-stage renal failure, that are characterised by persistently elevated CRP concentrations in some individuals, are associated with premature cardiovascular disease [278, 279].

Table 6 Present suggested use of CRP in cardiovascular disease

Summary box (1)
<ul style="list-style-type: none">• Raised baseline CRP values predict coronary events, stroke, and progression of peripheral disease• Relative risk of coronary event is 2.0 for single baseline CRP concentration >2.4 mg/l versus <1 mg/l• Increased CRP production may reflect:<ul style="list-style-type: none">- Inflammation in atherosclerotic plaques?- Total atherosclerotic burden?- Inflammation elsewhere in the body?- Metabolic status rather than inflammation?- Individual higher CRP responsiveness to prevalent endogenous or environmental acute phase stimuli?• CRP is not uniquely associated with cardiovascular disease; other systemic markers of inflammation show similar associations, though less marked than with CRP

8.6.1 C-reactive protein and pathogenesis of atherosclerosis

The original identification of the possibility that CRP may contribute to pathogenesis of atherosclerosis [110, 111], has lately become the focus of much work in this field [280]. The binding of CRP to lipids, especially lecithin (phosphatidyl choline), and to plasma lipoproteins, especially what was formerly

called β -lipoprotein, has been known for over 60 years [281, 282], and the first suggestion of a possible relationship to atherosclerosis came when it was demonstrated that aggregated, but not native, non-aggregated, CRP selectively bound just LDL and some VLDL from whole serum [111]. However native CRP does bind to partially degraded, so-called modified LDL [187], as it is found in atheromatous plaques, and to oxidised LDL [112, 283]. Furthermore CRP is present in most such plaques examined *ex vivo* [188, 189]. This CRP could contribute to complement activation in the plaques [56], and there is experimental evidence supporting a possible role of complement in atherogenesis [284-286]. CRP has also been reported to stimulate tissue factor production by peripheral blood monocytes and could thereby have important pro-coagulant effects [287, 288]. However this latter action of CRP has not been well defined or robustly controlled [289]; for example, all the published work has been done with commercially sourced CRP of incompletely defined provenance and purity, and there have been few robust specificity controls. Nevertheless if the phenomenon is reproducible it provides a possible direct link between increased CRP production and atherothrombotic events. Similarly it has been claimed that CRP is recognized by a subset of cellular Fc(γ) receptors and could thereby engage multiple processes of inflammation [189, 290-294]. One study using ultrasensitive confocal fluorescence microscopy claims only a weak direct interaction with the Fc(γ)RIIa receptor, which is significantly enhanced in the presence of antibodies to CRP [295] and more robustly controlled studies, using recombinant and highly purified CRP and avoiding use of whole IgG anti-CRP antibodies, do not confirm such interactions with human cells at all [296-298].

Endothelial dysfunction, a marker of atherosclerosis related to coronary events, is associated in epidemiological studies with markers of systemic inflammation including CRP production [299, 300], and CRP has been reported to have direct effects on both inducible and constitutive endothelial nitric oxide synthesis [301-303]. We for example demonstrated (with careful specificity controls) that pure human CRP has specific, direct effects on vascular function *in vitro* via increased NO production [304]. However the *in vivo* relevance of this is unclear. Expression of adhesion molecules in endothelial cell cultures is also reportedly increased by *in vitro* exposure to CRP [305-307], as are angiotensin type 1 receptors in vascular smooth muscle [308]. The expression and activity of plasminogen activator inhibitor-1 by human aortic endothelial cells has also been claimed to be upregulated by CRP [309]. CRP values are clearly related to the development, severity and progress of coronary artery disease in transplanted hearts, and immunohistochemical detection of arterial endothelial ICAM-1 was associated with elevated serum CRP concentrations [310]. In other studies addition of CRP to LDL in cell culture systems apparently stimulates the formation of foam cells, typical of atherosclerotic plaques. It is not known whether this reflects opsonisation of the LDL particles by CRP or an effect of CRP on the phagocytic cells themselves [311, 312].

All these experimental *in vitro* observations, and many others [313-325], must be treated with great caution [326] until the purity and integrity of the CRP used have been rigorously established and the specificity of the observed effects formally established, for example by the use of specific CRP absorbents, ligands, antibodies and inhibitors of binding. Thus for example the finding that sodium

azide and endotoxin in CRP preparations affects the apparent biology of CRP *in vitro* (see Chapter 14). Furthermore in extrapolating results from *in vitro* models, it is critical to bear in mind key facts about human CRP, in particular its systemic distribution as a plasma protein and its 10000 fold dynamic concentration range in the acute phase response. These properties would seem to make inherently unlikely some of the claims for CRP as a fine modulator of sophisticated cellular functions.

8.6.2 Myocardial infarction and stroke

Once arterial occlusion has occurred and there is ischaemic tissue damage with cellular necrosis and ensuing local inflammation, the possible pathogenetic contribution of CRP is much clearer. Apart from the epidemiological association between higher peak CRP values and poor prognosis, there is robust immunohistochemical evidence of CRP deposition within all acute myocardial infarcts, co-localised with activated complement components [197-199]. Although this suggests that CRP might have deleterious effects, investigation of such mechanisms in man will require a drug that selectively inhibits CRP effects *in vivo*. However, rat CRP does not activate rat complement, in contrast to human CRP which potently activates rat as well, of course, as human complement [75]. The rat model of myocardial infarction, produced by coronary artery ligation, could therefore be used to investigate specifically the complement dependent pro-inflammatory role of human CRP in myocardial infarction. When rats undergoing coronary artery ligation received daily injections of pure human

CRP some of the coronary artery ligated rats treated with human CRP died, and those surviving to day 5, when all animals were killed, had infarcts 40% larger than buffer or SAP-treated controls. This enhancement of infarct size by human CRP was completely abrogated by *in vivo* complement depletion of the rats using cobra venom factor, and hence was complement dependent [201]. It has long been known that *in vivo* complement depletion markedly reduces inflammation and infarct size in this and similar animal models [327-330]. A substantial proportion of final myocardial infarction size following acute coronary occlusion is determined by complement mediated inflammation, and human CRP, both in the rat model and perhaps also in the clinical situation, is responsible for at least some of this complement activation [331].

Work has also been performed with respect to ischaemic stroke, in which it is known that CRP values increase in concentration after the event [214]. Rats subjected to middle cerebral artery occlusion and then treated with human CRP develop significantly larger cerebral infarcts compared with control subjects receiving human serum albumin [332]. Human CRP may thus contribute to ischaemic tissue damage in the brain as well as in the heart.

8.7 Summary: C-reactive protein, a novel therapeutic target?

There is compelling epidemiological and laboratory evidence that CRP is a sensitive marker of the inflammation and/or metabolic processes associated with atherothrombotic events. The presence of CRP within most atherosclerotic plaques and all acute myocardial infarction lesions, coupled with binding of CRP

to lipoproteins and its capacity for pro-inflammatory complement activation, suggest that CRP may contribute to the pathogenesis and complications of cardiovascular disease (Table 7). Availability of drugs to block CRP binding and its effects *in vivo* would provide a powerful tool for determining whether CRP is just a marker or does indeed participate in the pathogenesis of atheroma and/or its complications. Such agents may also have cardioprotective effects in acute myocardial infarction as well as acutely in stroke. Existing knowledge of the structure and function of CRP, including its three dimensional structure alone and complexed with ligands, coupled with experience in developing an inhibitor of the related protein, SAP, establish an excellent platform for drug design.

Table 7 Possible specific associations of CRP with cardiovascular disease

Summary box (2)
<ul style="list-style-type: none">• CRP binds selectively to LDL, especially 'modified' LDL• CRP is deposited in atherosclerotic plaques• Aggregated and/or ligand complexed CRP activates complement and can be pro-inflammatory• CRP is co-deposited with activated complement in all acute myocardial infarction lesions• Human CRP and complement are reported to increase final myocardial infarction and stroke size in experimental models• CRP may therefore be a therapeutic target

This thesis addresses further the biology of CRP by tackling this interesting and important protein at an epidemiological, *in vitro*, *in vivo*, and pharmacological level. It includes a robust assessment of whether or not CRP is a useful clinical predictor of heart disease and attempts further clarification of whether or not CRP is, at times, a biological villain or just an innocent, albeit convenient to measure, bystander.

9 Laboratory Materials and Methods

9.2 General reagents and laboratory chemicals

Where not specified or described, reagents, laboratory chemicals, and antibodies were purchased from either Sigma-Aldrich Company Ltd (Gillingham, England) or VWR International Ltd (Poole, England).

9.3 Common buffers

Common buffers and solutions, and their compositions, are listed below. 0.1% w/v sodium azide was added unless the buffer was intended for *in vivo* studies.

TN	0.01 M Tris, 0.14 M NaCl, pH 8.0
TC	0.01 M Tris, 0.14 M NaCl, 0.002 M CaCl ₂ , pH 8.0
TE	0.01 M Tris, 0.14 M NaCl, 0.01 M EDTA, pH 8.0
PBS	0.01 M phosphate buffer, 0.0027 M KCl 0.137 M NaCl, pH 7.4
PEBT	PBS containing 10 mM EDTA, 1% BSA, 0.2% Tween 20, pH 7.4
BE	0.07 M Barbitone buffer containing 0.01 M EDTA, pH 8.6
BC	0.07 M Barbitone buffer containing Ca Lactate (4H ₂ O), pH 8.6
TCB	TC containing 1% w/v bovine serum albumin
TCB 4%	TC containing 4% w/v BSA
TEB	TE containing 1% w/v BSA

9.4 Protein preparation

CRP (human, rabbit and rat) and SAP (human and mouse) previously purified by affinity chromatography from either ascites or serum were available in the laboratory [75, 77, 333-335]. In particular human CRP was prepared from malignant effusion fluids removed for therapeutic purposes collected from patients without active infections (source material tested negative for hepatitis B and C, anti-HIV-1 and HIV-2). CRP was purified by sequential DEAE anion exchange and ligand elution calcium dependent affinity chromatography on phosphoethanolamine-carboxyhexyl Sepharose. After calcium chelation and concentration, the CRP was buffer exchanged into TC buffer with or without azide, depending on its ultimate use. The CRP was >99% pure by overloaded silver stained 8-18% reduced sodium dodecyl sulphate polyacrylamide electrophoresis, was all in its native pentameric form by analytical size exclusion chromatography, and was fully functional by calcium dependent binding to immobilised phosphoethanolamine.

Human CRP concentrations in final preparations were established by absorbance at 280 nm, corrected for light scattering at 320 nm, and using the specific extinction coefficient for pure CRP (1.75 for 1 mg/ml) established by interferometric measurement of refractive index [336]; similarly for human SAP where the extinction coefficient is 1.71 for 1mg/ml [336].

The protomers of human CRP are non-glycosylated and have an M_r of 23,027, as predicted by the amino acid sequence and confirmed by electrospray ionization

mass spectrometry measurement [25]. The mass of a pentamer is therefore M_r 115,135. The protomer mass of human SAP is 25,462 D, as predicted by the amino acid and complete, invariant, glycan sequence, and confirmed by electrospray ionization mass spectrometry measurement [90]. An SAP pentamer, therefore has an M_r of 127,310.

The Gram negative bacterial LPS content was 100 pg/mg of CRP, measured by the kinetic chromogenic *Limulus* amoebocyte lysate assay (BioWhittaker Europe S.P.R.L., Parc Industriel de Petit Rechain, B-4800, Verviers, Belgium).

9.5 PC- and PE-Sepharose

PC-Sepharose and PE-Sepharose formed the basis for affinity chromatography and demonstration of calcium dependent binding (as evident by changes in concentration of the pentraxin protein after incubation with a suspension of beads) of isolated proteins, radiolabelled pentraxins and pentraxins in the presence and absence of antagonists *in vitro* and *ex vivo*.

9.5.1 Preparation of PC-Sepharose

1 g of aminophenylphosphorylcholine (Sigma) was offered to 60 ml CH Sepharose 4B (Pharmacia) and coupled according to the manufacturer's instructions. The PC-Sepharose was then washed into TC.

9.5.2 Preparation of PE-Sepharose

28.3 g of phosphoethanolamine was offered to 800 ml CH Sepharose 4B (Pharmacia) and coupled according to the manufacturer's instructions.

9.6 SDS-PAGE electrophoresis

Under reduced and denatured conditions, purity of protein preparations was demonstrated by SDS-PAGE electrophoresis using two proprietary gels according to the manufacturers instruction; NOVEX 4-20% Tris Glycine gels (Invitrogen Ltd, Paisley, UK) and ExcelGels (Pharmacia Biotech, St Albans, UK). Appropriate molecular weight calibration standards were used.

9.7 Normal Human serum

Venous blood from healthy volunteers was allowed to clot at room temperature for approximately 1 hour, then centrifuged at 2000g (Sorvall RT7 centrifuge with RTH-750 rotor, Kendro Laboratory Products Ltd, Stevenage, England) and the sera collected. Pooled normal human serum (NHS) was stored at -70°C to avoid activation of the complement pathway. To inactivate the complement pathway, NHS was heat-treated in a 56°C water bath for 30 minutes.

9.8 Antibodies

Certain anti-sera were available within the laboratory [72, 334, 337]. Others were commercially sourced as above.

Monospecific rabbit and sheep anti-sera against human CRP, SAP, mouse SAP and rabbit CRP: These were raised by immunisation with isolated pure proteins emulsified in Freund's complete adjuvant (CFA; Difco Labs, Surrey) followed by booster injections in incomplete Freund's adjuvant (ICFA; Difco).

Anti-human C3 serum: A monospecific antiserum against human C3 ("A" and "D" determinants) was obtained by immunisation of a sheep with specific antigen-antibody precipitates formed in agarose gel between purified human C3 and a non-specific sheep anti-human C3.

Anti-mouse C3 serum: Monospecific antiserum against mouse C3 was raised in a sheep following immunisation with yeast-mouse complement complexes incorporated in Freund's incomplete adjuvant.

The specificity of all anti-sera was previously determined by double diffusion in agarose.

The monoclonal anti-human CRP 21F12 was a gift of Dr S. Eda (Roche Diagnostics, Basle).

9.9 Immunochemical Techniques

9.9.1 Double immunodiffusion

Double immunodiffusion was performed in 1% w/v agarose (Indubiose A 37, BioSeptra, France) in BE buffer or TC buffer (if calcium dependent interactions were being studied).

9.9.2 Electroimmunoassay

Concentrations of human CRP, human SAP and mouse SAP were measured by rocket immunoelectrophoresis in 1% w/v agarose (Indubiose A 37) gels in BE buffer, after the methods of Laurell [338]. Whole monospecific antisera incorporated into gels cast onto GelBond film (Flowgen Instruments Ltd., Sittingbourne, Kent), were incorporated at optimal concentrations for each protein respectively. Electrophoresis was performed at 15°C, 200 volts constant for 6-8 hours. For mouse C3, 1% w/v agarose C gel (Amersham-Pharmacia, Little Chalfont, England) was used, containing sheep anti-serum to mouse C3 and run for 20 hours, with BC buffer across a 100V gradient at 15°C.

2 µl samples and standards, which consisted of dilutions of whole sera containing or spiked with known concentrations of each protein, were used. Migration of rocket immunoprecipitates was compared against standards and the range of the

assays was in the order of 2-150 mg/l, and the coefficient of variation of replicate assays less than 10% [339].

9.9.3 Antigen-antibody crossed immunoelectrophoresis

A modification of the Laurell technique [340] was used to estimate conversion of C3 by assessment of C3 cleavage products, with anti-C3 (murine or human) in the second stage gel. Cobra Factor (CoF) from the venom of the *Naja naja* cobra, available in the laboratory, and prepared as previously described, was used to generate control C3 cleavage products (5U per experiment) [341, 342]. Murine and human sera prepared for complement by prompt centrifugation and subsequent storage at -70°C was used as a complement source, and complement activation took place at 37°C.

9.9.4 Staining of immunoprecipitates

After washing in 5% w/v NaCl, gels were pressed, dried and stained in 0.2% Coomassie blue (Brilliant Blue R250, Sigma) dissolved in acetic acid / ethanol / distilled water (1:4.5:4.5), and destained as required in a similar solution not containing dye.

9.10 Haemolytic complement fixation assay

Complement activation was also assessed by a standard haemolytic complement fixation assay; sheep red blood cells in Alseviars and anti-sheep red blood cell were purchased from TCS Biosciences and complement fixation test medium from Oxoid.

9.11 Measurement of human CRP by automated assays

Human CRP was measured in serum or buffer as appropriate using a high sensitivity automated microparticle enhanced latex turbidimetric immunoassay (COBAS MIRA; Roche Diagnostics, Basle). The lower limit of detection was 0.2 mg/l with an interassay CV of 4.2% at 4 mg/l and 6.3% at 1 mg/l [343, 344]. Specific properties of this assay, described in detail later (Chapter 13.6.3.3), mean that ligand bound human CRP loses its immunoreactivity in this assay. This therefore also allows the use of this particular assay to determine any specific fluid phase binding of potential calcium dependent ligands for CRP. The efficacy of novel CRP ligands was thus tested in this assay, in both buffer and serum, *in vitro* and *ex vivo*. Known concentrations of protein were mixed with know concentrations of drug; incubation took place at room temperature for 30 minutes. Results are expressed as the molar ratio of drug to pentraxin protomer.

Alternatively, the concentration of human CRP was analyzed with a sensitive latex-enhanced immunoassay performed on a Dade-Behring BNII Nephelometer. The run-to-run CVs, at CRP concentrations of 0.47, 10.5, and 54.9 mg/l are reported as 6.4%, 3.7%, and 2.9%, respectively [345].

Standardisation of both CRP assays is based on the WHO International Reference Standard (85/407) [346].

9.12 Protein radiolabelling

Purified proteins were iodinated by the N-bromosuccinimide (British Drug Houses, Poole) method as previously described [347]. For use *in vitro* and for *in vivo* clearance studies in mice, pure pentraxins were labeled with ^{125}I (Na^{125}I , Amersham International, Bucks). 0.005% w/v KI in distilled water was added to pure protein in TE or TC, followed by Na^{125}I . Following addition of N-bromosuccinimide 0.025% w/v in distilled water, the contents were mixed for 15 seconds before transfer to a Sephadex G25 prepacked column (PD10, Pharmacia) which was blocked (BSA) and equilibrated in TN or TC. The column was promptly eluted with 8 ml of TN or TC, which served to quench the reaction and to separate iodinated protein from free radioiodide. After this gel filtration step more than 95% of the radioactivity in the protein fraction was precipitable with 10% w/v trichloroacetic acid. Binding to PE-Sepharose demonstrated retained function and a CobraTMII Auto-Gamma Counter (Packard Bioscience) was used throughout for quantification of radioactivity.

9.13 Animals

9.13.1 Husbandry and routine testing

All mice used were maintained in a conventional animal house (Comparative Biology Unit, Royal Free Hospital), and use was in accordance with the Animals (Scientific Procedures) Act 1986. Wild type C57/BL6J mice were sourced from Charles River Laboratories, UK. Animals were age and sex matched.

Human CRP transgenic mice (CRPTg), on a C57/BL6J background, were a gift of Dr B. Kyewski (German Cancer Research Center, Heidelberg, Germany) [74]. After injection of LPS into CRPTg mice it has been reported that peak concentrations of serum IL-6 (170 ± 35 ng/ml) are attained within 2 hours. This is followed by hepatic production of human CRP with peak values occurring within 18 hours. CRPTg mice carry a 31-kb *Clal* fragment of human genomic DNA comprising the CRP gene, 17 kb of 5'-flanking sequence, and 11.3 kb of 3'-flanking sequence. It has been established that the *cis*-acting elements within the *CRP* transgene are responsible for liver-specific and for lipopolysaccharide (LPS)-induced acute phase expression of human CRP. CRPTg mice exhibit a sexually dimorphic pattern of constitutive expression of human CRP with basal, testosterone dependent expression of the transgene only in males [348-351]. In view of reported infertility of homozygote CRPTg mice (personal observation, Dr B. Kyewski), the colony was maintained as heterozygotes with respect to the human CRP transgene.

ApoE^{-/-tm1Unc} mice, originally from the Jackson Laboratories were obtained from Charles River Laboratories, having been backcrossed for six generations onto a C57BL/6J background and undergone three generations of brother/sister mating. ApoE^{-/-tm1Unc} mice were back crossed three times as appropriate, with human CRP transgenic mice until a colony of apoE^{-/-tm1Unc}/CRP Tg mice was generated.

Animals were identified at around 6 weeks of age by polymerase chain reaction of DNA extracted from tail biopsy, using PCR primers as described below.

9.13.2 DNA Extraction

Mouse tail tips were added to 300 µl of lysis buffer (100 mM Tris, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, pH 8.5) containing 100 mg/l proteinase K (Roche) and incubated for 48 hours at 37°C. Following digestion, samples diluted 1 in 5 in water, were heated at 88°C for 15 minutes prior to use.

9.13.3 Polymerase Chain Reaction

CRPTg mice were identified as follows. Using Ready-To-GoTM (RTG) PCR beads (Amersham Pharmacia Biotech) and the primers CCATGGAGAAGCTGTTGTG (sense) and GTACTGGAGCTACTGTGACT (antisense) the DNA was amplified under the following conditions : 95°C

2min30sec [1 cycle] (denaturation); 95°C 30sec, 60°C 30sec, 72°C 30sec [35 cycles] (denaturation, anneal, extension); and 72°C 5sec [1 cycle] (final extension). PCR amplified products were size fractionated by 1% agarose gel electrophoresis (20 minutes 70V; AquaPor LE [National Diagnostics] in 0.089M Tris-borate, 0.089M Boric acid). After staining with ethidium bromide, DNA bands were visualised with a UV transilluminator. Positive animals demonstrate a 600 base pair band. Apo E genetic testing was similarly carried out, using three primers: IMR180 (5'-GCCTAGCCGAGGGAGAGCCG-3' T_m=69.8°C), IMR181 (5'-TGTGACTTGGGAGCTCTGCAGC-3' T_m=67.4°C), IMR182 (5'-GCCGCCCGACTGCATCT-3' T_m=69.3°C). The first two amplify a 155bp wildtype band, while the IMR180 and IMR182 amplify a 245bp band. The latter band is present in both heterozygote and knockout mice. Using RTG tubes to 2µl of previously prepared DNA the following amount of primers were added: 1µl of IMR180, 1.3µl of IMR181 and 0.5 µl of IMR182. The volume was made to 25µl by adding water and run as following: 94°C 3 min [1 cycle] (denaturation); 94°C 30sec 68°C 30 sec 72°C 30 sec [35 cycles] (denaturation, anneal, extension); 72°C 7 sec [1 cycle] (final extension). The PCR products were run on an agarose gel and the results visualised by UV light as above.

9.13.4 Phlebotomy

Blood was collected by tail bleeding or at bleed out (under anaesthesia) into 1.5 ml vials (Eppendorf, Hamburg, Germany) and allowed to clot for 6 hours at room temperature. Serum was separated by centrifugation at 14000 rpm in a

microfuge (Sigma-Aldrich, Poole, UK) for 5 minutes, and stored in aliquots at -70°C prior to analysis. For sequential blood sampling mice were bled at different time points from the cut tip of tail after pre-warming them for 5 minutes under a hot lamp.

9.13.5 Insertion of mini osmotic pumps

Mice (20-25 g) maintained under isoflurane anaesthesia had primed osmotic displacement pumps (ALZET, Charles River Laboratories, UK) inserted into a sub-cutaneous pocket on the back. Pumps delivering 1 µl/hour for 7 days with a reservoir volume of approximately 221µl, filled with 0.76M RMM in TC or buffer were used. Animals were bled prior to pump insertion and as appropriate subsequently.

9.14 Experimental protocols

9.14.1 Generation of autologous acute phase response

Autologous sterile acute phase responses were generated by sub-cutaneous administration of either 0.5 ml of 10% (w/v) casein (ICN Pharmaceuticals) in 0.05 M NaHCO₃ buffer or 0.25-0.5ml 2% (w/v) aqueous silver nitrate [352].

Mouse acute phase serum was obtained by exsanguination from dissected subclavian vessels of anaesthetised animals 24-36 hours following stimulation. After clotting and centrifugation, serum was stored at -70°C. Alternatively animals were challenged with endotoxin as described below.

9.14.2 Local Shwartzman Reaction

A local Shwartzman reaction was elicited by intra-dermal administration of 10 or 20 µg *Salmonella enteritidis* endotoxin (1 mg/ml in azide free PBS). The affected skin was subsequently harvested 24 hours later and formalin fixed, prior to wax embedding and sectioning. Haematoxylin and eosin staining was then performed [353].

9.14.3 LPS lethality model

LPS (*E. coli* O111:B4, *E. coli* O55:B5 or *Salmonella typhimurium*, 1 mg/ml in azide free PBS) was administered, at doses between 5-10 mg/kg, by intra-peritoneal injection and lethality was documented over the subsequent 72 hours. In some experiments acute phase responses in wild type and human CRP transgenic mice were initiated 24 hours before LPS challenge as above. In other experiments isolated pure human CRP in TC solution was injected intra-peritoneally 15 minutes before and 4 hours after LPS challenge. Control animals challenged with LPS received injections of solvent alone.

9.14.4 Binding of LPS in the fluid phase to human CRP

LPS (*E. coli* O111:B4; *E. coli* J5 [Rc mutant]; *E. coli* O26:B6; in PBS at 1 mg/ml) were tested for their ability to be recognised by CRP, by incubation of human CRP with LPS for 30 minutes at room temperature, followed by determination of the apparent CRP concentration on the COBAS MIRA assay. Pneumococcal C-polysaccharide (Serum Statens Institute, Denmark) was used as a positive control for loss of immunoreactivity. All solutions and dilutions were buffered in TC.

9.14.5 Collagen arthritis

Murine type II collagen arthritis was generated in C57/BL6 mice based on the method of Campbell *et al.* [354]. Mice were immunised with chick collagen (in complete Freund's adjuvant), intra-dermally at several sites in the base of the tail, with a total of 100 µl emulsion, on day 0, with an identical boost on day 21. The chick collagen type II (Sigma) was dissolved overnight at 4°C in 10 mM acetic acid to make a 2 mg/ml solution. Complete Freund's adjuvant (CFA) was made by grinding 100 mg of heat killed *M. tuberculosis* (H37Ra; Difco Laboratories) in 20 ml incomplete Freund's adjuvant (Difco). A fresh emulsion of equal volumes of chick collagen at 2 mg/ml in 10 mM acetic acid with CFA was made prior to immunisation.

A macroscopic arthritis score was determined two to three times per week for up to 100 days, depending on whether any arthritis developed:

- 0 No swelling or redness;
- 1 Swelling/redness of paw or one joint;
- 2 Two joints involved;
- 3 More than two joints involved;
- 4 Severe arthritis of the entire paw and joints

The arthritis score for each mouse is the sum of the scores of all four paws.

The mice were sacrificed, and their limbs fixed in 10% buffered formalin and decalcified in 5% formic acid. The tissue was then processed and embedded in paraffin, prior to sectioning and staining with haematoxylin and eosin.

Serum samples stored at -70°C were assayed for transgenic human CRP, murine SAP, and mouse anti-collagen antibodies. The final assay was performed as follows. Microtitre plates with N-oxysuccinimide activated surfaces (Corning Costar, Bucks, UK) were coated by incubation with 100 μl per well of the antigen, chick Collagen Type II diluted to 0.1 mg/ml in PBS, for 1 hour at 21°C . After decanting, the wells were each washed with 200 μL PBS-Tween (PBS, pH 7.4, containing 0.05% Tween 20) three times and then subsequently blocked with 100 μL 0.2M Tris pH 8.0 for 1 hour at 21°C . After further washing with PBS-Tween, 100 μl samples diluted in PEBT, were added in triplicate and incubated at 37°C for 1 hour. The plates were then decanted and each well was washed three further times with 200 μl of PEBT buffer, after which 100 μL I^{125}

radiolabelled sheep-anti-mouse Ig (Amersham-Biosciences, Little Chalfont, UK), diluted in PEBT, was offered for 1 hour at 37°C. Following a final decanting and washing step, the wells were blotted dry and counted individually in the γ -counter. Bound radioactivity was quantified on the gamma counter.

9.14.6 Determination of Atherosclerosis area in mouse aorta

Male mice of the four different genotypes, apoE^{+/+}, apoE^{-/-}, apoE^{+/+}-hCRP⁺, and apoE^{-/-}-hCRP⁺, were housed at 4 animals per cage under conventional conditions and received normal mouse diet (RM1, Special Diet Services Ltd, Witham, Essex, England) *ad libitum* throughout. Groups were closely age matched and apoE^{-/-} and apoE^{-/-}-hCRP⁺ mice were litter mates. One cohort was studied from age 8 weeks with serial tail bleeds at 4 week intervals until being killed for measurement of atherosclerotic lesions at 20 weeks. A second cohort was killed at 12 weeks and a third at 56 weeks. Group sizes were as shown in the results. All blood samples were taken at least 4 hours after the start of the daily light phase when food consumption was minimal. Serum was separated after clotting overnight and was stored frozen at -70°C until analysed, except for gel filtration studies that were performed on unfrozen samples that were briefly kept at 4°C until tested.

9.14.7 Analysis of atherosclerotic lesions

The mice were euthanased by carbon dioxide asphyxiation and bled out from the inferior vena cava. The hearts and aortae were immediately perfused *in situ* with oxygenated Krebs-Henseleit buffer at 37°C under a pressure of approximately 110 cm of water via a cannula inserted in the left ventricle and an outlet created by cutting the right atrium. After approximately 30 minutes the buffer was replaced with buffered formal saline at 37°C and the perfusion was continued for a further 30 minutes. The hearts and aortae were then removed, cleaned of extravascular fat and stored in formalin until they were processed. Cross-sections of the aortic root were used for analysis. After equilibrating the tissue in OCT compound overnight the hearts were immersed in OCT compound in a syringe with the aorta facing the cork. The tissue was frozen by immersion in liquid nitrogen and the hearts were then sectioned (Bright Instrument Company Ltd, UK) perpendicular to the axis of the aorta, starting within the heart and proceeding in the direction of the aorta. After the aortic root had been identified by the appearance of the three-valve leaflets, alternate 10 µm sections were taken and mounted on Polysine slides (VWR). Sections were air dried overnight and stained with oil red O (VWR), counterstained with Mayer's haematoxylin and covered using an aqueous mountant (Shandon). Alternate 10 µm sections through the aortic sinus were examined in a Leica DM XRA2 microscope equipped with a 2.5× objective and total magnification of 40×, and the whole area within the outer perimeter of the oil red O stained zones was quantified. Quantification of the atherosclerotic lesion areas was performed by drawing

around the lesions by hand using the QWIN software. Some of the sections were torn or folded and the images of these were discarded. Colour images were captured by analogue video camera under identical lighting, microscope, camera and PC conditions and were analyzed using Leica QWIN software [355].

The mean lesion area (μm^2) for each animal was calculated from the sum of the areas in all sections in which the aortic valve leaflets were present, divided by the number of sections analyzed. Colour thresholds were set that quantified the areas that were stained red within the lesions. Absolute values for the cross-sectional areas of the lesions were obtained by previous calibration of the software using an image of a micrometer slide. Plaques were also examined after staining with haematoxylin and eosin as well as with Verhoeff Van-Gieson staining for elastin.

9.14.8 Immunohistochemistry

Immunoperoxidase immunohistochemical staining was performed using standard techniques on sections that were not used for oil red O staining. After blocking by incubation with 20% vol/vol normal rabbit serum and elimination of endogenous peroxidase activity by incubation with H_2O_2 , optimal dilutions of primary antisera (mono-specific goat anti-human CRP and sheep anti-mouse C3) determined by prior titration were applied. Tissue bound primary antibodies

were detected using rabbit anti-goat IgG antibody (DAKO Ltd, Ely, UK), that cross reacts with sheep IgG, followed by peroxidase-goat anti-peroxidase complexes (DAKO), visualisation with diaminobenzidine and hematoxylin counterstaining. The specificity of all immunostaining was established by its complete abolition when the primary antisera were absorbed before use with the respective pure antigens; human CRP that had previously been immobilised covalently on CNBr-activated Sepharose (Amersham), and mouse C3 was captured on plain Sepharose beads by complement fixation in fresh mouse serum, as previously described [356].

9.14.9 Gel filtration

Size exclusion chromatography to study CRP-lipoprotein interactions in murine serum was performed using the ÄKTATM explorer 100 HPLC modular system (Amersham Biosciences) with a 24.7ml Superdex 200 HR10/30 column (exclusion limit 1.3×10^6 Da and separation range 10^4 - 6×10^5 Da). Undiluted 100 μ L serum samples were eluted at 0.25 ml/min with TC buffer monitored at 280 nm, and 250 μ L fractions were collected for CRP and cholesterol assay.

Similarly using the ÄKTATM explorer isolated proteins were gel filtered under standardised conditions [357], at room temperature, to study novel pentraxin-ligand interactions. Column equilibration and elution was performed with TE or TC buffers as appropriate, with added ligand, quoted as an excess of drug to pentraxin protomer.

9.14.10 Determination of serum cholesterol and triglycerides

The MIRA automated analyser was used to measure, enzymatically, serum cholesterol and triglycerides in mouse samples (Roche Diagnostics, UK). Calibrators and controls were purchased from Roche (C.f.a.s. calibrator for automated systems and Precinorm L).

9.14.11 Protein clearance studies in mice

Known concentrations of protein, radiolabelled or cold, were injected (100 μ l volumes) intra-venously into the lateral tail veins of mice. Potential ligands or buffer controls, were either pre-incubated with the protein for 30 minutes at 37°C, injected intra-peritoneally (maximum volume 200 μ l) prior to the injection of tracer, or delivered by an osmotic pump. Serial tails bleeds were performed into pre-weighed tubes and using the gamma counter, counts per minute per gram of blood were calculated [26]. Alternatively the concentration of protein was directly measured. Sera was also used to assess for residual binding of pentraxins to PE-Sepharose; the concentration of pentraxin being measured before and after a 30 minute (37°C) incubation with PE-Sepharose beads.

9.14.12 *Streptococcus pneumoniae* lethality model

Streptococcus pneumoniae (strains WU2 and A66.1 were provided by Dr D. E. Briles, University of Alabama, USA; strains Wt and D39-6 were a gift of Dr J. Brown, Imperial College) was grown from frozen aliquots on pre-poured Columbia agar plates in a CO₂ enriched environment at 37°C (Oxoid). A single colony was selected and Todd Hewitt Broth inoculated. After 8 hours growth, 0.5 ml of broth was transferred to 5 ml of fresh broth, for incubation for a further 2-3 hours. Bacteria were harvested thus at mid-log phase. Bacterial numbers were estimated prior to inoculation in mice in sterile Ringers lactate solution by measurement of absorbance at 420 nm (A_{420} of 1 is $\sim 2 \times 10^8$ CFU), and subsequently by colony counting. Mice were inoculated intra-peritoneally with an inocula ranging from 50-1000 colony forming units, and lethality noted over the subsequent 72 hours. As appropriate passive administration of human CRP and/or putative selective antagonists of CRP binding, were give intra-peritoneally at varying doses and time intervals. *In vitro* studies looking at binding of CRP (native and radiolabelled) were performed using pelleted bacteria, in the presence and absence of calcium and other CRP ligands.

9.14.13 Lipoprotein isolation and modification

VLDL was isolated from heparinised plasma and was a gift of Miss N. Marshall (University of Liverpool) [358].

For LDL, blood withdrawn from volunteers was drawn into a syringe containing 63 mM EDTA and 42 μ M diethylenediamino-pentaacetic acid (DTPA) at a ratio (by volume) of 1:20 anticoagulant. Plasma was separated by centrifugation at 1000g. A potassium bromide density gradient ultracentrifugation method was utilised to isolate LDL from the plasma using a Beckman L-70 centrifuge with a fixed angle rotor (Ti-70) working at 150,000g (1.006 mg/mL <density< 1.063 mg/mL). The isolated lipoproteins were dialysed at 4°C against 12 mM Tris (pH 7.4) and the protein concentration of each lipoprotein preparation was determined using the modified method of Lowry [359], with stock solutions of BSA as standard.

9.14.13.1 Modification of LDL (Enzymatic [E] and Oxidative [Ox])

Native human LDL was isolated and enzymatic modification with trypsin, cholesterol esterase, and neuraminidase was undertaken. LDL was diluted to 3 mg/mL cholesterol in HEPES buffer (20 mmol/L Hepes, 150 mmol/L NaCl, 2 mmol/L CaCl₂, pH 7.0). Initially 6.6 μ g/mL trypsin (Sigma) and 40 μ g/mL cholesterol esterase (Boehringer Mannheim) were added for 8 hours at 37°C. Trypsin inhibitor (Sigma) was then added at 10 μ g/mL after the initial incubation with trypsin and cholesterol esterase. The pH of the solution was adjusted to 5.5 by addition of morpholinoethane sulfonic acid buffer, pH 5.0, and neuraminidase (Behringwerke) was added at 79 mU/mL for 14 hours, 37°C [360].

Oxidised LDL was prepared by incubation of LDL with copper sulphate. Varying concentrations of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in normal PBS (40 μM downwards) were incubated with equal volumes of LDL overnight at 37°C.

Modified lipoproteins were stored at 4°C and used within a week, after dialysis into appropriate buffers. The buffers included PBS, 0.1 M Carbonate ($\text{Na}_2\text{CO}_3/\text{NaHCO}_3$) pH9.0, and 0.1 M Boric acid buffer pH8.0 (KCl and H_3BO_3)

9.15 Novel CRP and SAP Ligands

Novel synthetic routes were developed by Professor S. Ley and colleagues (Department of Chemistry, University of Cambridge) to produce new pharmacological ligands for CRP and SAP. Ligands, expressed as the molarity with respect to pentraxin protomer, were used as solutions in TN or TC (described further in Chapter 13). CPHPC, a selective pharmacological inhibitor of SAP binding was manufactured by Roche Pharmaceuticals [94]. Other ligands were purchased commercially from Sigma.

9.15.1 Inhibition of CRP binding – plate based assay

Using microtitre plates (Costar) 100 μl volumes of either modified lipoproteins or pneumococcal C-polysaccharide (Serum Statens Institut, Denmark) were

attached to wells. This was either through hydrophobic interactions (EIA/RIA High Binding Type 1) or covalently (N-oxysuccinimide surface amine binding polystyrene stripwell plates). Incubation with 0.2 M Tris blocked residual binding sites and three buffer washes, 200 μ l, between each step was always performed. Radiolabelled human CRP (I^{125}) was offered to the immobilised ligands (incubated at 37°C for 30 minutes) and binding was shown to be calcium dependent. Comparison of inhibition by novel ligands was made against phosphorylcholine, the best-known natural ligand.

9.15.2 Electron microscopy

Transmission electron microscopy with negative staining by 2% aqueous uranyl acetate of pure human CRP on carbon coated grids was performed by the Electron Microscopy Department, Royal Free Hospital, to visualise CRP in the presence and absence of potential CRP ligands.

9.15.3 Mass spectrometry and X-ray crystallography

NanoESI MS measurements of CRP with and without ligand, were performed on an LCT mass spectrometer (Micromass), with Dr A. Aquilina in Professor C. Robinson's laboratory, University of Cambridge. Typically, 2 μ l of solution was electrosprayed from gold-coated glass capillaries prepared in-house. In order to

preserve non-covalent interactions, the following instrument parameters were used: capillary voltage, 1.5 kV; cone gas, 100 l/hour; sample cone, 140 V; extractor cone, 8 V; ion-transfer stage pressure, 8.0×10^{-3} mbar; ToF analyser pressure, 2.0×10^{-6} mbar. The pressure in the ion-transfer stage was decreased from 8.0×10^{-3} to 5.0×10^{-3} mbar to dissociate monomers from the pentamers and to remove excess solvent from the complex. All spectra were calibrated externally using a solution of CsI, and were processed with MassLynx software (Micromass). Buffer salts were removed by applying 20 μ l of stock solution to a Micro-BioSpin chromatography column (Bio-Rad Laboratories) previously equilibrated in an electrospray buffer (EB), 200 mM ammonium acetate, pH 8.0 (with or without 1 mM calcium). The process was repeated to achieve satisfactory buffer exchange [361].

X-ray crystallography, and subsequent data analysis, was performed by Professor S. Wood and colleagues (Southampton University), with purified human CRP in the presence and absence of ligand, using methods applied previously for the study of the SAP-CPHPC complex [94]. Crystals of the CRP-RMM complex were grown by hanging drop vapour diffusion from 11 mg/ml CRP with a ten-fold molar excess of RMM in 150 mM NaAc pH 4.6, 50 mM CaCl_2 , and 52% v/v 2-methyl-2,4-pentanediol.

9.16 Statistical analysis

Statistical analyses were carried out using SPSS (Version 10.0). Discrete variables were compared using χ^2 or Fisher's exact test, as appropriate. Continuous variables are expressed as mean \pm SD and compared by means of the unpaired, 2-sided *t* test or analysis of variance (ANOVA) for > two groups. Variables not normally distributed are reported as medians and inter-quartile ranges or geometric means \pm SD, and analysed using nonparametric tests. Kaplan-Meier survival analysis was performed and P values sought by the log rank test or Breslow test.

Section 10.5 describes in detail, separately, the methods for the Reykjavik Study.

10 Assessing the role of C-reactive protein determination in coronary heart disease prediction

10.2 Introduction

Atherosclerosis is the underlying cause of most cardiovascular disease, starting early in life and progressing for decades before being complicated with acute myocardial infarction, stroke or angina. The development of atherosclerosis involves a complex and self-reinforcing interaction between lipid accumulation and modification, the endothelium, smooth muscle cells and macrophages, inflammatory cytokines and various blood components [362]. Laboratory and epidemiological data show that baseline values of CRP, SAA, leukocyte count, and serum albumin, predict future risk of coronary heart disease [179]. It remains unclear whether such associations are causal or reflect confounding by classic risk factors, chronic infective processes, or early disease [363-365]. Refining the association by increasing the available data is important, in particular assessing whether or not so called new markers of risk add value to established markers. With Professor J. Danesh, Dr J. Wheeler, the Iceland Heart Association, Roche Diagnostics and the Royal Free Hospital Clinical Chemistry Department, this chapter describes the collaborative effort to further study the predictive power of CRP measurement in coronary heart disease risk prediction [168].

10.3 Risk Factors

There has been much excitement at the potential use of CRP as a new marker of cardiovascular disease but widespread changes to clinical practice must be based on good evidence [366, 367]. The concept of the risk factor for chronic diseases, such as cardiovascular disorders, is derived from the application of epidemiological methods initially developed for infectious and nutritional deficiency diseases; the aim being to identify factor(s) linked to the development of the disease.

Established risk factors (e.g. gender, smoking, diabetes, hypertension and hypercholesterolaemia) have enabled reasonably accurate assessment of risk among groups and individuals to provide guidance for management. In addition, risk factors have provided important clues to the biology of cardiovascular diseases [368]. Although the importance of conventional risk factors is well established, it is commonly suggested that more than 50% of patients with CHD lack any of the conventional risk factors [369, 370]. This implies that other factors play a significant role in the development of disease and, furthermore, that there is a substantial void in current understanding of the pathogenesis of CHD. This perceived void has led to considerable research on non-traditional risk factors and genetic causes of heart disease. However, data to support this "50%" belief are limited, and some have suggested that conventional risk factors play a much more significant role [371-374].

For clinical prediction models, a variety of criteria arise for risk factors that might be useful and practical (Table 8) [375]. These criteria importantly imply that to be useful, the factor must be an independent predictor of risk. That is, it must improve the predictive power of other risk factors already identified and used for prediction.

Table 8 Characteristics of biomarkers of disease

Ideal characteristics of biomarkers [375]
<ul style="list-style-type: none"> • The measure should add important independent information about the risk of prognosis, beyond that of current risk markers.
<ul style="list-style-type: none"> • The measure should account for a significant proportion of the risk associated with the given condition; for common conditions such as cardiovascular disease, this could be a modest proportion of risk, if the impact overall is clinically significant.
<ul style="list-style-type: none"> • The measure should be reproducible.
<ul style="list-style-type: none"> • The measure should be sensitive and specific, with a high predictive value.
<ul style="list-style-type: none"> • The test should be readily available and practical to implement.

10.4 Inflammation, C-reactive protein, and coronary heart disease risk

Inflammation occurs in the vasculature as a response to injury, lipid peroxidation, and perhaps infection [364]. Various risk factors, including hypertension, diabetes, and smoking, are amplified by the harmful effects of oxidised low-density-lipoprotein cholesterol, initiating a chronic inflammatory

reaction, the result of which is a vulnerable plaque, prone to rupture and thrombosis [363]. As noted already epidemiological and clinical studies have shown strong and consistent relationships between markers of inflammation and risk of future cardiovascular events. Inflammation can potentially be detected directly, locally, within vessels by imaging techniques as well as emerging techniques, such as identification of temperature or pH heterogeneity. It can also though be detected more easily, systemically, by measurement of inflammatory markers [376].

A number of different markers of inflammation are measurable in blood [377]. These include cytokines, chemokines, soluble adhesion molecules, and acute-phase reactants. Of these, the most reliable and readily accessible for clinical use is CRP, and in particular what some have termed *high sensitivity* CRP (hsCRP). The “high sensitivity” refers simply to the lower detection limit of the assay procedures being used. The analyte is the same regardless of the assay range and very sensitive CRP assays have been reported from research laboratories for 30 years [19, 378, 379]. The new development is the introduction of commercial and automated routine CRP immunoassay systems with greater sensitivity than before. CRP has several advantages over other markers. First, the analyte is stable (CRP in serum or plasma samples is stable when refrigerated for at least 7 days and for extended periods of time, many decades, when frozen). Second, many automated hsCRP methods are commercially available and third an international secondary serum reference material (CRM 470, produced by the International Federation for Clinical Chemistry [380]) is readily available for standardisation of kit calibrators.

hsCRP assays are those that have a low enough detection limit to permit CRP measurement in all, or nearly all, healthy subjects. This translates into a lower limit of quantification of ≤ 0.3 mg/L and assay imprecision of $<10\%$ at a CRP concentration <0.5 to 1.0 mg/L, although exact performance requirements have not been defined [381, 382].

To date, CRP values have been used prognostically in a variety of different cardiovascular settings [383-385]. Interpretation of such studies must bear in mind the differing pathophysiological processes involved in, for example, myocardial infarction, unstable angina and stable angina. Nevertheless CRP is invariably produced in large amounts in response to myocardial necrosis and the peak values of circulating CRP powerfully predict outcome after myocardial infarction. CRP production, as well, predicts poor prognosis in severe unstable angina or after angioplasty, and may have the potential to guide primary and secondary prevention strategies involving HMG CoA-reductase inhibitors. Finally CRP production also predicts coronary events in general populations. Figure 8 from John Danesh's work [179], shows the previous meta-analysis addressing the prognostic ability of CRP to predict future coronary heart disease events, in healthy individuals. On the basis of the data then available, comparing individuals with CRP concentrations in the top third with those in the bottom third at baseline, gave a combined risk ratio of 1.9 for coronary heart disease (95% CI 1.5 to 2.3). The estimated mean usual log CRP values in the top third and bottom thirds were 0.38 and 0.02 mg/l in the general population, which correspond to mean estimated usual values of 2.4 and 1.0 mg/l respectively.

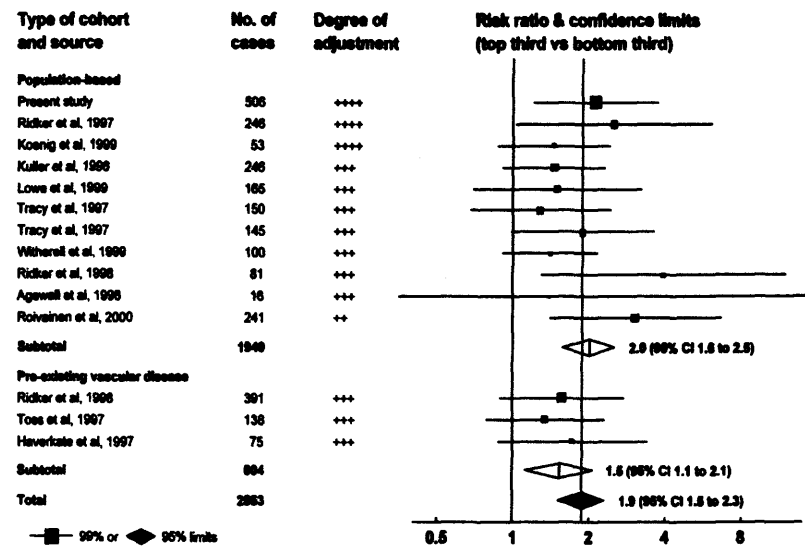


Figure 8 Previous meta-analysis of prospective studies of CRP and coronary heart disease prediction

Latterly guidelines from the American Heart Association/Center for Disease Control (2002-03) included the measurement of CRP as a class IIa recommendation for stratifying patients with known cardiovascular disease (CVD) at a moderate (10-20%) 10-year event risk and a class IIb recommendation for patients without known CVD (Figure 9) [383].

- High-sensitivity C-reactive protein is an independent marker of risk that may be used at the discretion of the physician in patients judged by global risk assessment to be at intermediate risk (10% to 20% risk of coronary heart disease per 10 years) for cardiovascular disease. hsCRP may help direct further evaluation and therapy in the primary prevention of CVD. The benefits of such therapy based on this strategy remain uncertain.
(Class IIa, Level of Evidence: B)
- hsCRP is an independent marker of risk and may be used at the discretion of the physician as part of a global coronary risk assessment in adults without known CVD. The benefits of this strategy remain uncertain.
(Class IIb, Level of Evidence: C)
- hsCRP levels may be useful in motivating patients to improve their lifestyle behaviours. The benefits of this strategy remain uncertain.
(Class IIb, Level of Evidence: C)
- Patients with persistently unexplained marked elevation of hsCRP (≥ 10 mg/L) after repeated testing should be evaluated for non-cardiovascular causes.
(Class IIa, Level of Evidence: B)
- Inflammatory markers (cytokines, other acute-phase reactants) other than hsCRP should not be measured for the determination of coronary risk.
(Class III, Level of Evidence: C)
- hsCRP measurement in patients with stable coronary disease or acute coronary syndromes may be useful as an independent marker of prognosis for recurrent events, including death, myocardial infarction, and restenosis after percutaneous coronary intervention. The benefits of therapy based on this strategy remain uncertain.
(Class IIa, Level of Evidence: B)
- Application of secondary prevention measures should not depend on hsCRP determination.
(Class III, Level of Evidence: A)
- Application of management guidelines for ACS should not depend on hsCRP levels.
(Class III, Level of Evidence: A)
- Serial testing of hsCRP should not be used to monitor the effects of treatment.
(Class III, Level of Evidence: C)

Figure 9 American Heart Association/Center for Disease Control Guidelines

The report acknowledged, however, that the epidemiological data to support this view were not entirely consistent and recommended that additional larger prospective studies be conducted to improve the reliability of the evidence. In particular, it remains uncertain whether CRP is an independent risk factor for coronary heart disease. For example, in the British Regional Heart Study, the odds ratio was reduced from 3.46 to 2.13 after adjustment for baseline confounding factors [179]. This substantial reduction suggests that more exact adjustment for confounding factors might produce a greater reduction.

This chapter presents data from the largest prospective study so far of CRP (Table 9), as well as discussing a subsequent updated meta-analysis [168], to help evaluate the relevance of CRP determination in the prediction of coronary heart disease. The lead investigator was Professor J. Danesh of the Department of Public Health and Primary Care, Institute of Public Health, University of Cambridge.

Table 9 Prospective observational studies of CRP and coronary heart disease: details of study populations (pre-2003)

Study	Geographical location	Population/sampling method*	Year of baseline survey	No. CHD cases	Total no. participants	Age range (years)	% male	Follow-up: mean duration (years)
Reykjavik [168]	Iceland	Population register/complete birth cohorts	1967-1991	2 406	18 569	39-59	70	20
ARIC [386]	USA	Listing of household/random	1987-89	615	15 792	45-64	43	8
WOSCOPS [387]	Scotland	Population register/complete	1989-95	580	6 595	45-64	100	6
BRHS [179]	UK	General practitioners list/random	1978-80	506	5 661	40-59	100	16
Womens Health (WHI) [276]	USA	RN/LPN register/complete	1992-95	371	28 345	45+	0	8
WHIOS [388]	USA	40 clinic lists/complete	1994-98	280	93 724	50-79	0	3
Caerphilly [389]	Wales	Electoral rolls/random	1979-83	249	2 512	45-59	100	14
MRFIT [174]	USA	Industry & government employees/complete	1973-76	246	12 866	35-57	100	10
Physicians Health [177]	USA	Physicians register/complete	1982	246	22 071	40-84	100	14
Helsinki Heart [390]	Finland	Industry employees/complete	1981-82	241	4 081	40-55	100	10
AFCAPS/TEXCAPS [273]	USA	Civilian and military clinics/complete	1990-93	216	6 605	45-73	ns	5
Speedwell [391]	England	General practitioners list/complete	1979-82	165	1 690	47-67	100	6
CHS [186]	USA	Medicine eligibility lists/complete	1989-90	150	5 888	>=65	61	3
Helsinki Aging [392]	Finland	Population register/random from birth cohorts	1989	147	651	75-85	28	10
RHPP [186]	USA	Medicine eligibility lists/complete	1995	145	3 884	65-79	43	3
Glostrup [393]	Denmark	Population register/random from birth cohorts	1976-84	133	5 637	30-50	100	12
Quebec [394]	Canada	Population register/random	1985	105	2 100	45-77	100	5
Kaiser Permanente [395]	USA	Health check list/random	1967-79	100	261	ns	ns	5
Iowa 65+ [396]	USA	Population register/complete age 65+ years	1982	74	3 673	65+	41	4
MONICA Augsburg [178]	Germany	Population register/random	1984-92	53	5 069	25-74	100	7
HOORN [397]	Netherlands	Population register/random	1989-92	24	2 484	50-75	48	5
Göteborg Intervention [398]	Sweden	Population register/complete	1993	16	508	50-72	100	3

* Sampling method: random = a randomly selected subset of eligible persons was invited to participate; complete = all eligible persons in the study population were invited to participate. Eligibility was based on more restrictive criteria in the following studies: WOSCOPS (persons with LDL of 174-232mg/dl); MRFIT2 (persons with a high risk for CHD based on blood pressure, cholesterol and smoking profiles); Helsinki Heart (persons with high non-HDL levels); AFCAPS/TEXCAPS (persons with below average HDL); Göteborg Intervention (persons with hypertension). ARIC=Atherosclerosis Risk in Communities; WOSCOPS=West of Scotland Coronary Prevention Study; BRHS=British Regional Heart Study; WHIOS=Womens Health Initiative Observational Study; MRFIT=Multiple Risk Factor Intervention Trial; AFCAPS/TexCAPS=Air Force/Texas Coronary Atherosclerosis Prevention Study; CHS=Cardiovascular Health Study; RHPP=Rural Health Promotion Project.

10.5 Study Methods

We measured CRP concentrations in about 2400 incident coronary heart disease cases and in about 4000 controls “nested” within the Reykjavik prospective cohort study of about 19 000 middle-aged men and women without a history of myocardial infarction, thereby involving about four times as many coronary heart disease cases as the previous largest such study. In addition to reducing random error because of its larger sample size, the present study was able to assess the impact of within-individual variation of inflammatory markers because serial blood samples were taken over several years in several hundred of its participants.

10.5.1 Patients and Controls

Iceland has a total population of around 280,000 with over half the population living in the capital Reykjavik. The Reykjavik Study is a large population-based cohort study that started in 1967 [399-402]. All men born between 1907 and 1934 and all women born between 1908 and 1935 who were resident in Reykjavik and its adjacent communities on 1 December 1966 were identified in the national population register and then invited to participate in the study during five stages of recruitment between 1967 and 1991, yielding 8888 male and 9681

female participants without a history of myocardial infarction (72 percent response rate) [403]. Nurses administered questionnaires, made physical measurements, recorded an electrocardiogram, performed spirometry (forced expiratory volume in 1 second), and collected fasting venous blood samples for measurement of ESR, and for preparation of aliquots of serum, which were stored at -20°C for subsequent analysis. Factors measured either by the nurses or in the laboratory included systolic and diastolic blood pressure, fasting total cholesterol, triglycerides, smoking habits (classified here only either as smoking or non-smoking or current/ex-smoker and never smoked), height, and weight, as well as assessment for the presence of diabetes. A standard glucose tolerance test was performed, with a fasting and 90 minute glucose concentration determined [404]. All participants have been monitored subsequently for all cause mortality and for cardiovascular morbidity, with a loss to follow up of only about 0.6 percent to date [403].

A total of 2459 men and women with available serum samples had major coronary events between the beginning of follow-up and 31 December 1995, yielding mean durations of follow-up among CHD cases of 17.5 (SD 8.7) years and of 20.6 (SD 8.2) years among controls. Among men, 1073 CHD deaths and 701 non-fatal myocardial infarctions (MIs) were recorded (including 564 confirmed MIs and 137 possible MIs) and 385 CHD deaths and 300 non-fatal MIs among women (including 237 confirmed MIs and 63 possible MIs). Deaths from coronary heart disease were ascertained from central registers on the basis of a death certificate with International Classification of Diseases codes 410-414,

and the diagnosis of non-fatal myocardial infarction was based on MONICA criteria. We selected 3969 controls “frequency” matched to cases on calendar year of recruitment, sex, and age in five year bands from among participants who had survived to the end of the study period without a myocardial infarction. A national ethics panel approved the study protocol and participants provided informed consent.

10.5.2 Laboratory Methods

Laboratory measurements were made without knowledge of the participants' disease status, and thus samples (approximately 100 μ l volumes) from patients and controls were randomly distributed during the assay process. Concentrations of CRP were measured by latex-enhanced immunoturbidimetry, with a lower limit of detection of 0.02 mg/l (Roche Diagnostics) [405], in the Clinical Chemistry Department of the Royal Free Hospital. The variation in CRP values within runs was less than 1%, and the between-day variability was 1% at a concentration of 14 mg/l and 3.7% at a concentration of 3.8 mg/l (Table 10).

Table 10 Quality control data for CRP measurement

Average of all runs over 4 weekends i.e. between batch variability		
<i>Control</i>	“SPS-13”	“CRP T-S”
<i>n</i>	106	132
<i>Quoted (mg/l)</i>	14.3	3.762
<i>Mean (mg/l)</i>	14.218	3.794
<i>SD</i>	0.1437	0.1407
<i>CV (%)</i>	1.011	3.708

The Wintrobe method was used to measure the erythrocyte sedimentation rate in fresh blood samples obtained at the time of base-line venesection [402]. Other biochemical and haematologic measurements involved the use of standard assays. Previous reports describe these in more detail [399]. To determine self-correlation for the markers of interest [406], measurements were made in pairs of samples obtained from 379 participants a mean of about 11.5 years apart.

10.5.3 Statistical Analysis

For ethical, social and political reasons (e.g. the deCODE project), the issue of confidentiality in research in Iceland is topical, and subject to legislation [407-409]. This had bearing on how the data was handled by myself and our collaborators. As a result the analysis is clearly divided into two sections.

Baseline analysis: The statistical analysis of the baseline data was performed in London using SPSS10.0 for Windows (SPSS Inc.). Log-transformation was used as appropriate for variables not normally distributed. Baseline associations were assessed by simple correlation, linear regression, analysis of variance and binary logistic regression. Adjustment was based upon gender, smoking status and diabetes as fixed factors, with other factors representing covariates (ESR, age, FEV1, BMI, fasting glucose, glucose tolerance at 90 minutes, cholesterol, triglycerides, systolic BP, diastolic BP, haematocrit, haemoglobin, creatinine). % FEV1 was calculated as the FEV1/Predicted FEV1 x 100. Predicted FEV1 for men and women was calculated as defined previously [410]. For graphical representation the boxes represent the interquartile range around the median (horizontal line); whiskers show the highest and lowest values within one interquartile interval from the box. Extreme values/outliers are not shown, but are included in the analysis. Error bar charts plot the mean and 95% confidence intervals for variables. Histograms demonstrate frequency, according to the variable/category assigned.

Case-control analysis: The case-control analysis, with access to further aspects of the dataset, was performed in Cambridge. Small differences in the datasets exist between the baseline and the case-control analysis and reflect independent parallel collaborations on going at the same time, but do not affect the overall conclusions. The analysis employed unmatched stratified logistic regression fitted according to the unconditional maximum likelihood (Stata software, version 7). To maximise the ability to compare the results with those of previous reports, primary analyses of values of CRP and ESR, were prespecified to

compare extreme thirds of patients and controls with respect to the distribution of values in the controls. Odds ratios were sequentially adjusted for the following variables: age, sex, calendar year of enrollment, smoking status, systolic blood pressure, total cholesterol level, triglyceride level, body-mass index, forced expiratory volume in one second, presence or absence of diabetes, socioeconomic status, and the concentrations of ESR.

10.6 Results

The analysis herein is focussed towards CRP in keeping with our contribution to the Reykjavik Study. ESR as the most robust alternative inflammatory marker is used for reference where appropriate.

10.7 Baseline data

The important baseline characteristics of our population are shown in Tables 11, 12, and 13. Nearly 70% of subjects were male, and the rate of active smoking at the time of recruitment was just over one-third. The mean age at recruitment was just over 55 years old. There were 152 diabetic patients. In keeping with previous population studies of CRP, the median CRP was 1.41 mg/l, the 90th centile being 5.82 mg/l and the 99th centile 27.14 mg/l. The geometric mean of log transformed CRP was 1.45 mg/l. Just over 4% of the individuals had a CRP ≥ 10 mg/l, the cut off previously used to indicate active inflammation. If these individuals are excluded the median CRP falls slightly to 1.32 mg/l, with the 90th centile being 4.64 mg/l and the 99th centile 8.85 mg/l. However specifically to avoid introduction of any extra bias, the data is analysed as a whole, without exclusion of patients with a CRP ≥ 10 mg/l; the objective of analysis was to ascertain baseline associations for CRP in *all* objectively well individuals.

Table 11 Summary of population attributes at baseline

Baseline statistics									
	N		Mean	Median	Std. Deviation	Skewness	Kurtosis	Percentiles	
	Valid	Missing						50	95
Age at bleed (yr)	6760	0	55.68	54.63	9.20	.49	.25	51.25	58.24
CRP (mg/l)	6620	140	2.86	1.41	5.21	6.80	70.90	.87	2.32
log CRP	6620	140	.16	.15	.49	.19	.17	-.07	.36
ESR (mm/1st hr)	6713	47	9.74	7.00	10.02	2.40	9.32	4.00	10.00
log ESR + 1	6713	47	.90	.90	.40	-.20	-.30	.70	1.04
Height (m)	6733	27	1.72	1.72	.09	-.21	-.39	1.68	1.76
Weight (kg)	6731	29	75.69	75.20	13.54	.39	.77	69.60	80.60
Body mass index (BMI)	6720	40	25.65	25.42	3.80	.76	2.60	23.92	26.87
Systolic BP (mmHg)	6735	25	143.33	140.00	22.66	.89	1.40	130.00	150.00
Diastolic BP (mmHg)	6730	30	89.00	88.00	11.85	.63	1.32	84.00	92.00
FEV1 (ml in 1 second)	6632	128	2848.43	2850.00	859.02	-.02	-.45	2450.00	3250.00
Fasting glucose (mmol/l)	6704	56	4.57	4.44	.89	5.79	59.51	4.22	4.66
Glucose tolerance: 90 minutes (mmol/l)	6455	305	5.89	5.55	2.08	2.14	9.67	4.94	6.27
Haemoglobin (mmol/l)	6705	55	9.11	9.18	.81	-.34	.91	8.81	9.43
Haematocrit (%)	6707	53	44.47	45.00	3.55	-.03	1.45	43.00	46.00
Cholesterol (mmol/l)	6752	8	6.58	6.50	1.18	.75	2.86	6.03	6.94
Triglycerides (mmol/l)	6354	406	1.22	1.05	.69	3.76	31.89	.87	1.28
log Triglycerides	6354	406	.04	.02	.20	.49	.70	-.06	.11
Creatinine (µmol/l)	6704	56	87.98	88.40	15.69	1.56	18.57	79.56	88.40
log Creatinine	6700	60	1.94	1.95	.07	-.12	3.17	1.90	1.95

Table 12 Gender differences in baseline parameters

	Gender							
	Female				Male			
	Valid No.	Mean	Median	Std Deviation	Valid N	Mean	Median	Std Deviation
CRP (mg/l)	1938	2.75	1.35	4.79	4682	2.91	1.44	5.38
log CRP **	1938	.14	.13	.50	4682	.17	.16	.48
ESR (mm/1st hr)	1948	14.57	12.00	11.83	4785	7.77	5.00	8.42
Log ESR + 1 *	1948	1.07	1.11	.34	4785	.78	.78	.38
Height (m) *	1954	1.62	1.62	.06	4779	1.76	1.76	.06
Weight (kg) *	1955	66.09	64.70	12.03	4776	79.62	78.70	12.09
Body mass index (BMI) *	1949	25.28	24.91	4.42	4771	25.82	25.63	3.51
Systolic BP (mmHg)	1953	143.64	140.00	24.42	4782	143.20	140.00	21.91
Diastolic BP (mmHg) *	1952	86.15	86.00	12.00	4778	90.16	90.00	11.59
FEV1 (ml in 1 second) *	1892	2084.26	2100.00	577.73	4740	3153.46	3150.00	757.05
Fasting glucose (mmol/l) *	1954	4.42	4.27	.92	4750	4.63	4.50	.87
Glucose tolerance: 90 minutes (mmol/l) *	1827	6.05	5.66	2.03	4628	5.83	5.49	2.10
Haemoglobin (mmol/l) *	1955	8.42	8.44	.68	4750	9.40	9.37	.67
Haematocrit (%) *	1954	41.69	42.00	3.17	4753	45.62	46.00	3.03
Cholesterol (mmol/l) *	1964	6.95	6.84	1.30	4788	6.43	6.37	1.09
Triglycerides (mmol/l)	1948	1.15	1.01	.58	4406	1.26	1.08	.74
log Triglycerides *	1948	.02	.00	.18	4406	.05	.04	.20
Creatinine (µmol/l)	1960	78.00	79.58	14.03	4744	92.10	88.40	14.44
log Creatinine *	1959	1.89	1.90	.07	4741	1.96	1.95	.06

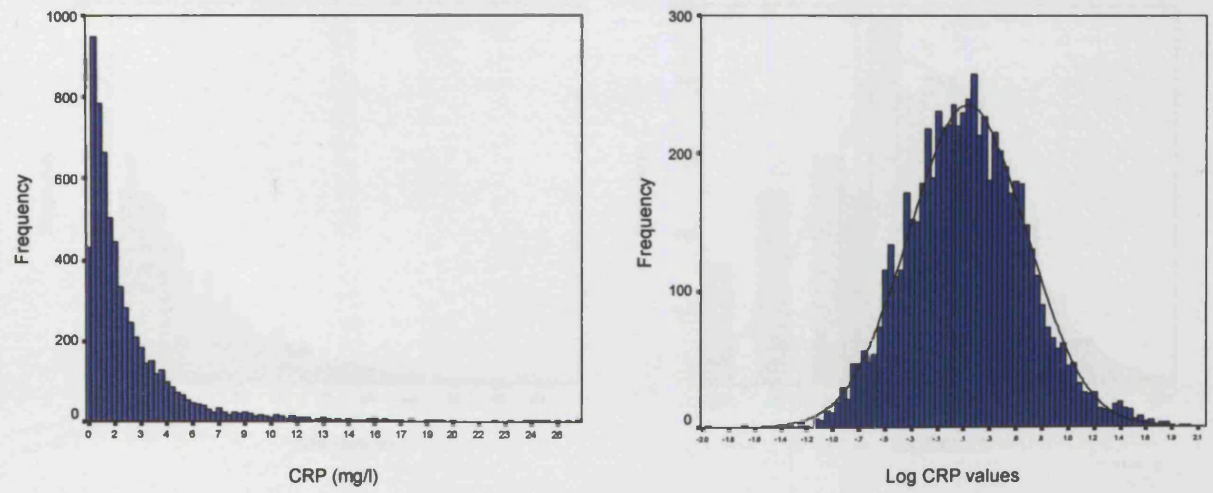
* P<0.001 for difference between females and males; ** P<0.01 for difference between females and males

Table 13 Breakdown of gender and crude smoking history at baseline

		Crude smoking history		Total	Age
		Non-smoker	Current smoker	%	Mean (SD)
		N (%)	N (%)		
Sex	Male	3337 (69.6)	1458 (30.4)	69.6	54.67 (8.93)
	Female	1093 (55.6)	872 (44.4)	30.4	58.15 (9.39)
%		65.5	34.5		55.68 (9.2)

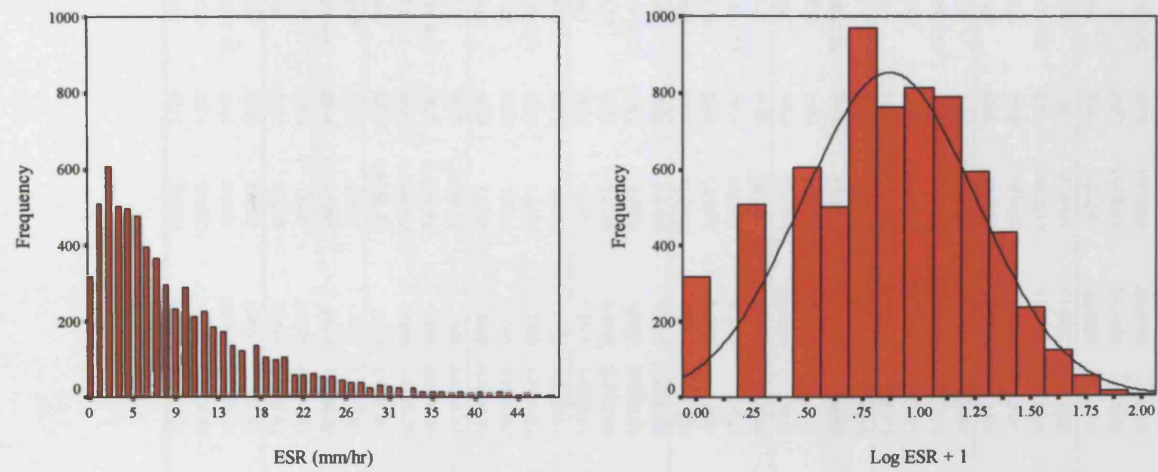
As with previous studies the distribution of CRP is highly skewed (Figure 10). When values are log transformed, the distribution assumes a Gaussian distribution. ESR values are less precise by analytical nature and the distribution is less skewed (Figure 11). Since ESR values can be zero, in this case the log transformation is adjusted by adding 1 to all the values. Triglycerides and creatinine distributions benefited in the same way from log transformation.

The cut off points for tertiles in the whole population for CRP were 0.87 mg/l and 2.32 mg/l. This differs very slightly from the case-control analysis, wherein the tertile cut points relate to the control group only, and therefore are, as one would expect, slightly lower (0.78 mg/l and 2 mg/l). Table 14 summarises the crude baseline differences for some of the important markers, when classified by CRP tertile. This is discussed in detail later.



Population distribution of CRP

Figure 10 Histogram demonstrating population distribution of CRP values



Population distribution of ESR

Figure 11 Histogram demonstrating population distribution of ESR values

Table 14 Summary of differences in baseline parameters according to CRP tertiles

	CRP Tertile	N	Descriptives						
			Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
BMI	<0.87	2179	24.5823	3.3075	7.088E-02	24.4433	24.7212	13.40	40.13
	0.87-2.32	2211	25.9218	3.5401	7.529E-02	25.7742	26.0694	14.79	41.77
	>2.32	2191	28.4501	4.2597	9.100E-02	28.2716	28.6286	15.12	53.91
	Total	6581	25.6542	3.8059	4.691E-02	25.5622	25.7461	13.40	53.91
Systolic BP (mm Hg)	<0.87	2183	140.3156	21.1961	.4537	139.4260	141.2053	92.00	290.00
	0.87-2.32	2215	143.8388	22.3210	.4743	142.9088	144.7689	90.00	240.00
	>2.32	2197	145.8081	24.1193	.5146	144.7970	146.8152	86.00	276.00
	Total	6595	143.3280	22.6998	.2794	142.7803	143.8757	86.00	290.00
Diastolic BP (mm Hg)	<0.87	2181	87.8446	11.0341	.2363	87.3812	88.3079	28.00	150.00
	0.87-2.32	2214	89.2913	11.8845	.2526	88.7960	89.7866	60.00	142.00
	>2.32	2195	89.8907	12.5625	.2681	89.3648	90.4165	50.00	164.00
	Total	6590	89.0121	11.8745	.1463	88.7254	89.2989	28.00	164.00
FEV1 (ml)	<0.87	2152	3027.6380	870.5115	18.7852	2990.8382	3064.4378	500.00	5800.00
	0.87-2.32	2182	2890.2821	827.4938	17.7148	2825.5224	2895.0019	650.00	5150.00
	>2.32	2180	2856.6289	841.6344	18.1091	2821.1138	2892.1399	500.00	5200.00
	Total	6494	2847.9955	859.9480	10.6713	2827.0783	2888.9147	500.00	5800.00
% FEV1 Predicted	<0.87	2150	90.9033	19.6619	.4240	90.0718	91.7349	20.31	220.37
	0.87-2.32	2178	87.9714	19.0793	.4088	87.1696	88.7731	19.51	158.82
	>2.32	2154	82.7667	20.4685	.4410	81.9019	83.6315	19.11	154.14
	Total	6482	87.2143	20.0224	.2487	86.7288	87.7018	19.11	220.37
Age at bleed (yrs)	<0.87	2188	53.9867	8.5860	.1836	53.6068	54.3267	33.30	85.80
	0.87-2.32	2224	55.8281	9.1319	.1936	55.4484	56.2079	33.38	85.97
	>2.32	2208	57.0977	9.5899	.2041	56.6975	57.4979	33.41	86.29
	Total	6620	55.6363	9.2016	.1131	55.4146	55.8580	33.30	86.29
Cholesterol (mmol/l)	<0.87	2186	6.5475	1.1559	2.472E-02	6.4991	6.5960	3.42	12.85
	0.87-2.32	2221	6.6426	1.2057	2.558E-02	6.5924	6.6928	2.80	18.98
	>2.32	2205	6.5722	1.1676	2.487E-02	6.5234	6.6209	3.08	12.12
	Total	6612	6.5877	1.1773	1.448E-02	6.5593	6.6161	2.80	18.98
Triglycerides (mmol/l)	<0.87	2070	1.0890	.5482	1.200E-02	1.0455	1.0925	.15	7.01
	0.87-2.32	2083	1.2855	.7465	1.636E-02	1.2335	1.2976	.20	11.29
	>2.32	2078	1.3340	.7461	1.637E-02	1.3019	1.3661	.32	11.29
	Total	6231	1.2231	.6953	8.809E-03	1.2058	1.2403	.15	11.29
log Triglycerides	<0.87	2070	-1.3485E-02	.1849	4.085E-03	-2.1437E-02	-5.4938E-03	-.83	.85
	0.87-2.32	2083	5.188E-02	.1988	4.358E-03	4.333E-02	6.042E-02	-.69	1.05
	>2.32	2078	7.731E-02	.1986	4.313E-03	6.885E-02	8.576E-02	-.50	1.05
	Total	6231	3.865E-02	.1973	2.499E-03	3.375E-02	4.355E-02	-.83	1.05
Fasting glucose (mmol/l)	<0.87	2172	4.4941	.7285	1.563E-02	4.4634	4.5248	.39	15.54
	0.87-2.32	2204	4.5857	.8627	1.838E-02	4.5296	4.6017	2.22	15.78
	>2.32	2188	4.6432	1.0509	2.247E-02	4.5991	4.6873	2.39	18.98
	Total	6564	4.5678	.8628	1.102E-02	4.5462	4.5894	.39	18.98
Glucose tolerance: 90 minutes (mmol/l)	<0.87	2124	5.6198	1.8518	4.018E-02	5.5410	5.6906	.00	21.65
	0.87-2.32	2117	5.6851	2.0786	4.518E-02	5.7985	5.9737	2.22	25.96
	>2.32	2074	6.1670	2.2647	4.973E-02	6.0895	6.2645	1.94	22.20
	Total	6315	5.8884	2.0820	2.620E-02	5.8371	5.9398	.00	25.96

10.8 Associations

Previous epidemiological studies have described a number of associations for baseline CRP values. The tables below show unadjusted non-parametric (Table 15) and parametric (Table 16) associations for CRP and log transformed CRP. Factors that stand out as being correlated with CRP are increasing age, smoking status, reductions in FEV1 and increases in BMI. Other associations include gender (male vs. female), fasting glucose, glucose tolerance at 90 minutes, triglycerides and systolic blood pressure. ESR values, precisely because they are another measure of inflammation, are strongly associated with baseline CRP.

Table 15 Spearman Rank Correlations for CRP

Spearman Rank Correlations	
	CRP
CRP	1.000
ESR	.351**
Age at bleed	.146**
Gender	.031*
Smoking status	.165**
FEV1	-.187**
BMI	.202**
Diabetic status	.025*
Fasting glucose	.068**
Glucose tolerance: 90 minutes	.109**
Cholesterol	.007
Triglycerides	.202**
Systolic BP	.101**
Diastolic BP	.065**
Haematocrit	.080**
Haemoglobin	.046**
Creatinine	.043**

** Correlation is significant at the .01 level

* Correlation is significant at the .05 level (2-tailed).

Table 16 Pearson correlations for CRP

Pearson correlations	
	log CRP
log CRP	1.000
Log ESR + 1	.359**
Age at bleed	.149**
Gender	.033**
Smoking status	.160**
FEV1	-.189**
BMI	.199**
Diabetic status	.025*
Fasting glucose	.067**
Glucose tolerance: 90 minutes	.115**
Cholesterol	.003
log Triglycerides	.182**
Systolic BP	.105**
Diastolic BP	.072**
Haematocrit	.078**
Haemoglobin	.050**
log Creatinine	.054**

** . Correlation is significant at the 0.01 level

* . Correlation is significant at the 0.05 level (2-tailed).

Simple adjustment for age, gender and smoking modifies the correlations further (Table 17). At this stage, other than cholesterol, all the variables appear to have, at least statistical association with baseline CRP values. The partial correlations are shown for all the other variables in Table 18.

Table 17 Partial correlations for CRP (adjusted for age, gender, smoking)

Variable	Adjusted correlation for log CRP
log CRP	1.000
log ESR + 1	0.388 **
FEV1	-0.169 **
BMI	0.220 **
Fasting glucose	0.060 **
Glucose tolerance: 90 minutes	0.113 **
Cholesterol	0.023
log Triglycerides	0.159 **
Systolic BP	0.098 **
Diastolic BP	0.095 **
Haematocrit	0.072 **
Haemoglobin	0.047 **
log Creatinine	0.039 **

** Correlation is significant at the 0.001 level (2 tailed)

Table 18 Partial correlations for all variables (adjusted for age, gender and smoking)

	log CRP	Log ESR + 1	FEV1	BMI	Fasting glucose	Glucose tolerance: 90 minutes	Cholesterol	Log Triglycerides	Systolic BP	Diastolic BP	Haematocrit	Haemoglobin	log Creatinine
Log CRP	-	0.388*	-0.169*	0.220*	0.060*	0.113*	0.023	0.156*	0.098*	0.095*	0.072*	0.047*	0.040 ⁺
Log ESR + 1	0.388*	-	-0.115*	0.066*	0.067*	0.082*	0.135*	0.069*	0.106*	0.078*	-0.268*	-0.230*	-0.046*
FEV1	-0.169*	-0.115*	-	0.028	-0.024	-0.090*	0.018	-0.067*	-0.087*	-0.079*	-0.116*	-0.036 ⁺	0.030
BMI	0.220*	0.066*	0.028	-	0.210*	0.164*	0.063*	0.325*	0.239*	0.274*	0.153*	0.202*	0.081*
Fasting glucose	0.060*	0.067*	-0.024	0.210*	-	0.588*	0.031	0.161*	0.162*	0.122*	0.014*	0.048*	-0.011
Glucose tolerance: 90 minutes	0.113*	0.082*	-0.090	0.164*	0.588*	-	0.022	0.214*	0.158*	0.128*	0.050*	0.092*	0.035 ⁺
Cholesterol	0.023	0.140*	0.018	0.063*	0.031	0.022	-	0.223*	0.110*	0.106*	0.130*	0.146*	0.036 ⁺
log Triglycerides	0.159*	0.069*	-0.067*	0.330*	0.161*	0.214	0.223*	-	0.135*	0.162*	0.160*	0.190*	0.180*
Systolic BP	0.098*	0.106*	-0.087*	0.239*	0.162*	0.158*	0.110*	0.135*	-	0.748*	0.168*	0.153*	0.050*
Diastolic BP	0.095*	0.078*	-0.079*	0.274*	0.122*	0.128*	0.106*	0.162*	0.748*	-	0.216*	0.210*	0.074*
Haematocrit	0.072*	-0.268*	-0.116*	0.153*	0.014	0.050*	0.130*	0.160*	0.168*	0.216*	-	0.828*	0.078*
Haemoglobin	0.047*	-0.230*	-0.036 ⁺	0.202*	0.048*	0.092*	0.146*	0.190*	0.153*	0.210*	0.828*	-	0.076*
log Creatinine	0.039 ⁺	-0.046*	0.030	0.081*	-0.011	0.035 ⁺	0.036 ⁺	0.180*	0.049*	0.074*	0.078*	0.076*	-

Two tailed significance indicated at * $P \leq 0.001$ or ⁺ $P \leq 0.01$.

To further explore the associations three different analyses have been performed: simple linear regression, multivariate analysis and finally binary logistic regression. Categorical variables are coded 0 and 1 to enable insertion in the models. Caution must be exercised in the interpretation of statistical associations, as the biological relevance may not be important. Therefore in depth discussion of every statistical association is deliberately avoided. The different approaches show how changes in models/assumptions can alter strengths of associations, but strong associations remain regardless, namely, smoking, BMI and FEV1.

10.8.1 Linear regression

When entered into a linear regression model, the correlations adjust further, such that diabetic status and blood pressure are no longer significant. Other associations become significant, albeit with very low correlations e.g. cholesterol. Those with correlations $\geq \pm 0.1$ are ESR, age, gender (male), smoking, FEV1, BMI, cholesterol, triglycerides and haematocrit. The analysis is repeated for males and females separately, with only minor changes to the findings (Tables 19, 20 and 21). The comparison for ESR is shown in Table 22 (gender is assigned to female, as ESR is positively correlated with female sex).

Table 19 Linear regression analysis for CRP: males and females

Linear regression: coefficients ^a

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95% Confidence Interval for B		Correlations		
		B	Std. Error	Beta			Lower Bound	Upper Bound	Zero-order	Partial	Part
1	(Constant)	-2.224	.200		-11.122	.000	-2.616	-1.832			
	Log ESR + 1	.530	.016	.426	32.627	.000	.498	.562	.356	.397	.367
	Age at bleed	5.813E-03	.001	.099	7.171	.000	.004	.007	.139	.095	.081
	Gender	.197	.019	.183	10.384	.000	.160	.234	.040	.136	.117
	Smoking status	.143	.013	.140	11.385	.000	.119	.168	.166	.149	.128
	FEV1	-7.765E-05	.000	-.134	-8.304	.000	.000	.000	-.180	-.109	-.093
	BMI	2.146E-02	.002	.164	12.933	.000	.018	.025	.196	.169	.145
	Diabetic status	-2.926E-02	.043	-.008	-.677	.498	-.114	.055	.031	-.009	-.008
	Fasting glucose	-2.337E-02	.009	-.038	-2.643	.008	-.041	-.006	.069	-.035	-.030
	Glucose tolerance: 90 minutes	1.297E-02	.003	.055	3.718	.000	.006	.020	.117	.049	.042
	Cholesterol	-2.988E-02	.005	-.071	-5.923	.000	-.040	-.020	.010	-.078	-.067
	log Triglycerides	.146	.032	.058	4.598	.000	.084	.208	.179	.061	.052
	Systolic BP	-6.836E-05	.000	-.003	-.180	.857	-.001	.001	.104	-.002	-.002
	Diastolic BP	-9.859E-04	.001	-.024	-1.354	.176	-.002	.000	.078	-.018	-.015
	Haematocrit	3.070E-02	.003	.222	9.034	.000	.024	.037	.092	.119	.101
	Haemoglobin	-4.070E-02	.015	-.066	-2.628	.009	-.071	-.010	.056	-.035	-.030
	log Creatinine	.209	.088	.031	2.362	.018	.035	.382	.052	.031	.027

a. Dependent Variable: log CRP

Table 20 Linear regression analysis for CRP: males only

Linear regression: Coefficients ^{a,b}

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	Correlations		
		B	Std. Error	Beta			Zero-order	Partial	Part
1	(Constant)	-2.145	.254		-8.431	.000			
	Log ESR + 1	.532	.019	.420	28.700	.000	.401	.413	.384
	Age at bleed	4.375E-03	.001	.074	4.540	.000	.132	.072	.061
	Smoking status	.136	.015	.130	9.083	.000	.184	.142	.122
	FEV1	-7.712E-05	.000	-.119	-7.482	.000	-.246	-.117	-.100
	BMI	2.083E-02	.002	.150	9.834	.000	.167	.153	.132
	Diabetic status	-7.999E-02	.052	-.022	-1.533	.125	.009	-.024	-.021
	Fasting glucose	-1.889E-02	.010	-.031	-1.812	.070	.058	-.029	-.024
	Glucose tolerance: 90 minutes	1.251E-02	.004	.054	3.069	.002	.114	.048	.041
	Cholesterol	-1.739E-02	.006	-.039	-2.779	.005	.030	-.044	-.037
	log Triglycerides	9.660E-02	.036	.040	2.660	.008	.147	.042	.036
	Systolic BP	-1.501E-04	.000	-.007	-.326	.745	.104	-.005	-.004
	Diastolic BP	-4.067E-04	.001	-.010	-.468	.640	.077	-.007	-.006
	Haematocrit	3.970E-02	.004	.246	10.201	.000	.083	.159	.136
	Haemoglobin	-7.829E-02	.018	-.106	-4.366	.000	.022	-.069	-.058
	log Creatinine	.217	.107	.028	2.033	.042	.015	.032	.027

a. Dependent Variable: log CRP

b. Selecting only cases for which Gender = Male

Table 21 Linear regression analysis for CRP: females only

Linear regression: Coefficients ^{a,b}

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	Correlations		
		B	Std. Error	Beta			Zero-order	Partial	Part
1	(Constant)	-1.813	.355		-5.113	.000			
	Log ESR + 1	.534	.034	.363	15.806	.000	.378	.361	.325
	Age at bleed	8.049E-03	.002	.132	5.185	.000	.179	.126	.106
	Smoking status	.160	.023	.157	6.885	.000	.151	.166	.141
	FEV1	-9.548E-05	.000	-.107	-4.394	.000	-.259	-.107	-.090
	BMI	2.247E-02	.003	.192	8.279	.000	.246	.199	.170
	Diabetic status	5.891E-02	.078	.016	.759	.448	.079	.019	.016
	Fasting glucose	-3.926E-02	.017	-.061	-2.344	.019	.079	-.057	-.048
	Glucose tolerance: 90 minutes	1.590E-02	.007	.064	2.377	.018	.134	.058	.049
	Cholesterol	-5.441E-02	.009	-.140	-6.307	.000	-.004	-.153	-.130
	log Triglycerides	.252	.065	.092	3.869	.000	.250	.094	.079
	Systolic BP	8.136E-05	.001	.004	.120	.904	.104	.003	.002
	Diastolic BP	-1.260E-03	.001	-.030	-.936	.349	.063	-.023	-.019
	Haematocrit	6.752E-03	.007	.041	.959	.338	.084	.023	.020
	Haemoglobin	5.252E-02	.032	.069	1.663	.097	.081	.041	.034
	log Creatinine	.154	.157	.021	.986	.325	.085	.024	.020

a. Dependent Variable: log CRP

b. Selecting only cases for which Gender = Female

Table 22 Linear regression analysis for ESR

Linear regression: Coefficients ^a

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	Correlations		
		B	Std. Error	Beta			Zero-order	Partial	Part
1	(Constant)	2.974	.153		19.445	.000			
	Age at bleed	-5.671E-03	.001	-.120	-9.373	.000	.051	-.123	-.098
	Gender (Female)	5.041E-02	.014	.058	3.524	.000	.338	.047	.037
	Smoking status	4.303E-02	.010	.052	4.528	.000	.141	.060	.047
	FEV1	-4.698E-05	.000	-.101	-6.698	.000	-.283	-.088	-.070
	BMI	-4.848E-04	.001	-.005	-.385	.700	.022	-.005	-.004
	Diabetic status	-1.262E-02	.032	-.004	-.390	.697	.012	-.005	-.004
	Fasting glucose	6.050E-03	.007	.012	.913	.361	.015	.012	.010
	Glucose tolerance: 90 minutes	4.576E-03	.003	.024	1.751	.080	.083	.023	.018
	Cholesterol	5.310E-02	.004	.158	14.265	.000	.192	.186	.149
	log Triglycerides	1.607E-02	.024	.008	.677	.499	.041	.009	.007
	Systolic BP	1.119E-03	.000	.064	3.937	.000	.082	.052	.041
	Diastolic BP	1.335E-03	.001	.040	2.450	.014	.003	.032	.026
	Haematocrit	-3.989E-02	.003	-.359	-15.912	.000	-.380	-.206	-.167
	Haemoglobin	-1.445E-02	.012	-.029	-1.246	.213	-.363	-.017	-.013
	log Creatinine	-.259	.066	-.048	-3.924	.000	-.203	-.052	-.041
	log CRP	.297	.009	.370	32.627	.000	.356	.397	.342

a. Dependent Variable: Log ESR + 1

10.8.2 Univariate analysis

Similarly to the linear regression model, a simple univariate analysis for CRP (General Linear Model: Univariate; Type IV Sum of Squares) confirms the independent associations as smoking, age, FEV1, BMI, fasting glucose (weak), glucose tolerance at 90 minutes, cholesterol, triglycerides, haematocrit, haemoglobin, creatinine (weak) and ESR; Tables 23, 24 and 25. There were no strongly significant interactions between factors, although gender and diabetes status was weakly significant, $P=0.027$. In this analysis, gender was not significantly associated with CRP values. By assigning gender to be either 0 or 1 in the weighted least squares option, analysis restricted to males or females was also performed.

Table 23 Between subject effects for CRP: males and females**Univariate analysis: Tests of Between-Subjects Effects**

Dependent Variable: log CRP

Source	Type IV Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	387.323 ^a	20	19.366	112.031	.000
Intercept	16.926	1	16.926	97.913	.000
Age bled	8.889	1	8.889	51.423	.000
FEV1	11.917	1	11.917	68.939	.000
BMI	28.681	1	28.681	165.919	.000
Fasting glucose	1.091	1	1.091	6.310	.012
Glucose tolerance: 90 minutes	2.413	1	2.413	13.960	.000
Cholesterol	6.015	1	6.015	34.794	.000
log Triglycerides	3.514	1	3.514	20.328	.000
Systolic BP	6.841E-03	1	6.841E-03	.040	.842
Diastolic BP	.304	1	.304	1.758	.185
Haematocrit	14.044	1	14.044	81.243	.000
Haemoglobin	1.202	1	1.202	6.951	.008
log Creatinine	.933	1	.933	5.396	.020
log ESR + 1	183.471	1	183.471	1061.365	.000
Gender (Male)	.562	1	.562	3.249	.072
Smoking status	2.545	1	2.545	14.721	.000
Diabetic status	3.596E-02	1	3.596E-02	.208	.648
Error	982.902	5686	.173		
Total	1502.575	5707			
Corrected Total	1370.225	5706			

a. R Squared = .283 (Adjusted R Squared = .280)

Table 24 Between subject effects for CRP: females only**Univariate analysis (Females only): Tests of Between-Subjects Effects ^b**

Dependent Variable: log CRP

Source	Type IV Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	128.176 ^a	16	8.011	44.140	.000
Intercept	4.024	1	4.024	22.171	.000
Age bled	4.851	1	4.851	26.730	.000
FEV1	3.488	1	3.488	19.217	.000
BMI	12.290	1	12.290	67.717	.000
Fasting glucose	1.039	1	1.039	5.725	.017
Glucose tolerance: 90 minutes	1.087	1	1.087	5.990	.014
Cholesterol	7.167	1	7.167	39.492	.000
log Triglycerides	2.745	1	2.745	15.126	.000
Systolic BP	3.388E-03	1	3.388E-03	.019	.891
Diastolic BP	.157	1	.157	.867	.352
Haematocrit	.158	1	.158	.869	.351
Haemoglobin	.509	1	.509	2.802	.094
log Creatinine	.180	1	.180	.990	.320
log ESR + 1	45.168	1	45.168	248.866	.000
Smoking status	1.438	1	1.438	7.922	.005
Diabetic status	.223	1	.223	1.227	.268
Error	302.186	1665	.181		
Total	455.254	1682			
Corrected Total	430.362	1681			

a. R Squared = .298 (Adjusted R Squared = .291)

b. Weighted Least Squares Regression - Weighted by Gender: Male =0; Female =1

Table 25 Between subject effects for CRP: males only

Univariate analysis (Male only): Tests of Between-Subjects Effects ^b

Dependent Variable: log CRP

Source	Type IV Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	264.860 ^a	16	16.554	98.619	.000
Intercept	11.487	1	11.487	68.436	.000
Age bled	3.464	1	3.464	20.635	.000
FEV1	9.390	1	9.390	55.941	.000
BMI	16.200	1	16.200	96.509	.000
Fasting glucose	.546	1	.546	3.251	.071
Glucose tolerance: 90 minutes	1.582	1	1.582	9.424	.002
Cholesterol	1.297	1	1.297	7.725	.005
log Triglycerides	1.185	1	1.185	7.062	.008
Systolic BP	1.748E-02	1	1.748E-02	.104	.747
Diastolic BP	3.712E-02	1	3.712E-02	.221	.638
Haematocrit	17.442	1	17.442	103.911	.000
Haemoglobin	3.199	1	3.199	19.057	.000
log Creatinine	.694	1	.694	4.137	.042
log ESR + 1	138.235	1	138.235	823.535	.000
Smoking	1.227	1	1.227	7.307	.007
Diabetic status	.318	1	.318	1.896	.169
Error	672.764	4008	.168		
Total	1047.321	4025			
Corrected Total	937.625	4024			

a. R Squared = .282 (Adjusted R Squared = .280)

b. Weighted Least Squares Regression - Weighted by Gender: Male =1
Female =0

10.8.3 Multivariate analysis

Table 26 shows a multivariate analysis for CRP and ESR (General Linear Model: Multivariate; Type IV Sum of squares). In this analysis, the fixed factors chosen are smoking, gender and diabetic status, whilst the other variables are treated as covariates. For CRP it is notable that cholesterol, gender, and diabetic status, along with creatinine and both systolic and diastolic blood pressure are no longer significantly associated. Note this analysis clearly does not include ESR/CRP as covariates.

Table 26 Multivariate analysis for CRP and ESR

Multivariate analysis : Tests of Between-Subjects Effects

Source	Dependent Variable	Type IV Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	log CRP	203.851 ^a	19	10.729	52.312	.000
	Log ESR + 1	229.837 ^b	19	12.097	105.167	.000
Intercept	log CRP	1.174	1	1.174	5.725	.017
	Log ESR + 1	35.142	1	35.142	305.523	.000
Age Bled	log CRP	2.940	1	2.940	14.337	.000
	Log ESR + 1	5.841	1	5.841	50.784	.000
FEV1	log CRP	29.855	1	29.855	145.566	.000
	Log ESR + 1	13.912	1	13.912	120.949	.000
BMI	log CRP	39.529	1	39.529	192.734	.000
	Log ESR + 1	3.014	1	3.014	26.200	.000
Fasting glucose	log CRP	1.136	1	1.136	5.539	.019
	Log ESR + 1	1.643E-03	1	1.643E-03	.014	.905
Glucose tolerance: 90 minutes	log CRP	4.803	1	4.803	23.417	.000
	Log ESR + 1	1.444	1	1.444	12.552	.000
Cholesterol	log CRP	2.674E-02	1	2.674E-02	.130	.718
	Log ESR + 1	19.270	1	19.270	167.529	.000
log Triglycerides	log CRP	5.579	1	5.579	27.203	.000
	Log ESR + 1	.843	1	.843	7.330	.007
Systolic BP	log CRP	.440	1	.440	2.146	.143
	Log ESR + 1	1.986	1	1.986	17.262	.000
Diastolic BP	log CRP	2.850E-02	1	2.850E-02	.139	.709
	Log ESR + 1	.522	1	.522	4.539	.033
Haematocrit	log CRP	1.972	1	1.972	9.615	.002
	Log ESR + 1	20.566	1	20.566	178.801	.000
Haemoglobin	log CRP	2.383	1	2.383	11.619	.001
	Log ESR + 1	.712	1	.712	6.193	.013
log Creatinine	log CRP	.145	1	.145	.706	.401
	Log ESR + 1	1.225	1	1.225	10.649	.001
Smoking status	log CRP	4.501	1	4.501	21.944	.000
	Log ESR + 1	.983	1	.983	8.545	.003
Gender (Male)	log CRP	.262	1	.262	1.276	.259
	Log ESR + 1	.202	1	.202	1.757	.185
Diabetic status	log CRP	3.472E-02	1	3.472E-02	.169	.681
	Log ESR + 1	3.894E-05	1	3.894E-05	.000	.985
Error	log CRP	1166.374	5687	.205		
	Log ESR + 1	654.137	5687	.115		
Total	log CRP	1502.575	5707			
	Log ESR + 1	5132.393	5707			
Corrected Total	log CRP	1370.225	5706			
	Log ESR + 1	883.974	5706			

a. R Squared = .149 (Adjusted R Squared = .146)

b. R Squared = .260 (Adjusted R Squared = .258)

10.8.4 Binary logistic regression

Finally, binary logistic regression, assessing the odds of CRP being in the upper third of the population distribution, was performed. Only subtle differences result, particularly depending on whether ESR is included as a covariate (Tables 27 and 28).

Table 27 Binary logistic regression: association with CRP value being in the upper tertile of distribution - ESR excluded

		Binary logistic regression: ESR excluded						95.0% C.I. for EXP(B)	
		B	S.E.	Wald	df	Sig.	Exp(B)	Lower	Upper
Step 1	Smoking status	.793	.066	144.247	1	.000	2.210	1.942	2.516
	Gender (Male)	.622	.101	37.598	1	.000	1.863	1.527	2.272
	Diabetic status	-.222	.225	.973	1	.324	.801	.515	1.245
	Age bleed	.013	.004	9.472	1	.002	1.013	1.005	1.022
	FEV1	.000	.000	75.754	1	.000	1.000	.999	1.000
	BMI	.085	.009	92.102	1	.000	1.089	1.070	1.108
	Fasting glucose	-.069	.047	2.155	1	.142	.933	.851	1.023
	Glucose tolerance: 90 minutes	.067	.018	13.234	1	.000	1.069	1.031	1.109
	Cholesterol	-.027	.027	1.003	1	.317	.974	.924	1.026
	log Triglycerides	.641	.169	14.356	1	.000	1.898	1.363	2.645
	Systolic BP	.001	.002	.092	1	.762	1.001	.997	1.005
	Diastolic BP	.001	.004	.033	1	.856	1.001	.993	1.008
	Haematocrit	.071	.018	14.995	1	.000	1.073	1.036	1.112
	Haemoglobin	-.292	.085	11.694	1	.001	.747	.632	.883
	log Creatinine	.163	.470	.121	1	.728	1.178	.468	2.960
	Constant	-4.064	1.045	15.112	1	.000	.017		

a. Variable(s) entered on step 1: SMOK, SEX01, DIABHIST, AGEbled, FEV1, BMI, GLUFMMOL, GLU90MMO, CHOLMMOL, LOGTRI, BPSYS, BPDIA, HCT, HBMMOL, LOGCREAT.

Table 28 Binary logistic regression: association with CRP value being in the upper tertile of distribution - ESR included

Binary logistic regression: ESR included

		B	S.E.	Wald	df	Sig.	Exp(B)	95.0% C.I. for EXP(B)	
								Lower	Upper
Step 1	Smoking status	.644	.071	83.221	1	.000	1.905	1.659	2.188
	Gender (Male)	.687	.108	40.240	1	.000	1.988	1.608	2.458
	Diabetic status	-.176	.238	.546	1	.460	.839	.527	1.336
	Age bled	.026	.005	30.408	1	.000	1.026	1.017	1.036
	FEV1	.000	.000	27.808	1	.000	1.000	1.000	1.000
	BMI	.080	.009	70.696	1	.000	1.083	1.063	1.103
	Fasting glucose	-.079	.050	2.453	1	.117	.924	.837	1.020
	Glucose tolerance: 90 minutes	.051	.020	6.801	1	.009	1.053	1.013	1.094
	Cholesterol	-.154	.029	28.112	1	.000	.857	.810	.907
	log Triglycerides	.527	.180	8.619	1	.003	1.695	1.192	2.410
	Systolic BP	-.002	.002	1.148	1	.284	.998	.993	1.002
	Diastolic BP	-.002	.004	.252	1	.616	.998	.990	1.006
	Haematocrit	.167	.020	66.883	1	.000	1.182	1.136	1.231
	Haemoglobin	-.218	.092	5.577	1	.018	.804	.672	.964
	log Creatinine	.701	.508	1.901	1	.168	2.016	.744	5.459
	log ESR + 1	2.482	.106	551.092	1	.000	11.961	9.723	14.715
Constant	-11.674	1.175	98.758	1	.000	.000			

a. Variable(s) entered on step 1: SMOK, SEX01, DIABHIST, AGEbled, FEV1, BMI, GLUFMMOL, GLU90MMO, CHOLMMOL, LOGTRI, BPSYS, BPDIA, HCT, HBMMOL, LOGCREAT, LOGESR1.

If the analysis is restricted to males or females the results are as indicated in Tables 29 and 30. The only differences of note appear as adjusted associations with haemoglobin and haematocrit.

Table 29 Binary logistic regression: association with CRP value being in the upper tertile of distribution - ESR included, males only

Binary logistic regression: ESR Included, Males only

		B	S.E.	Wald	df	Sig.	Exp(B)	95.0% C.I. for EXP(B)	
								Lower	Upper
Step 1	Smoking status	.618	.084	53.741	1	.000	1.855	1.572	2.188
	Diabetic status	-.488	.300	2.652	1	.103	.614	.341	1.104
	Age bled	.019	.006	11.872	1	.001	1.019	1.008	1.031
	FEV1	.000	.000	20.111	1	.000	1.000	1.000	1.000
	BMI	.074	.012	36.128	1	.000	1.077	1.051	1.104
	Fasting glucose	-.057	.060	.896	1	.344	.945	.839	1.063
	Glucose tolerance: 90 minutes	.049	.023	4.487	1	.034	1.051	1.004	1.100
	Cholesterol	-.109	.036	8.952	1	.003	.897	.835	.963
	log Triglycerides	.409	.210	3.789	1	.052	1.506	.997	2.273
	Systolic BP	-.003	.003	1.689	1	.194	.997	.991	1.002
	Diastolic BP	.000	.005	.002	1	.966	1.000	.990	1.010
	Haematocrit	.215	.025	76.103	1	.000	1.240	1.181	1.301
	Haemoglobin	-.365	.110	11.073	1	.001	.694	.560	.861
	log Creatinine	.575	.627	.842	1	.359	1.778	.520	6.077
	log ESR + 1	2.522	.123	422.876	1	.000	12.458	9.796	15.844
	Constant	-11.486	1.508	58.040	1	.000	.000		

a. Variable(s) entered on step 1: SMOK, DIABHIST, AGEbled, FEV1, BMI, GLUFMMOL, GLU90MMO, CHOLMMOL, LOGTRI, BPSYS, BPDIA, HCT, HBMMOL, LOGCREAT, LOGESR1.

Table 30 Binary logistic regression: association with CRP value being in the upper tertile of distribution - ESR included, females only

Binary logistic regression: ESR Included, Females only

		B	S.E.	Wald	df	Sig.	Exp(B)	95.0% C.I. for EXP(B)	
								Lower	Upper
Step 1	Smoking status	.738	.133	30.914	1	.000	2.091	1.612	2.713
	Diabetic status	.369	.421	.789	1	.381	1.446	.634	3.298
	Age bled	.039	.009	19.373	1	.000	1.040	1.022	1.059
	FEV1	.000	.000	11.222	1	.001	1.000	.999	1.000
	BMI	.090	.015	34.863	1	.000	1.094	1.062	1.128
	Fasting glucose	-.145	.096	2.257	1	.133	.865	.716	1.045
	Glucose tolerance: 90 minutes	.069	.037	3.423	1	.064	1.072	.996	1.153
	Cholesterol	-.260	.051	25.483	1	.000	.771	.697	.853
	log Triglycerides	.686	.360	3.634	1	.057	1.986	.981	4.022
	Systolic BP	.000	.004	.016	1	.900	1.000	.992	1.007
	Diastolic BP	-.001	.008	.015	1	.903	.999	.984	1.014
	Haematocrit	.060	.040	2.256	1	.133	1.062	.982	1.148
	Haemoglobin	.106	.183	.333	1	.564	1.111	.776	1.591
	log Creatinine	.850	.891	.909	1	.340	2.339	.408	13.416
	log ESR + 1	2.474	.219	127.655	1	.000	11.874	7.730	18.240
	Constant	-10.461	2.065	25.671	1	.000	.000		

a. Variable(s) entered on step 1: SMOK, DIABHIST, AGEbled, FEV1, BMI, GLUFMMOL, GLU90MMO, CHOLMMOL, LOGTRI, BPSYS, BPDIA, HCT, HBMMOL, LOGCREAT, LOGESR1.

Further analysis, with reclassification of some of the variables, again in a binary logistic regression model, was performed specifically to calculate the odds of having a CRP in the upper tertile of the distribution (Table 31). This shows how for example a smoker has twice the chance of having an elevated CRP; similarly so for someone who is morbidly obese. The FEV1 and %FEV1 interaction is more complex, as the number of individuals with severely impaired FEV1 at baseline is small, and FEV1 itself needs to be interpreted with respect to age, sex and height. The pooled analysis of %FEV1 shows that those with severely impaired lung function have around a two fold increase in likelihood of having a raised CRP (one greater than 2.32 mg/l).

Table 31 Odds ratio of having a CRP in the upper tertile of population distribution

<i>Condition</i>	Females only	Males only	Pooled
	Odds ratio (95% CI) of being in upper third of CRP distribution; P<0.001 unless indicated		
Smoker vs. Non-smoker	2.091 (1.612 – 2.713)	1.855 (1.572 – 2.188)	1.905 (1.659 – 2.188)
Male vs. Female			1.988 (1.608 - 2.458)
%FEV1 <40 (Severe) vs. Normal	3.221 (1.676 – 6.192)	1.656 (0.765 – 3.587) n.s.	2.415 (1.482 – 3.933)
%FEV1 40-60 (Moderate) vs. Normal	1.469 (1.029 – 2.098)	1.394 (0.973 – 1.997) n.s.	1.401 (1.097 – 1.789)
FEV1 >75th centile vs. 25th centile	0.011 (0 – 1414) n.s.	0.609 (0.463 – 0.803)	0.600 (0.473 – 0.761)
FEV1 50-75th centile vs. 25th centile	0.772 (0.473 – 1.258) n.s.	0.831 (0.643 – 1.075) n.s.	0.787 (0.638 – 0.972)
BMI > 30 vs. < 25	2.655 (1.814 – 3.887)	2.006 (1.536 – 2.620)	2.183 (1.758 – 2.712)
BMI 25-30 vs. < 25	1.443 (1.114 – 1.870)	1.356 (1.147 – 1.603)	1.375 (1.196 – 1.581)
Age > 70 vs. <50	2.130 (1.225 – 3.703)	2.210 (1.440 – 3.392)	2.043 (1.474 – 2.832)
Age 60-70 vs. <50	2.197 (1.461 – 3.3041)	1.171 (0.922 – 1.488) n.s.	1.42 (1.161 – 1.737)

10.8.5 Individual associations

Studies such as ours shed detailed light on the factors associated with baseline CRP values. Although some associations do not persist after adjustment, it nevertheless demonstrates that classical descriptions of what constitutes inflammatory states may need to be revised. The following analysis (Figures 12-

15) shows clearly the associations of age, gender, diabetes, and smoking on baseline CRP values. In doing such analysis, it remains important to note that association does not imply causality.

10.8.5.1 Age

This study was skewed to patients over 50 (as the object was to investigate future cardiovascular events) and therefore does not have an adequate spread of individuals across all ages, to fully address the relationship between age and CRP. It is not surprising nonetheless that there was an increase in baseline CRP as individuals aged, with a mean difference in age of + 3 yrs when comparing individuals in the upper and lower tertiles of CRP distribution ($P < 0.001$); see Table 14 and Figure 12. This equates to a mean difference of 0.82mg/l when comparing those over 70 with those under 50 ($P < 0.001$). After full adjustment age remains correlated with log CRP ($P < 0.001$), and has a partial correlation coefficient of 0.1 in the linear regression analysis.

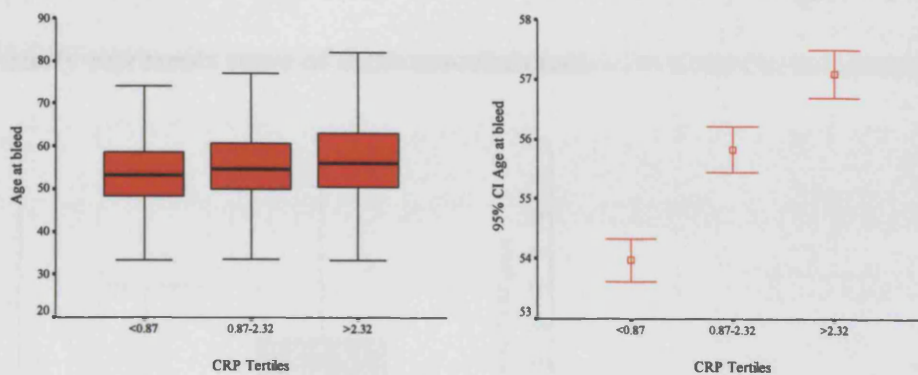


Figure 12 CRP values and age

10.8.5.2 Smoking

The baseline data categorises individuals into active smokers (includes cigarettes, pipes and cigars) and non-smokers. The non-smoker category includes ex-smokers. Quantification of smoking habits was not available for this part of the analysis. Current smokers have 50% greater baseline CRP values as compared to non-smokers with geometric means of 1.85 mg/l (95% CI 1.77 - 1.94) vs. 1.27 mg/l (95% CI 1.23 - 1.32); $P < 0.001$. When fully adjusted for the other covariate smokers had a geometric mean CRP of 1.73mg/l whilst for non-smokers it was 1.12 mg/l ($P < 0.001$). It appears the effect of smoking on CRP values equates to an active process, since if the analysis is repeated, classifying individuals into active or previous smokers and those that have never smoked, there is no difference in baseline CRP values. Fully adjusted values reveal for

those that smoke or who have smoked a geometric mean CRP of 1.21 mg/l vs. 1.35 mg/l for those that have never smoked ($P>0.05$). This is in keeping, for example, with another large study recently published [411]. Figure 13 below graphically represents some of these associations.

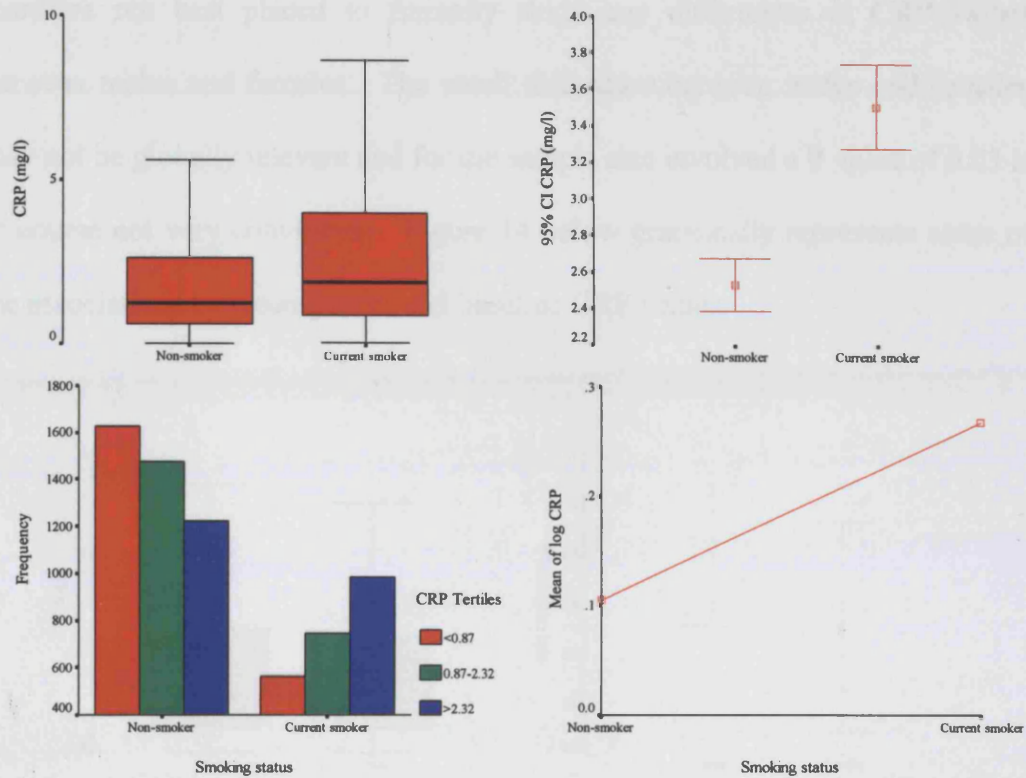


Figure 13 CRP values and smoking

10.8.5.3 Gender

The effects of gender in this study are not very striking, particularly when fully adjusted, as already shown above. The unadjusted geometric mean for men was 1.48 mg/l (95% CI 1.44 – 1.53) and for women was 1.36 mg/l (95% CI 1.30 – 1.44); $P = 0.006$. Full adjustment reveals a geometric mean CRP for men of 1.63 mg/l vs. for women 1.29 mg/l; this is however only just significant with a P

value of 0.033. Of note our study does not have equal numbers of males and females, we lack a complete drug history (information was not recorded on the use of aspirin or “statins” and fewer than 5% of the women reported use of hormone replacement treatment), and the age range of subjects is limited. It is therefore not best placed to formally study any differences in CRP values between males and females. The small difference between males and females may not be globally relevant and for the sample size involved a P value of 0.03 is of course not very convincing. Figure 14 below graphically represents some of the associations between gender and baseline CRP values.

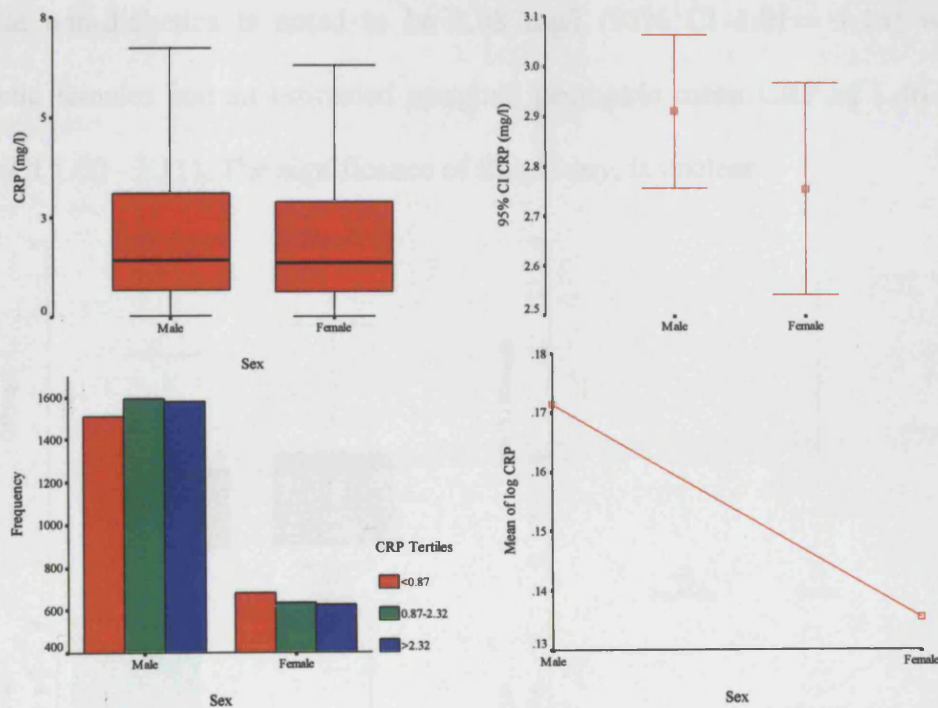


Figure 14 CRP values and gender

10.8.5.4 Diabetes

The total number of diabetics in this study is small (n=152; M 98 : F 54) but the geometric mean CRP for a diabetic patient was 1.74 mg/l (95% CI 1.45- 2.04) as opposed to 1.44 mg/l (95% CI 1.40 – 1.48) for a non-diabetic individual; P= 0.04 (Figure 15). As noted already on further adjustment, the association does not hold for diabetes, but this study has too few diabetics individuals for a truly robust analysis of this important subgroup. However interestingly in the multivariate analysis there was an interaction noted between diabetes and gender (P =0.027), and with full adjustment the estimated marginal geometric mean for female non-diabetics is noted to be 1.08 mg/l (95% CI 1.01 - 1.15) whilst diabetic females had an estimated marginal geometric mean CRP of 1.46 mg/l (95%CI 1.00 - 2.11). The significance of this, if any, is unclear.

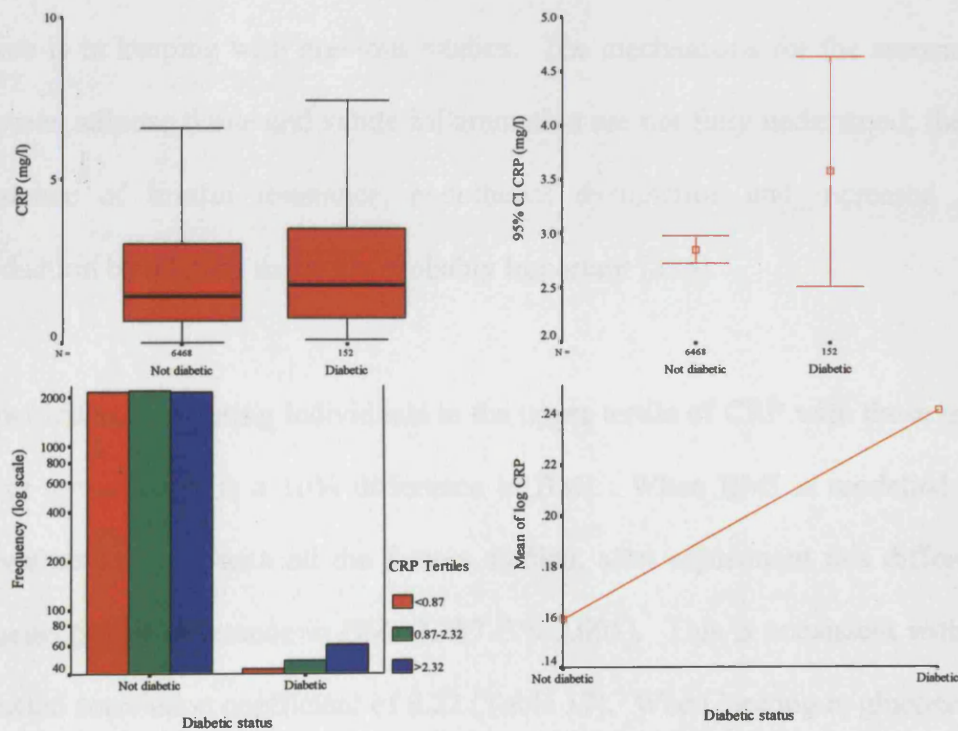


Figure 15 CRP values and diabetes status

More involved associations with parameters such as BMI, FEV1 and blood pressure have also been described before, and we have found similar associations.

10.8.5.5 BMI and fasting glucose/glucose tolerance

Obesity, impaired glucose tolerance and the metabolic syndrome are important, modifiable, cardiovascular risk factors. The body mass index (weight/height²) is a good marker of adjusted body mass, although it doesn't distinguish the distribution of the weight. The WHO has classified BMI as follows: "normal weight" is defined as BMI \geq 18.5, but less than 25; "overweight" equals BMI \geq 25, but less than 30; and "obese" individuals have BMIs \geq 30. In our study, BMI remained significantly associated with baseline CRP values throughout analysis, which is in keeping with previous studies. The mechanisms for the association between adipose tissue and subtle inflammation are not fully understood; the co-existence of insulin resistance, endothelial dysfunction and increased IL-6 production by adipose tissue are probably important [236].

In particular, comparing individuals in the upper tertile of CRP with those in the lower tertile, there is a 10% difference in BMI. When BMI is modelled in a univariate analysis with all the factors studied, after adjustment this difference remains (mean difference in BMI 2.287 P < 0.001). This is consistent with the adjusted correlation coefficient of 0.22 (Table 17). When looking at glucose and glucose tolerance at baseline, before adjustment, the difference between tertiles of CRP in fasting glucose is 0.149 mmol/l. After adjustment (not including

diabetes status or 90 minute glucose tolerance), the adjusted mean difference between the upper tertile and lower tertile of CRP in value is 0.026 mmol/l and is not significant. Excluding the small number of diabetics doesn't change this. However the relationship between glucose tolerance at 90 minutes, a dynamic physiological challenge, is stronger. At baseline the difference between upper and lower tertiles of CRP for the 90 minute glucose tolerance value is 0.54 mmol/l ($P < 0.001$). After adjustment (including for the baseline glucose value but not diabetic status), the mean difference falls to 0.188 mmol/l but remains highly significant ($P = 0.005$). If you exclude all diabetics, the difference becomes 0.201 mmol/l ($P = 0.002$). Whilst the magnitude of the change is small, the association of baseline CRP values with a dynamic function of insulin sensitivity is intriguing and in keeping with the concept of dysregulated inflammation in glucose intolerance and diabetes. More specific studies on restricted groups of individuals would be needed to extend these observations.

Figures 16- 20 illustrate the associations described above.

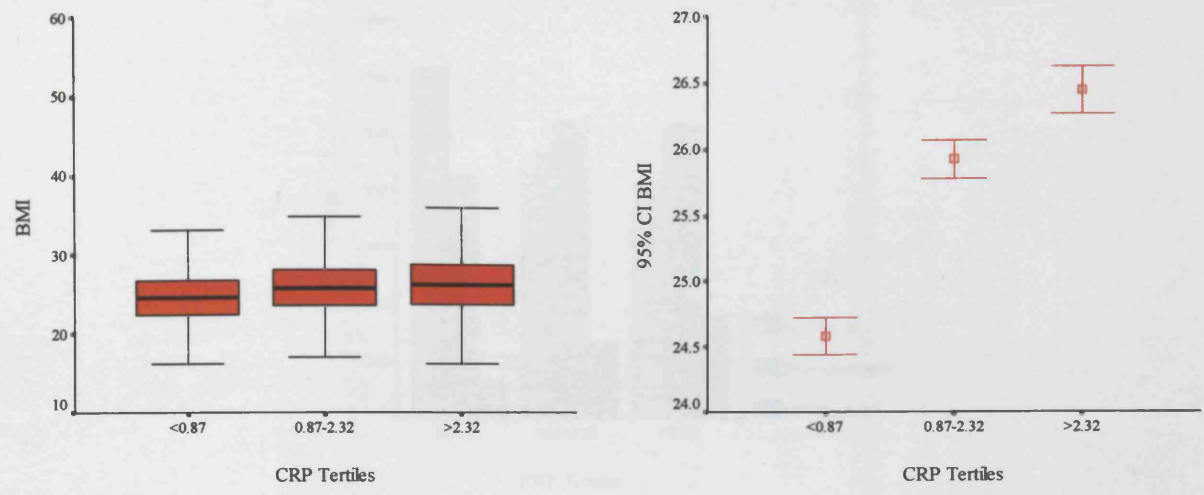


Figure 16 BMI and CRP values

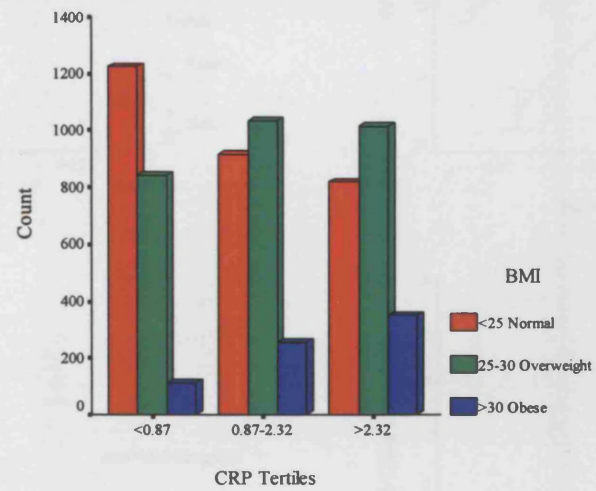


Figure 17 WHO Categories of obesity and CRP values

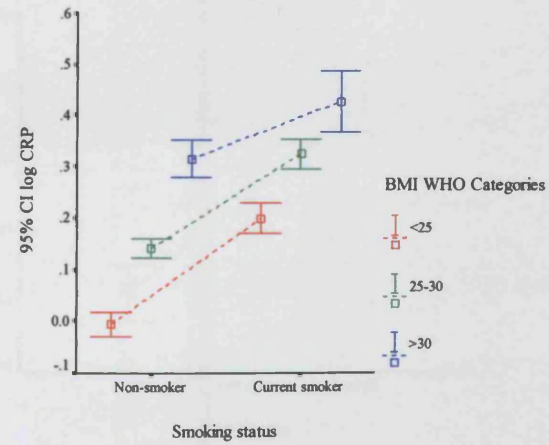
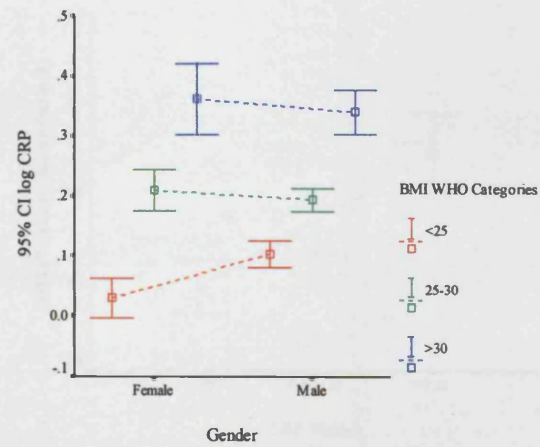
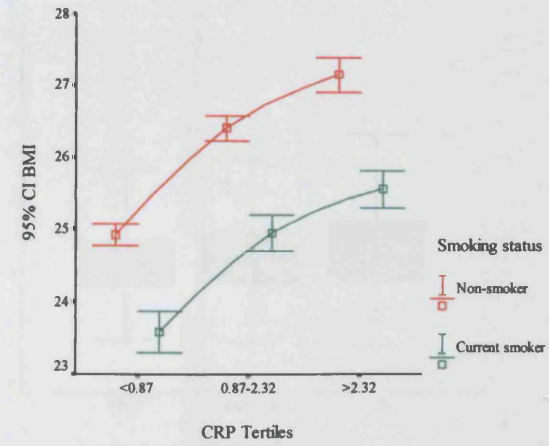
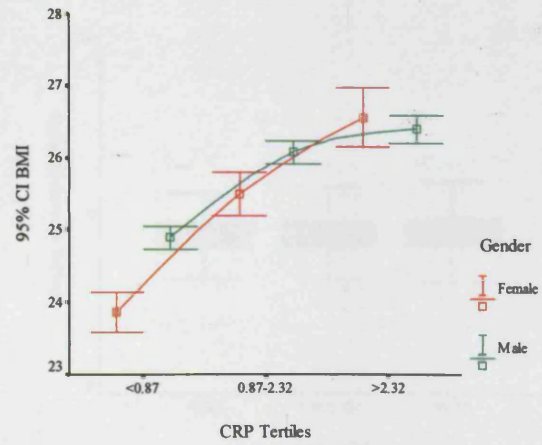


Figure 18 BMI and CRP values: effect of smoking and gender

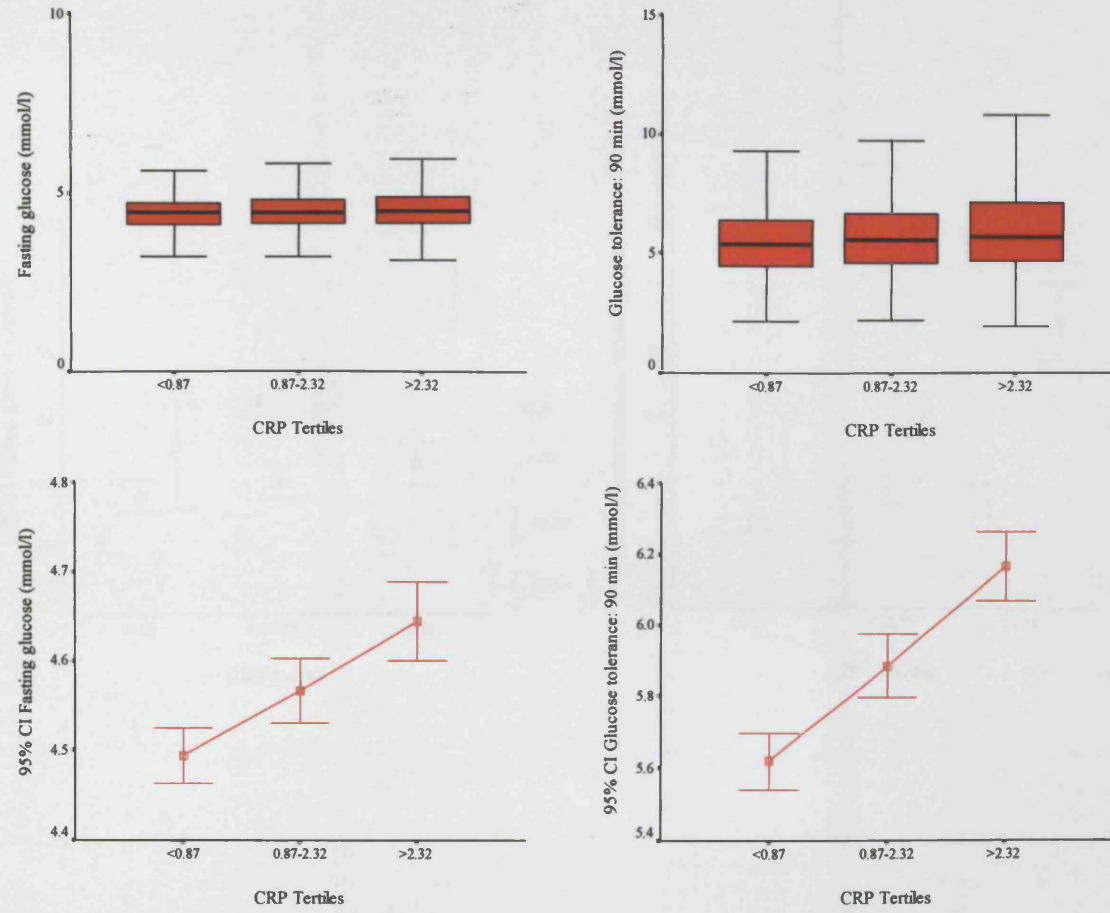


Figure 19 Fasting glucose and glucose tolerance and CRP values

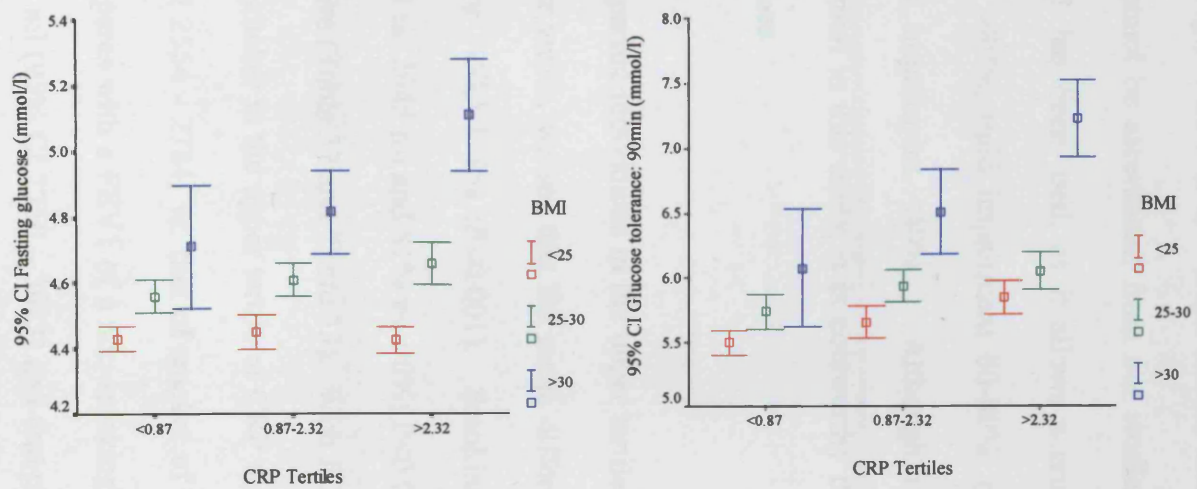


Figure 20 Fasting glucose and glucose tolerance and CRP values: effect of BMI

10.8.5.6 FEV1 and %FEV1

Lung function is an interesting association for CRP (Figure 21). Although at first thought it may be assumed to just reflect smoking habits, this is not the case, as demonstrated in Figures 22 and 23. Other factors must be important, although precisely what cannot be ascertained from our studies. To add some clinical correlate, %FEV1 has been used, as it allows a crude classification of lung function: normal >80%, mild impairment 60-80%, moderate impairment 40-60%, and severe impairment <40%. Although the numbers with severe impairment are small in this study, it is noteworthy that this is the group with elevated CRP values.

Specifically, comparing individuals in the upper tertile of CRP distribution with those in the lower tertile, we see that the mean difference in FEV1 is -371 ml ($P<0.001$) and for %FEV1 8% ($P<0.001$). Smoking does affect FEV1 and %FEV1 (2662 ml vs. 2945 ml and 81% vs. 90%; $P<0.001$) but the differences in CRP values persists (Table 32 and Table 33). With full adjustment for the other covariates a non-smoker in the upper tertile of CRP distribution has an FEV1 of 2669 ml (95% CI 2554 - 2784) vs. that of smoker of 2375 ml (95% CI 2216 - 2534). This compares with a FEV1 of a non-smoking individual with a CRP < 0.87mg/l of 2881 ml (95% CI 2750 – 3013) and that of a smoker 2956 ml (95% CI 2681 – 3231). There is therefore more to the relationship between reduced crude lung function and CRP values, than just smoking. Interestingly when one reclassifies smokers into those that smoke or have smoked as opposed to those that have never smoked, whilst the difference in FEV1/%FEV1 with respect to

CRP remain, there is no effect of smoking history. Adjusted values for FEV1 for example, in those that have never smoked are 2651 ml (95% CI 2565 – 2737) compared to 2715 ml (95% CI 2602 – 2827) for those that are current or ex-smokers.

Table 32 FEV1, smoking and CRP distribution

CRP Tertiles vs Smoking status

Dependent Variable: FEV1

CRP Tertiles	Smoking status	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
<0.87	Non-smoker	3098.329	20.969	3057.223	3139.435
	Current smoker	2821.231	35.831	2750.991	2891.472
0.87-2.32	Non-smoker	2935.392	22.010	2892.246	2978.538
	Current smoker	2709.900	31.137	2648.861	2770.938
>2.32	Non-smoker	2750.625	24.266	2703.056	2798.194
	Current smoker	2539.788	27.054	2486.753	2592.823

Table 33 %FEV1, smoking and CRP distribution

CRP Tertiles vs Smoking status

Dependent Variable: % FEV1

CRP Tertiles	Smoking status	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
<0.87	Non-smoker	92.743	.484	91.794	93.692
	Current smoker	85.525	.828	83.903	87.147
0.87-2.32	Non-smoker	90.741	.508	89.744	91.737
	Current smoker	82.433	.719	81.023	83.842
>2.32	Non-smoker	86.623	.561	85.524	87.722
	Current smoker	77.970	.625	76.745	79.196

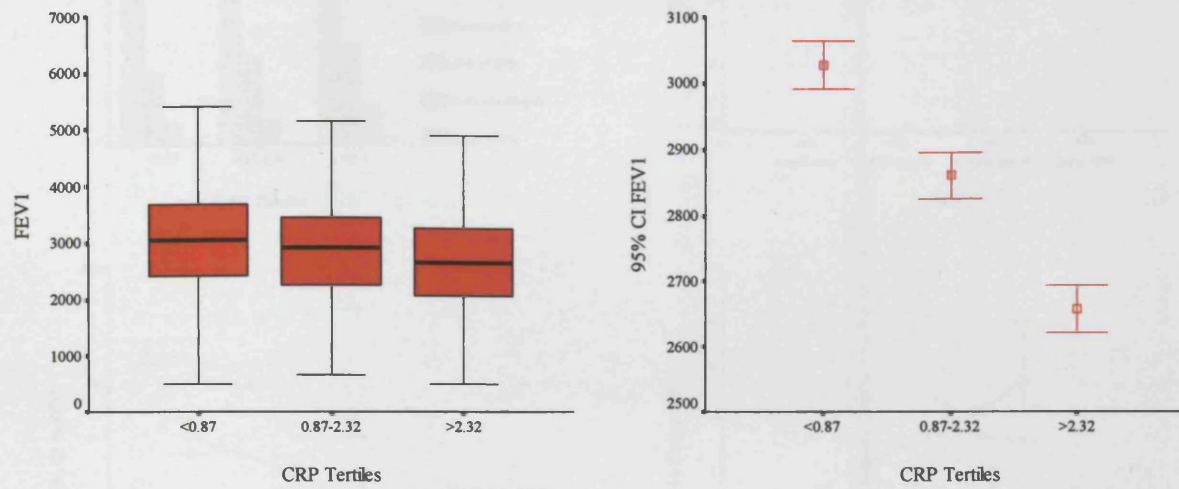


Figure 21 Crude lung function and CRP values

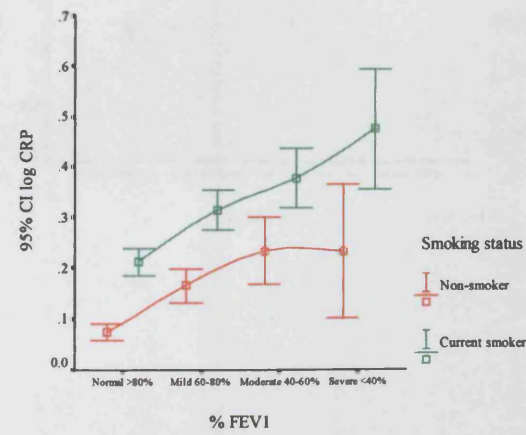
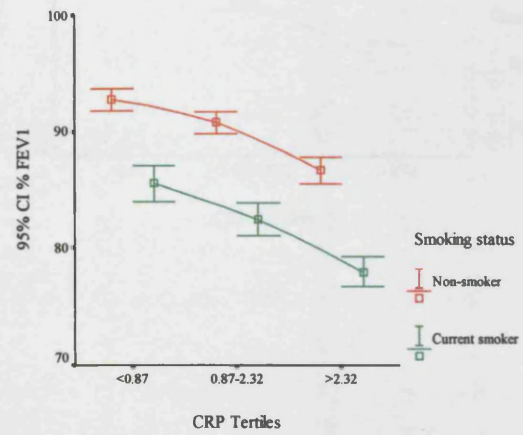
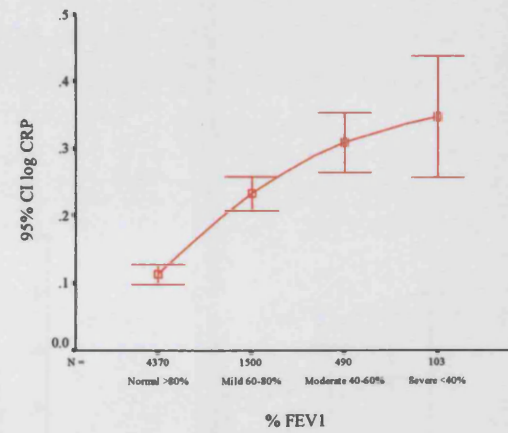
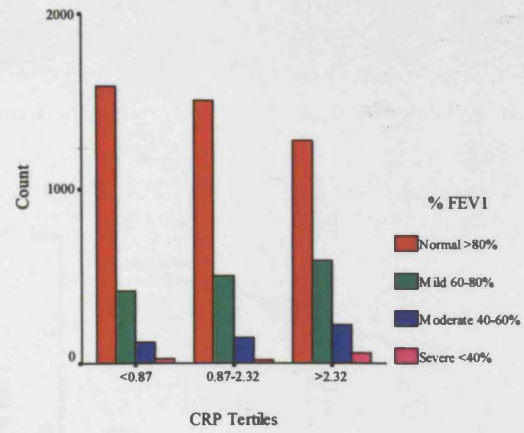


Figure 22 Crude lung function and CRP values: effect of smoking

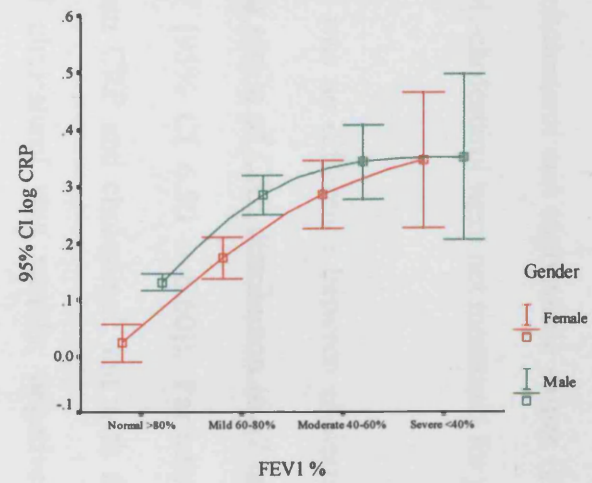
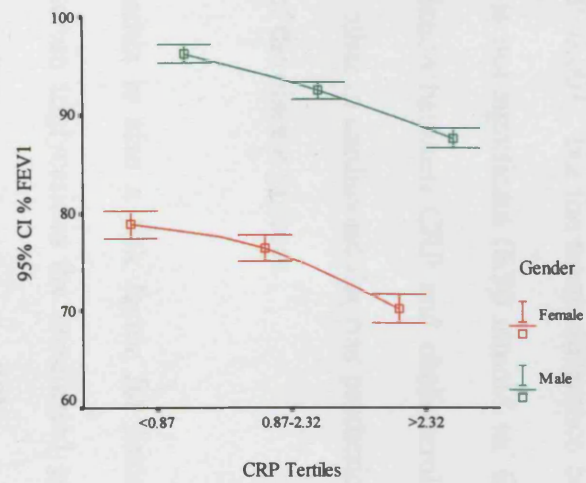


Figure 23 Crude lung function and CRP values: effect of gender

10.8.5.7 Lipid parameters

Hyperlipidaemia, in particular raised cholesterol is clearly an established major cardiovascular risk marker. Our study was able to assess direct relationships with total serum cholesterol and triglyceride values (Figures 24 and 25). LDL-cholesterol or HDL-cholesterol were not measured for practical reasons.

Unadjusted, there was no difference between cholesterol values for patients in the upper or lower tertile of CRP distribution (6.57 mmol/l [95% CI 6.52-6.62] and 6.55 mmol/l [95% CI 6.50 – 6.60]). Partially adjusted, there was no correlation between CRP and cholesterol, but with full adjustment in a linear regression model cholesterol was weakly, negatively, associated with CRP values (B -0.08; P<0.001), but the mean difference between extreme tertiles of CRP distribution is not significant (6.50 mmol/l vs. 6.66 mmol/l). The lack of any strong association between CRP and cholesterol suggests that they could complement each other in cardiovascular risk prediction, if they are representing different aspects of the disease process.

Hypertriglyceridaemia is also a risk factor for future cardiovascular disease. With respect to serum triglycerides the unadjusted geometric means for upper and lower tertiles of CRP were significantly different: 1.19 mmol/l (95% CI 1.17 – 1.22) vs. 0.97 mmol/l (95% CI 0.95 – 0.99). Fully adjusted values were not however significantly different (1.11 mmol/l [95% CI 1.04 – 1.19] vs. 1.03 mmol/l [95% CI 0.93 – 1.13]), although when correlated as continuous

values, and adjusted for other variables, log triglycerides has a regression coefficient of 0.06 ($P < 0.001$). This association with CRP is unexplained, but must reflect subtle metabolic and inflammatory changes associated with an excess level of triglycerides. Notably the partially adjusted correlations (age, sex, diabetes) between triglycerides and BMI is 0.33 ($P < 0.001$).

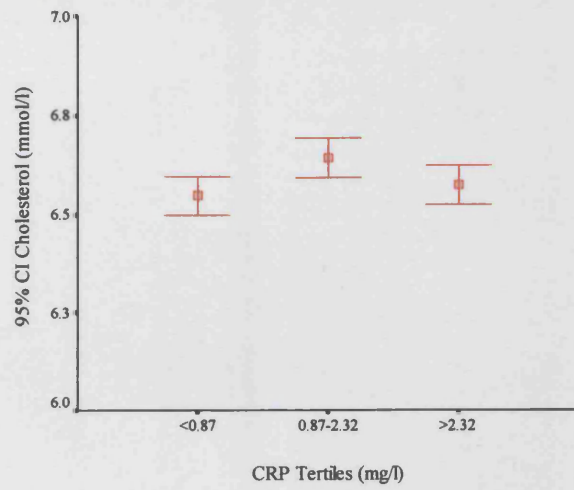
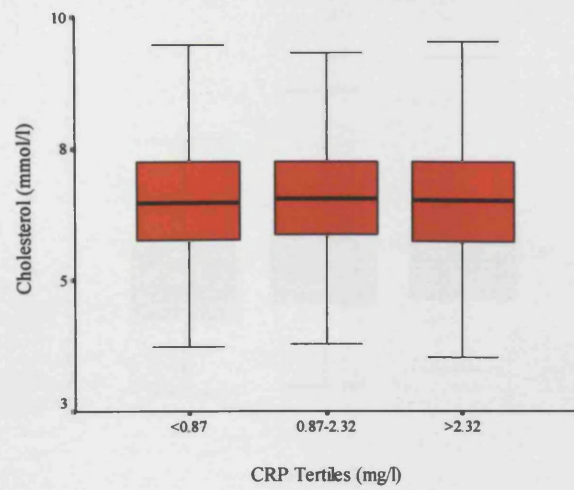


Figure 24 Cholesterol and CRP values

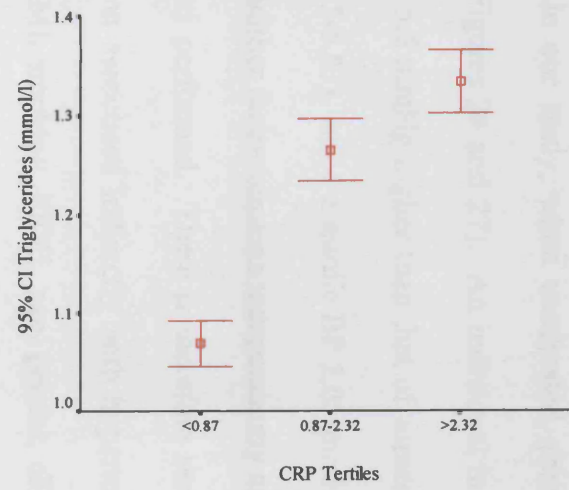
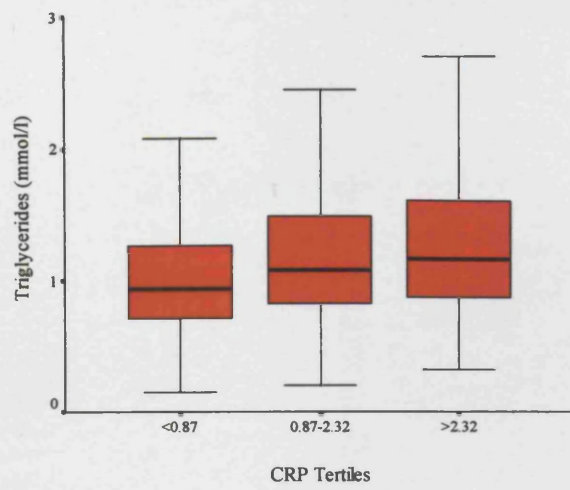


Figure 25 Triglycerides and CRP values

10.8.5.8 Blood pressure

Finally blood pressure is another important cardiovascular risk factor, for which other studies have reported associations with baseline CRP values [167, 412, 413]. Certainly in our study, when unadjusted, there is an association with baseline values (Figures 26 and 27). An individual in the upper tertile of CRP has a systolic BP 5.5 mmHg higher than that of someone in the lower tertile of CRP (95% CI 4.15-6.83), and a diastolic BP 2.05 mmHg higher (95% CI 1.34 – 2.75). However neither factor remains independently associated with CRP in the regression analyses performed. There is therefore serological evidence of low grade inflammation associated indirectly with hypertension but adjustment for factors such as BMI, smoking, FEV1, age, gender, eliminate the association in our population.

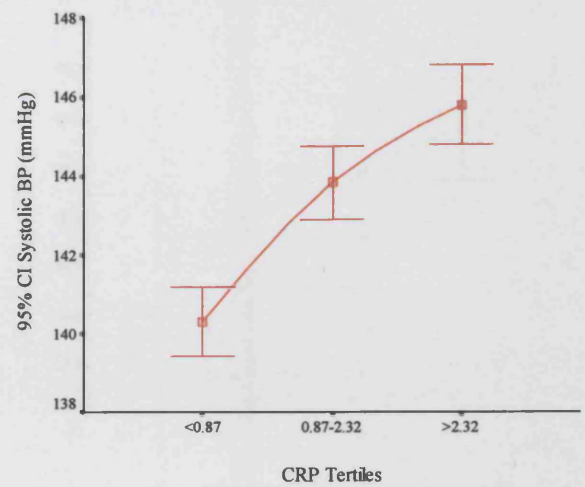
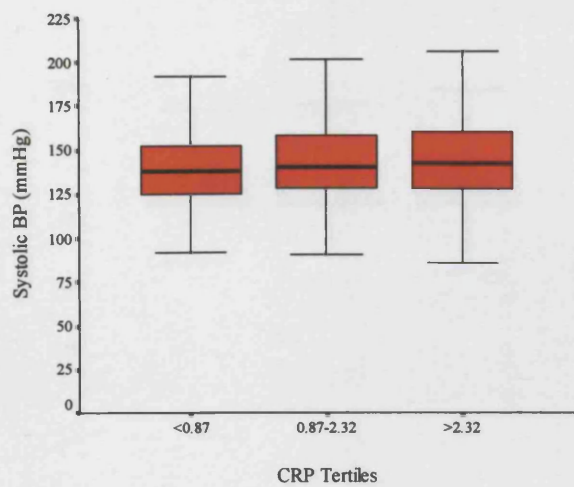


Figure 26 Systolic blood pressure and CRP values

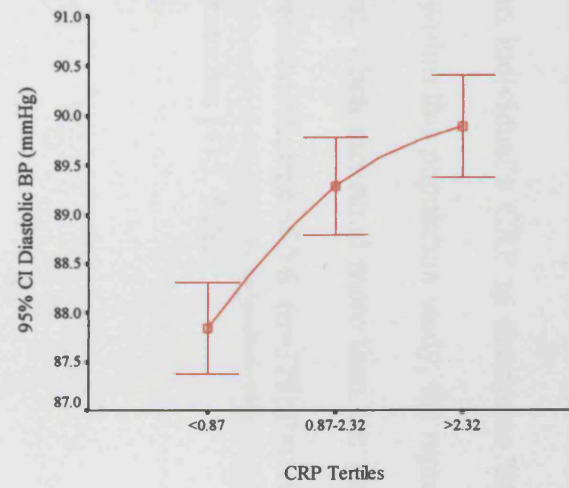
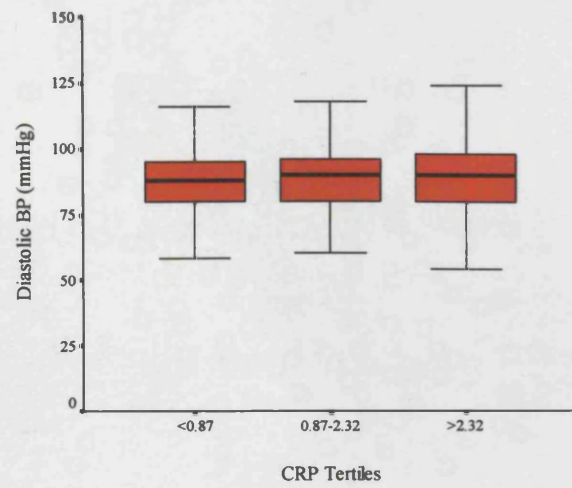


Figure 27 Diastolic blood pressure and CRP values

10.9 Reproducibility

One obvious criticism placed at using CRP in risk prediction has been the wide variation in values possible because of its manifest sensitivity to all causes of inflammation. This impacts indirectly on the question of how many times one should measure an individual's CRP to determine their "true" baseline value [414]. However, within this population study, the reproducibility of CRP values in the same patient, when measured more than ten years apart was very good, with a Spearman rank coefficient of 0.6 ($n=379$; $P<0.001$); Figure 28. This is in keeping with other studies [415, 416].

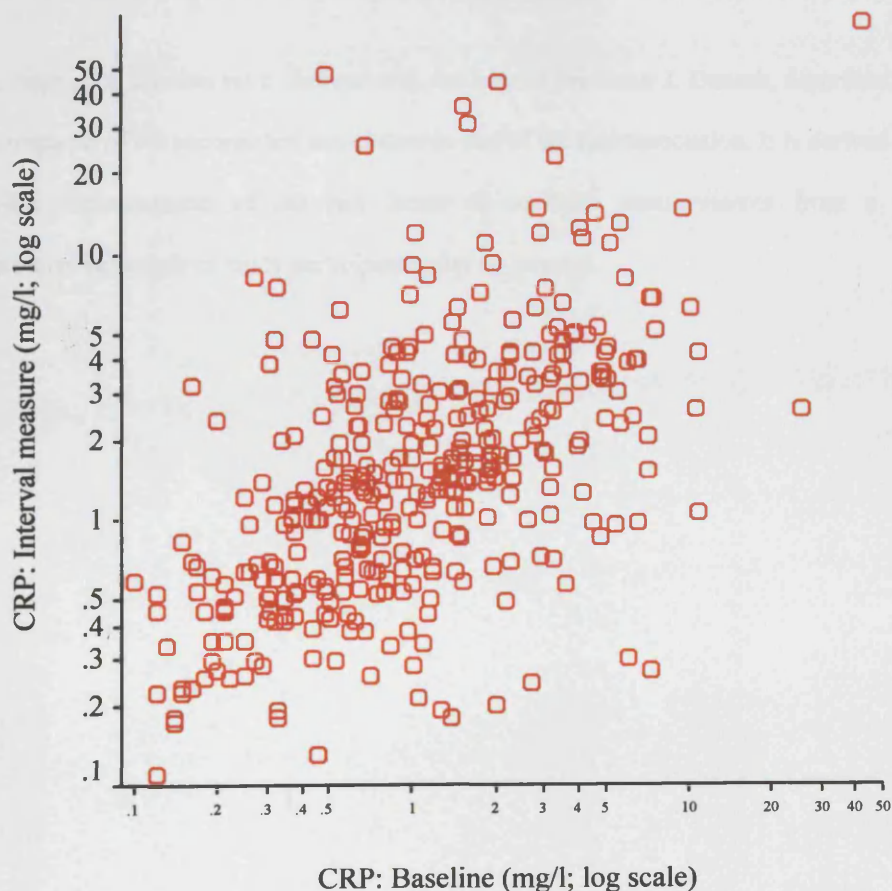


Figure 28 Inter-individual reproducibility for CRP
- mean interval between samples 11.43 years [SD 2.04]

The reasonably high decade-to-decade consistency of CRP values recorded compares well with other traditional cardiovascular risk factors. The regression dilution ratio, a self-correlation coefficient, is shown in Table 34 for the important cardiovascular risk factors, based on the reproducibility data from the sequential samples.

Table 34 Self-correlation coefficients for cardiovascular risk factors

Risk factors	Self-correlation coefficient (95% CI)
C-reactive protein	0.59 (0.52 – 0.66)
ESR	0.67 (0.61 – 0.73)
Blood pressure	0.66 (0.60 – 0.72)
Cholesterol	0.60 (0.64 – 0.66)

- the regression dilution ratio, derived with the help of Professor J. Danesh, describes the ratio of the steepness of the uncorrected association to that of the real association. It is derived by relating baseline measurements of the risk factor to replicate measurements from a reasonably representative sample of study participants after an interval.

10.10 Case-control analysis: Inflammatory Markers and Incident Coronary Heart Disease

The mean age at the time of the coronary heart disease event was 70.2 ± 9.7 years. There were significant differences between patients and controls with respect to established coronary risk factors, such as smoking status, body-mass index, blood pressure, and serum lipid concentrations (Table 35). Information on occupation was available for only 1742 cases and 2888 controls respectively whilst information on education was available for only 1292 cases and 2157 controls respectively. There were no significant differences in occupation, education beyond high school, or home ownership/nature of house. No information on alcohol use was available, nor on physical activity.

As a first observation it remains quite striking that the value of CRP in individuals who subsequently go on to have coronary events is, unadjusted, 37% higher, than matched controls. Our study, regardless of addressing the independent predictive power of CRP, constitutes further evidence of the apparent inflammation that accompanies future atherothrombotic events, and underscores the need to further understand the processes at play.

Table 35 Baseline comparison of risk factors: cases vs. controls

Comparison of Baseline Values of Risk Factors and Other Characteristics in Cases and Controls			
	Cases (n=2459)	Controls (n=3969)	P value
Demographic			
Age (years)	55.8 (9.3)	55.7 (9.1)	Matched
Male, n (%)	1774 (72)	2743 (69)	Matched
Questionnaire			
Current smokers (incl. cigarettes, cigars, pipe), n (%)	1417 (58)	1941 (49)	<0.001
Current cigarette smokers, n (%)	962 (39)	1266 (32)	<0.001
History of diabetes, n (%)	83 (3)	63 (2)	<0.001
Physical measurements			
Height (m)	1.71 (0.087)	1.72 (0.087)	0.07
Weight (kg)	76 (14)	75 (14)	<0.001
Body mass index (kg/m ²)	26 (3.9)	25 (3.7)	<0.001
Systolic blood pressure (mm Hg)	146 (22)	141 (20)	<0.001
Diastolic blood pressure (mm Hg)	89 (11)	87 (11)	<0.001
Forced expiratory volume (ml/1 st sec)	2800 (85)	2900 (86)	0.002
Blood sample			
Total serum cholesterol (mmol/l)	6.82 (1.18)	6.40 (1.14)	<0.001
Serum triglycerides (mmol/l)‡	1.19 (0.79)	1.03 (0.62)	<0.001
Fasting glucose (mmol/l)	4.6 (1.1)	4.5 (0.8)	<0.001
Serum creatinine (µmol/l)	77 (14)	75 (13)	<0.001
Haemoglobin (mmol/l)	9.2 (0.80)	9.1 (0.81)	<0.001
Haematocrit (%)	45 (3.6)	44 (3.5)	<0.001
Inflammatory markers			
C-reactive protein (mg/l)‡	1.75 (5.3)	1.28 (5.2)	<0.001
ESR (mm/1 st h)‡	7.4 (10.6)	6.3 (9.7)	<0.001
Values are mean (SD) unless indicated otherwise. ‡ Factor log transformed for analysis and presented as geometric mean (SD).			

The odds ratio for coronary heart disease was 1.92 (95% CI 1.68 - 2.18; $\chi^2=105$, with 1 df) among patients with values in the top third (cutoff value, 2.0 mg/l), as compared with the bottom third (cutoff value, 0.78 mg/l), of base-line CRP concentrations in the control group. The odds ratio fell to 1.45 (95% CI 1.25 - 1.68; $\chi^2=28$, with 1 df) after adjustment for smoking status, other established coronary risk factors (systolic blood pressure, total cholesterol, triglycerides,

body mass index, FEV1, history of diabetes), and indicators of socioeconomic status (Table 36 and Figure 29).

Comparisons between the top and bottom thirds of patients and controls with respect to ESR gave adjusted odds ratios for coronary heart disease (cutoff value of 10 mm/hr for the top third and 4 mm/hr for the bottom third), of 1.30 (95% CI 1.13 -1.51; $\chi^2=13$, with 1 df); (Table 36 and Figure 29).

Receiver operator curves (ROC) curves display the relationship between sensitivity (true positive rate) and 1-specificity (false-positive rate) across all possible threshold values that define the positivity of a disease or condition. Summary measures of ROC curves, such as the area under the curve (AUC), can summarise into a single statistic the inherent capacity of a test for discriminating a diseased from a non-diseased subject across all possible levels of positivity. The area under the receiver operating characteristic curve is the most commonly used measure of the ability of a biomarker to distinguish between two populations. Logistic-regression analysis was used to calculate the areas under the receiver-operating-characteristic curve after adjustment for age, sex, and period, with data on major established risk factors and inflammatory markers added to the model in the order of the strength of each variable's association with coronary heart disease.

In our case we wished to specifically address the question of whether or not our study suggests CRP adds information, above and beyond that of standard cardiac

risk factors. In Figure 29 one can see that the calculated areas under receiver-operating-characteristic curves indicate that information on the CRP concentration provided comparatively little additional predictive value over that provided by assessment of other major established risk factors.

These findings were not materially changed in analyses restricted to the 2083 patients without evidence of coronary heart disease at base line, to the 2206 patients with CRP values who had a confirmed myocardial infarction or died of coronary heart disease, or to the participants without evidence of acute-phase reactions at the base-line examination (i.e. this analysis excluded 132 patients and 152 controls with a CRP concentration of more than 10 mg/l or an erythrocyte sedimentation rate of more than 30 mm/hr). The findings were also unaffected by changes in the cutoff values (e.g. analyses of quarters or fifths, or according to increases of 1 SD) (Table 36).

Table 36 Relative odds of coronary heart disease

Relative Odds of Coronary Heart Disease in Individuals Who Had Inflammatory Markers in Top Third of Distribution of Controls Relative to Those Who Had Values in Bottom Third of this Distribution											
Factor (tertile cutoffs)	No. of Cases			No. of Controls			Odds Ratio (95% CI) Adjusted For:				
	Top	Middle	Bottom	Top	Middle	Bottom	Age, sex and period	Age, sex, period and smoking	Age, sex, period, smoking and risk factors*	Age, sex, smoking, risk factors and socioeconomic status	Age, sex, smoking, risk factors, socioeconomic status and other inflammatory markers
Inflammatory markers All 2459 cases and 3969 controls											
C-reactive protein (2 and 0.78 mg/l)	1090	742	574	1294	1300	1297	1.92 (1.68-2.18)	1.76 (1.54-2.01)	1.45§ (1.25-1.69)	1.45 (1.25-1.68)	1.36 (1.16-1.58)
ESR (10 and 4 mm/hr)	922	733	785	1220	1201	1521	1.64 (1.44-1.87)	1.55 (1.36-1.78)	1.31 (1.13-1.51)	1.30 (1.13-1.51)	1.18 (1.01-1.38)
2083 cases without evidence of coronary heart disease at entry and 3969 controls†											
C-reactive protein (2 and 0.78 mg/l)	887	635	520	1294	1300	1297	1.81 (1.58-2.07)	1.65 (1.43-1.89)	1.37 (1.17-1.60)	1.37 (1.17-1.59)	1.30 (1.10-1.52)
ESR (10 and 4 mm/hr)	766	633	671	1220	1201	1521	1.53 (1.34-1.77)	1.45 (1.26-1.68)	1.22 (1.05-1.43)	1.22 (1.04-1.42)	1.10 (0.95-1.28)
Some established CHD risk factors All 2459 cases and 3969 controls											
Total cholesterol (6.80 and 5.85 mmol/l)	1150	826	482	1309	1320	1334	2.62 (2.29-3.00)	2.68 (2.3-3.08)	2.33 (2.01-2.70)	2.35 (2.03-2.74)	2.38 (2.05-2.77)
Smoking‡ (current vs. never)	1417	544	498	1941	851	1177	1.74 (1.53-1.98)	1.74 (1.53-1.98)	1.87 (1.63-2.22)	1.87 (1.62-2.16)	1.75 (1.51-2.03)
Systolic blood pressure (147 and 131 mm Hg)	1041	742	670	1308	1240	1402	1.72 (1.51-1.96)	1.85 (1.62-2.10)	1.51 (1.32-1.74)	1.50 (1.30-1.73)	1.50 (1.30-1.74)
<small>Markers of socio-economic status = non-manual occupation, education beyond high school, home ownership and living in an apartment block. * Systolic blood pressure, total cholesterol, triglycerides, body mass index, smoking (former or current, including number cigarettes per day), FEV1, history of diabetes. † These analyses exclude individuals with ECG evidence of CHD or a history of angina. Individuals with a history of MI were excluded from the baseline survey. ‡ Smoking is not represented as thirds of a continuous distribution: case and control frequencies are tabulated for current-, ex- and non-smokers, and odds ratios are calculated comparing current and never smokers. § The adjusted odds ratio of CRP and CHD using alternative comparisons were: 1.55 (1.31-1.84) top quarter vs. bottom quarter; 1.65 (1.36-2.00) top fifth vs. bottom fifth; 1.20 (1.12-1.27) per standard deviation (log scale).</small>											

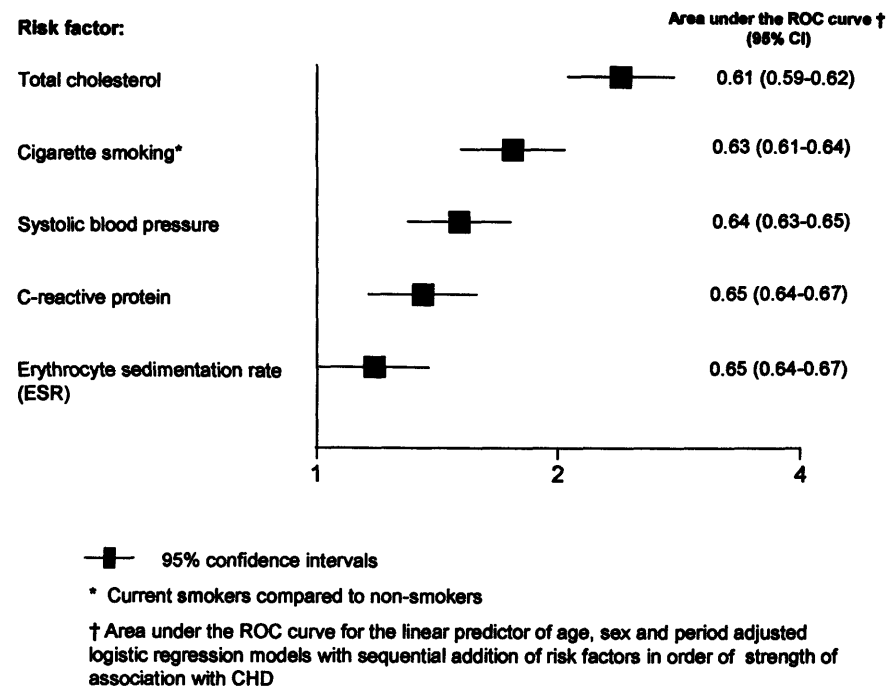


Figure 29 Comparison of the strength of associations of several factors with coronary heart disease

- 2459 incident cases and 3969 controls in the Reykjavik study (comparisons involve top third vs. bottom third in the population)

10.11 Discussion and Meta-analysis

CRP is an extremely sensitive marker of classical inflammatory processes such as infection or tissue necrosis. This study demonstrates, with great statistical power, that serological evidence of low grade inflammation is an important accompanying feature of many markers of health and disease, not thought of as classically inflammatory processes i.e. active smoking, increased body mass index, reduced forced expiratory volume, increasing age, increasing blood pressure, increasing triglycerides and impaired glucose tolerance are associated with a highly sensitive marker of systemic inflammation. These associations on further analysis can be both direct and indirect (as assumed by loss of association with adjustment), and reflect the presumed very complicated interaction between host genetic factors and variable production of inflammatory mediators.

Whilst association and causation manifestly differ, this study clearly confirms as a striking principle, the important association of biochemical evidence of inflammation with future coronary artery disease. Healthy individuals, who subsequently go on to have coronary heart disease events, are more likely to have raised systemic biochemical markers of inflammation, many years prior to clinical manifestations of disease.

In some respects it is therefore a secondary question, whether or not CRP is an independent marker of cardiac disease, because clinically that will depend on how one chooses to use the information gained. However the question is

important because resources are limited and should be efficiently targeted to maximal effect. If we can already identify those at risk then the extra costs of a new test are not justifiable, particularly if that cost can be channelled more effectively elsewhere.

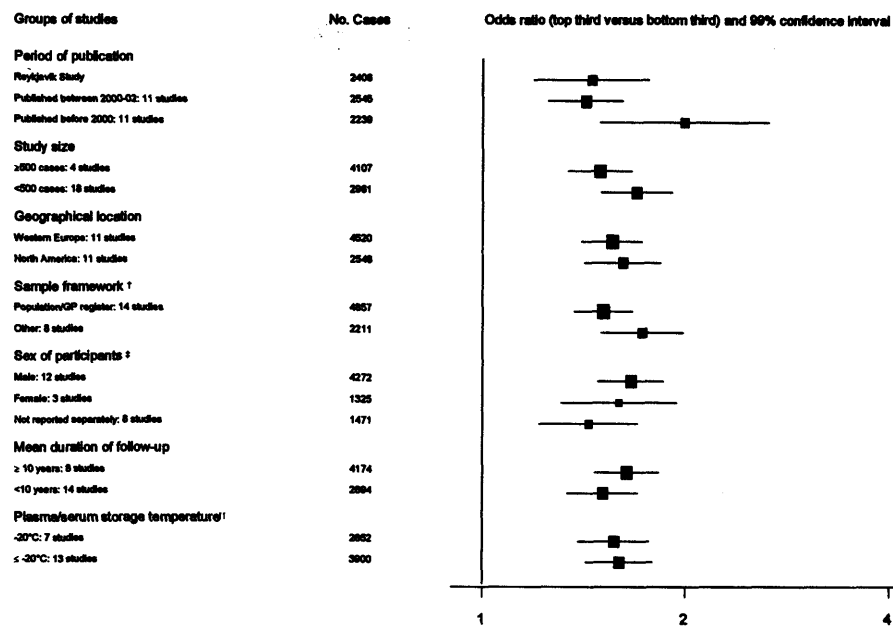
In order to help put the new data from the present study in context, John Danesh and Jerry Wheeler went on to update the meta-analysis of previous relevant studies. We reported this in *The New England Journal of Medicine* [168] (Figure 30). Twenty-two prospective studies of CRP (including the present study) were included, involving a total of 7068 patients, with a weighted mean age at entry of 57 years and a weighted mean follow-up of 12 years. All studies used high-sensitivity assays, and all but two reported adjustment for at least smoking status and some other established risk factors for coronary heart disease. There was evidence of heterogeneity between these studies ($\chi^2=46$, with 21 df; $P=0.001$), but with the exception of the date of publication ($\chi^2=15$, with 2 df; $P<0.001$), characteristics such as sample size ($\chi^2=4.0$, with 1 df; $P=0.04$), location ($\chi^2=0.3$, with 1 df; $P=0.58$), sampling method ($\chi^2=5.2$, with 1 df; $P=0.02$), sex of participants ($\chi^2=3.4$, with 2 df; $P=0.18$), mean duration of follow-up ($\chi^2=1.6$, with 1 df; $P=0.20$), and sample storage temperature ($\chi^2=0.1$, with 1 df; $P=0.77$) did not account for much of the overall heterogeneity.

The previous meta-analysis, found the odds ratio for non-fatal myocardial infarction or death from coronary heart disease to be about 2.0 in a comparison of extreme thirds of CRP values [179], and in a few, relatively small studies, the odds ratio was even greater. By contrast, the best current evidence suggests that

the odds ratio is about 1.5 (95% CI 1.4 - 1.6). A number of studies subsequent to ours are now reporting odds ratios of the same magnitude [203, 204]. The tendency toward more extreme findings in studies published before 2000 is consistent with the preferential publication of positive results in earlier studies. For example restriction of analyses to the four studies involving more than 500 patients, comprising 4107 cases of coronary heart disease, which should limit any such bias, yielded a combined odds ratio of 1.49 (95% CI 1.37 - 1.62; $\chi^2=10.6$, with 3 df; $P=0.01$). This value is somewhat smaller than the overall odds ratio of 1.58 (95% CI 1.48 - 1.68) derived from combining all 22 studies.

Moreover, contrary to some earlier suggestions, the current data indicate that established coronary risk factors are generally stronger predictors than are CRP values. For example, in our study, the adjusted odds ratios among people with raised total cholesterol concentrations and among smokers were approximately 2.4 (95% CI 2.0 - 2.7) and 1.9 (95 % CI 1.6 - 2.2), respectively.

Further clarification of the predictive ability of CRP in CHD will require the pooling of studies based on individual participant data from each of the available prospective studies, enabling more complete adjustment for other risk factors, more precise quantification of the associations in particular subgroups (such as age- and sex-specific associations as well as assessments of combinations of inflammatory markers), more reliable characterisation of the shape of any dose-response relationship, and more detailed investigation of potential sources of heterogeneity.



- * The figure is based on 22 studies, apart from the data under "period of publication", which lists 23 studies to show that one study published in two separate reports, with the first published before 2000 and the update published in 2002
- †† "Other" sample frameworks include participants selected under various criteria (eg, individuals without a history of coronary disease recruited into randomised trials, individuals identified by their occupation, etc); GP=general practice
- ‡ The Reykjavik Study provided estimates for males and females separately
- † Such information was unavailable for 2 studies

Figure 30 Twenty-two prospective studies of CRP and coronary heart disease

10.11.1 Limitations

The validity of this study's measurements has been demonstrated by the reasonably high decade-to-decade consistency of CRP values recorded in paired samples in several hundred study participants. This consistency was at least as good as that recorded in previous studies based on much briefer intervals (ranging from one to five years). Further validation of measurement is suggested by the expected baseline associations observed between CRP and other inflammatory markers with one another and with established risk factors. Mean values and distributions of several established coronary risk factors (and their strength of association with CHD) were also generally similar in the present study to those seen in other Western European populations. Whilst the Reykjavik population's relative homogeneity will minimise certain residual biases (such as differences in socioeconomic status), the findings do have wider relevance. Measurements of only total serum cholesterol concentrations (rather than of LDL and HDL cholesterol subfractions, which have opposing effects on CHD risk) were available in the present study, thereby under-estimating the predictive ability of lipid levels (and potentially over-estimating the adjusted predictive ability of CRP). Information was not recorded in this study on the use of aspirin, "statins", or hormone replacement treatments, but they were relatively uncommonly used in the general adult population of Reykjavik between 1967 and 1991. The analysis was not altered by comparisons among extreme quarters or extreme fifths rather than among extreme thirds. The use of any particular cutoff point (such as 3 mg/l), consistently applied among studies, would also

yield similar contrasts with previous estimates, provided the shape of the relationship between CRP values and coronary risk were approximately log-linear.

10.12 Summary

Our study confirms the presence of raised CRP concentrations years before evidence of coronary artery disease. This does not equate with evidence that inflammatory markers should be measured in risk stratification. The data from our large prospective study (involving 2406 patients who had non-fatal myocardial infarction or who died from coronary heart disease) and from an updated meta-analysis of 21 previous reports (including a total of 4662 such patients) indicate that the value of CRP measurement in the prediction of the risk of coronary disease has previously been overestimated. The best current evidence, suggests that the odds ratio is about 1.5 (95% CI 1.4 - 1.6). Measurement of CRP added only marginally to the predictive value of established risk factors: the area under the receiver-operating-characteristic curve increased from 0.64 (95% CI 0.63 - 0.65) to only 0.65 (95% CI 0.64 - 0.67) when it was included. The current data confirm that established coronary risk factors are generally stronger predictors than are CRP values. The adjusted odds ratios among people with raised total cholesterol concentrations and among smokers were approximately 2.4 (95% CI 2.0 - 2.7) and 1.9 (95% CI 1.6 - 2.2), respectively in our cohort.

11 C-reactive protein and animal models of disease

11.2 Introduction

An array of studies largely confined to passive administration of CRP or transgenic production of rabbit or human CRP in mice (Table 37), points to a role for CRP in host defence against microbial infection and in the physiological and pathophysiological handling of autologous constituents; under various circumstances CRP may also function as a pro-inflammatory mediator [34]. Indeed the capacity of human CRP to bind to phosphocholine and related residues that are widely present in cell membranes and bacteria and then to precipitate soluble ligands, aggregate particulate ligands and activate the classical complement pathway, all resemble closely the classical properties of antibodies. The subtleties of animal models of disease however, along with species differences in CRP glycosylation, capacity to activate autologous complement, and regulation of basal and acute phase synthesis (the acute phase production of murine CRP never exceeds trace amounts and no mouse CRP knockout has been made), suggest extreme caution in extrapolating from animal models to man. Aware of the limitations of heterologous systems, we studied further the *in vivo* role of human CRP following endotoxin administration and in the context of experimental arthritis, making particular use of a human CRP transgenic mouse strain, in which CRP production is under the control of its human promoter.

Table 37 Array of animal studies by 2004 published in the investigation of the biological properties of CRP

Authors	Authors observations/claims	Transgenic/Passive CRP
Kushner I & Kaplan MH 1961 [417]	Immunohistochemical localisation of Cx-reactive protein in rabbits: association with necrosis	Endogenous rabbit
Kushner I <i>et al.</i> 1963 [196]	Localisation of Cx-reactive protein in heart in induced myocardial infection in rabbits	Endogenous rabbit
Hurliman J <i>et al.</i> 1966 [10]	The liver as the site of C-reactive protein formation	Endogenous rabbit
Parish WE 1977 [418]	Cutaneous reactions in rabbits and guinea pigs following intracutaneous injection of aggregated CRP	Passive hCRP
du Clos TW <i>et al.</i> 1981 [419]	Localisation of rabbit CRP to inflammatory lesions in experimental allergic encephalomyelitis	Endogenous rabbit
Mold C <i>et al.</i> 1981 [10]	Protective against <i>S. pneumoniae</i> infection in mice	Passive hCRP
Nakayama S <i>et al.</i> 1982 [420]	Increase in splenic sequestration of erythrocytes coated with PC	Passive hCRP
Nakayama S <i>et al.</i> 1984 [421]	Inhibition of antibody responses to phosphocholine	Passive hCRP
Rowe IF <i>et al.</i> 1984 [76]	Reduced clearance of rabbit CRP during acute phase response	Passive rabbit CRP
Baltz ML <i>et al.</i> 1985 [422]	Half life of hCRP ~4hrs in rodents and not increased by CPS, though increased splenic localisation	Passive hCRP
Rowe IF <i>et al.</i> 1985 [423]	Rabbit CRP not detected in catheter-induced aortic endothelial injuries in the rabbit	Endogenous rabbit CRP
Ciliberto <i>et al.</i> 1987 [74]	Inducible and tissue-specific expression of human CRP in transgenic mice	hCRPTg
Horowitz J <i>et al.</i> 1987 [424]	Blood clearance of <i>S. pneumoniae</i>	Passive hCRP
Renia L <i>et al.</i> 1993 [425]	No protection against infection with <i>Plasmodium yoelii</i> sporozoites	Rabbit CRP Tg
du Clos TW <i>et al.</i> 1994 [426]	Decreased autoantibody levels and enhanced survival of (NZB x NZW) F1 mice	Passive hCRP
Hutchinson W <i>et al.</i> 1994 [26]	Hepatocytes are the single major site of pentraxin clearance and catabolism <i>in vivo</i>	Passive hCRP
Lin CS <i>et al.</i> 1995 [73]	Expression of rabbit CRP in transgenic mice	Rabbit CRP Tg
Szalai AJ <i>et al.</i> 1995 [427]	Protective against fatal <i>S. pneumoniae</i> infection	hCRPTg
Ahmed N <i>et al.</i> 1996 [428]	Diminished chemotactic factor-induced alveolitis	Rabbit CRP Tg
Weinhold B <i>et al.</i> 1997 [350]	Interleukin-6-dependent and -independent regulation of the human CRP gene	hCRPTg
Xia D and Samols D 1997 [429]	Resistance to endotoxaemia	Rabbit CRP Tg
Szalai AJ <i>et al.</i> 1998 [351]	Testosterone and IL-6 requirements for human CRP gene expression in transgenic mice	hCRPTg
Grisselli M <i>et al.</i> 1999 [201]	Human CRP is an important mediator of tissue damage in acute myocardial infarction	Passive hCRP
Noursadeghi M <i>et al.</i> 2000 [89]	Significantly reduced morbidity and mortality in murine endotoxaemia	Passive hCRP
Szalai AJ <i>et al.</i> 2000 [430]	Protective against fatal <i>S. enterica serovar typhimurium</i>	hCRPTg
Mold C <i>et al.</i> 2002 [431]	Protection from lipopolysaccharide (through interactions with Fcγ receptors)	Passive hCRP
Mold C <i>et al.</i> 2002 [432]	Protection from <i>S. pneumoniae</i> infection requires complement (but not Fcγ receptors)	Passive hCRP
Szalai AJ <i>et al.</i> 2002 [433]	Experimental allergic encephalomyelitis is inhibited in transgenic mice	hCRPTg
Danenbergh HD <i>et al.</i> 2003 [434]	Increased thrombosis after arterial injury in transgenic mice	hCRPTg
Szalai AJ <i>et al.</i> 2003 [435]	Delayed lupus onset in (NZB x NZW)F1 transgenic mice	hCRPTg
Gill R <i>et al.</i> 2004 [332]	Increases cerebral infarct size after middle cerebral artery occlusion	Passive hCRP
Paul A <i>et al.</i> 2004 [436]	Accelerates the progression of atherosclerosis in apolipoprotein E-deficient transgenic mice	hCRPTg

11.3 Endotoxin

The outer leaflet of the outer membrane of Gram-negative bacteria is composed primarily of lipopolysaccharides (LPS). These molecules all contain an endotoxic lipid A with which the LPS is anchored in the bacterial membrane and a polysaccharide unit presented at the cell surface that is specific to the bacterial species. The polysaccharide unit consists of the core oligosaccharide and the O-specific chain (Figure 31) [437]. Lipopolysaccharides are essential for the survival of Gram-negative bacteria, providing a permeation barrier for harmful substances. However LPS plays an important role in a number of recognition processes and the LPS of Gram-negative bacteria potently induce acute pro-inflammatory responses and endotoxic shock [438]. Endotoxaemia has been associated with a variety of human diseases, including sepsis [439], inflammatory bowel disease [440], liver failure [441], and pancreatitis [442].

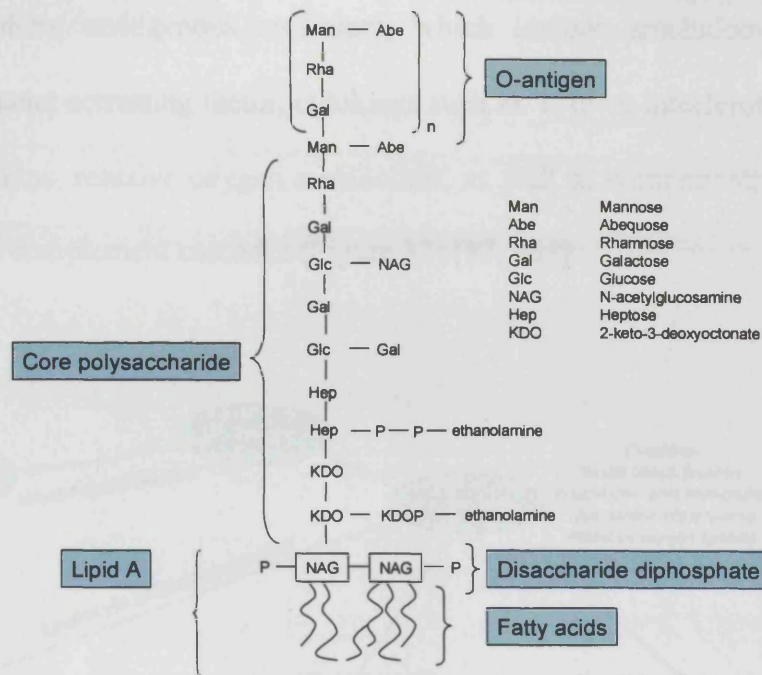


Figure 31 Schematic structure of lipopolysaccharide

11.3.1 Systemic endotoxaemia

Systemic endotoxin administration to animals generates a lethal model of endotoxic shock [443]. LPS binds to the acute phase protein, LPS-binding protein (LBP) and CD14, a glycosylphosphatidylinositol-anchored protein found on the surface of monocytes/macrophages, binds the complex of LPS and LBP. Recognition of LPS is initialised by the interplay between LBP, the membrane-bound or soluble forms of CD14 and the toll like receptor-4 (TLR4) MD-2 complex (MD-2 being an 160-amino acid protein associated with TLR4 on the cell surface, that enables TLR4 to respond to LPS). This leads to the rapid activation of an intracellular signalling pathway, highly homologous to the signalling pathway of IL-1, which results in the release of pro-inflammatory mediators. The many adverse effects of endotoxic shock are dependent on the

generation of these endogenous mediators, which include arachidonic acid metabolites, platelet activating factor, cytokines such as TNF- α , interferon- γ and various interleukins, reactive oxygen metabolites, as well as components of the coagulation and complement cascades (Figure 32) [97, 438].

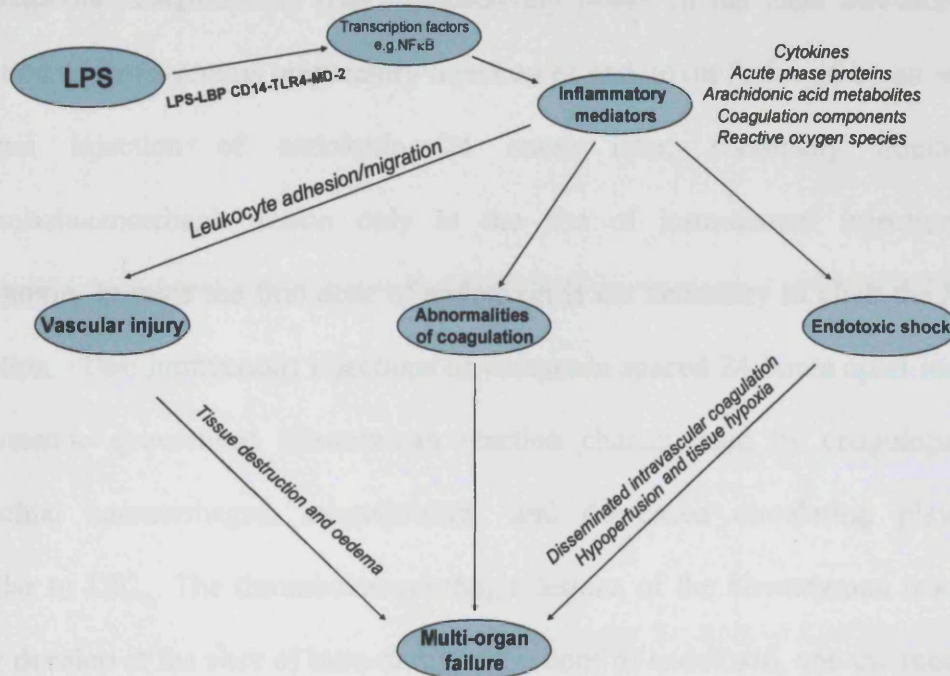


Figure 32 Physiological responses to endotoxaemia

The changes described following endotoxin challenge, represent one very specific example of an acute phase response. Although not fully understood, the acute phase response is generally assumed to be protective, and CRP has been reported to contribute to resistance against the toxicity of LPS; one study in transgenic mice expressing rabbit CRP [429], and subsequently also for wild type mice passively administered human CRP [89, 431].

11.3.2 The Shwartzman reaction

Local responses to endotoxin can also be generated. The Shwartzman reaction was first described more than 50 years ago and the two types, local and generalised, are models of thrombohaemorrhagic skin necrosis and disseminated intravascular coagulopathy (DIC) respectively [444]. In the local Shwartzman reaction an intra-venous preparatory injection of endotoxin followed by an intra-dermal injection of endotoxin 24 hours later, classically elicits a thrombohaemorrhagic lesion only at the site of intra-dermal injection of endotoxin. In mice the first dose of endotoxin is not necessary to elicit the local reaction. Two intravenous injections of endotoxin spaced 24 hours apart induce a systemic generalised Shwartzman reaction characterised by coagulopathy, petechial haemorrhages, microthrombi, and decreased circulating platelets similar to DIC. The thrombohaemorrhagic lesions of the Shwartzman reaction only develop at the sites of intra-dermal injections of endotoxin, and the reaction has been reproduced by administration of TNF- α and interferon- γ [445-447].

The acute phase response and the pentraxins in particular, were studied some time ago in the context of the local Shwartzman reaction [353]. Following generation of the local Shwartzman reaction in mice, there was a marked acute phase response (murine SAP and C3). Neither steroid therapy nor *in vivo* complement depletion had an effect on either the cutaneous lesion or the accompanying acute phase response. Interestingly though, administration of colchicine at the same time as LPS suppressed both the acute phase response and the Shwartzman reaction. Inhibition of the cutaneous reaction by colchicine was

abrogated by injecting mice with casein, an unrelated acute phase stimulus, the day before LPS challenge. We were particularly interested to readdress this model, to see if human CRP might increase the size of the lesion, potentially by binding to damaged cells and enhancing complement activation.

11.4 Collagen arthritis

Rheumatoid arthritis (RA) is a chronic progressive auto-inflammatory disease of the joints associated with significant morbidity, deformity, and impaired quality of life [448, 449]. CRP concentrations correlate with disease severity and radiographic progression in RA, as well as predicting disease onset, prior to clinical signs being evident [450-453]. Complement has been repeatedly implicated in the pathogenesis of RA based on studies showing reduced levels of native complement components and increased levels of complement metabolites in plasma, synovial fluid, and synovial tissue of RA patients; this is thought to be secondary to immune complex complement activation [454-456]. Ligand bound CRP can activate the complement system *in vivo*, and complexes between CRP and the activated complement components C3d (C3d-CRP) and C4d (C4d-CRP), which in part reflect CRP-mediated complement activation, as well as the overall levels of activated C3 and C4, have been correlated with disease [457, 458]. Finally CRP has been detected immunohistochemically in rheumatoid synovium [459]. All these lines of evidence, at the least, suggest the possibility of a direct pathogenic role for CRP in RA.

However it is as conceivable that CRP is anti-inflammatory in this auto-immune disease. Less appreciated and not as well understood as CRP's ability to bind antigen and aid in the elimination of microbes, is its known ability to bind autoantigens and presumed capacity to promote clearance of apoptotic cells [460]. These latter properties of CRP have been suspected to contribute to homeostasis and to autoimmune disease. In addition to binding to subcellular particles from lysed or permeabilised cells (small nuclear ribonucleoprotein particles in particular) [84, 461, 462], CRP binds apoptotic cells in a Ca^{2+} -dependent manner [59]. In so doing it appears to enhance opsonisation and phagocytosis of apoptotic cells by macrophages. Controversially in lupus, a condition characterised by an inappropriate CRP response for the amount of tissue damage present [28, 463], there are preliminary observations for a protective role for CRP [426]: recent reports for example in human CRP transgenic mice, suggested that CRP protected against SLE by increasing blood and mesangial clearance of immune complexes and by preventing their accumulation in the renal cortex [435], and one study claimed treatment of NZB/NZW mice with human CRP prior to disease onset delayed the onset of high-grade proteinuria and prolonged survival [464].

Attempting to further understand the role of CRP in arthritis is therefore of interest. Collagen-induced arthritis (CIA) is an animal model of autoimmunity that has been studied extensively because of its similarities to RA [465, 466]. Severe polyarticular arthritis, with synovitis, pannus formation, cartilage and bone erosion are hallmarks of CIA and histologically, murine CIA is characterised by an intense synovitis that corresponds precisely with the clinical

onset of arthritis. CIA is induced in genetically susceptible strains of mice by immunisation with type II collagen (CII), the major constituent protein of articular cartilage, and both T cell and B cell immunity to CII are required for disease manifestation. While the direct role of T cells in the pathogenesis of CIA is unclear, the B cell response in terms of anti-CII immunoglobulin is critical to the development of the disease. This response, predominated by the IgG2 isotype, requires the activation of the complement cascade. Transfer studies have shown that autoantibodies are directly pathogenic and can provoke at least some of the manifestations of joint inflammation. Antibodies, particularly as constituents of antibody-antigen immune complexes, play a central role in triggering inflammation; notably the DBA/1LacJ mouse strain, which represents one of the most sensitive models for the induction of autoreactive joint disease, became almost completely resistant to collagen arthritis when rendered congenic for C5 deficiency, despite abundant Ig and C3 deposition within the joints [467].

This chapter describes our experiments looking at the role of CRP *in vivo*, following local or systemic endotoxin challenge, and during development of collagen arthritis.

11.5 Results

11.5.1 The local Shwartzman Reaction

Intra-dermal injection of LPS (*Salmonella enteritidis*) all led to a Shwartzman reaction, regardless of whether a second systemic injection of endotoxin was given. At doses of LPS as low as 10 µg the reaction was evident histologically on haematoxylin and eosin staining (Figures 33 and 34). Intra-dermal injection of 20 µg LPS was also, as expected, associated with a systemic acute phase response at bleed out, with mouse SAP values >150 mg/l.

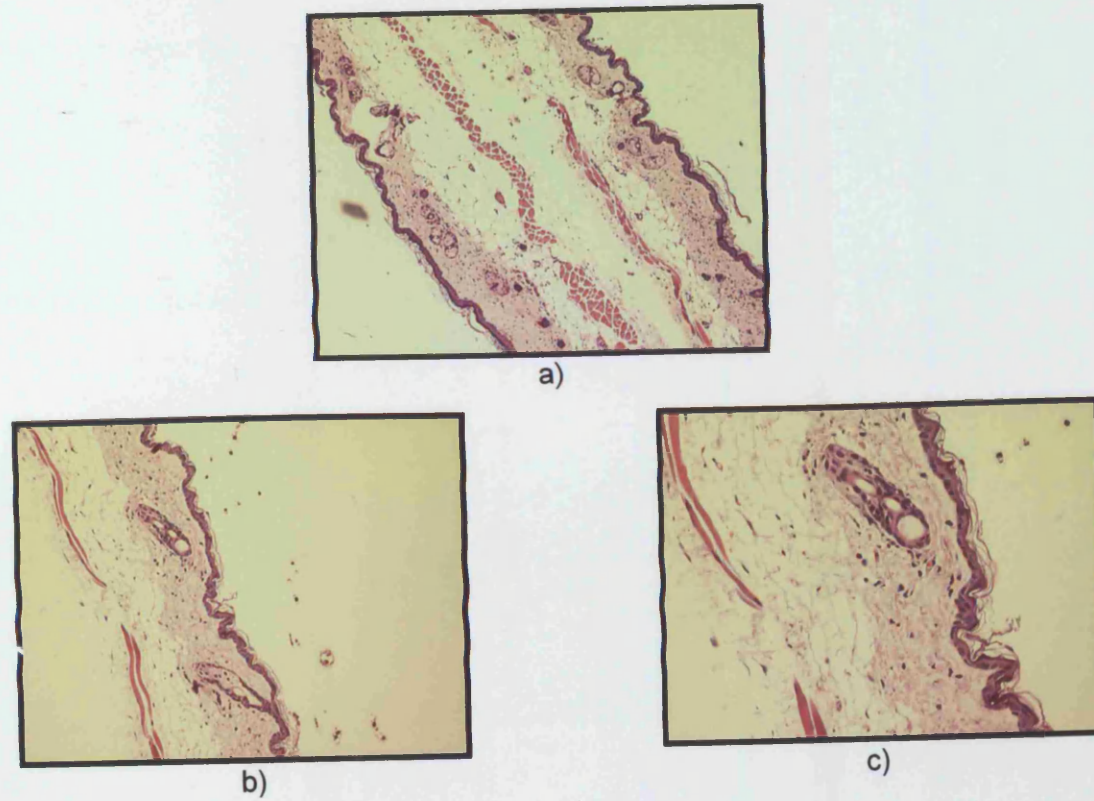


Figure 33 Normal histology of murine skin

- a) x5, b) x10, c) x20 (Objective magnification; Haematoxylin and eosin)

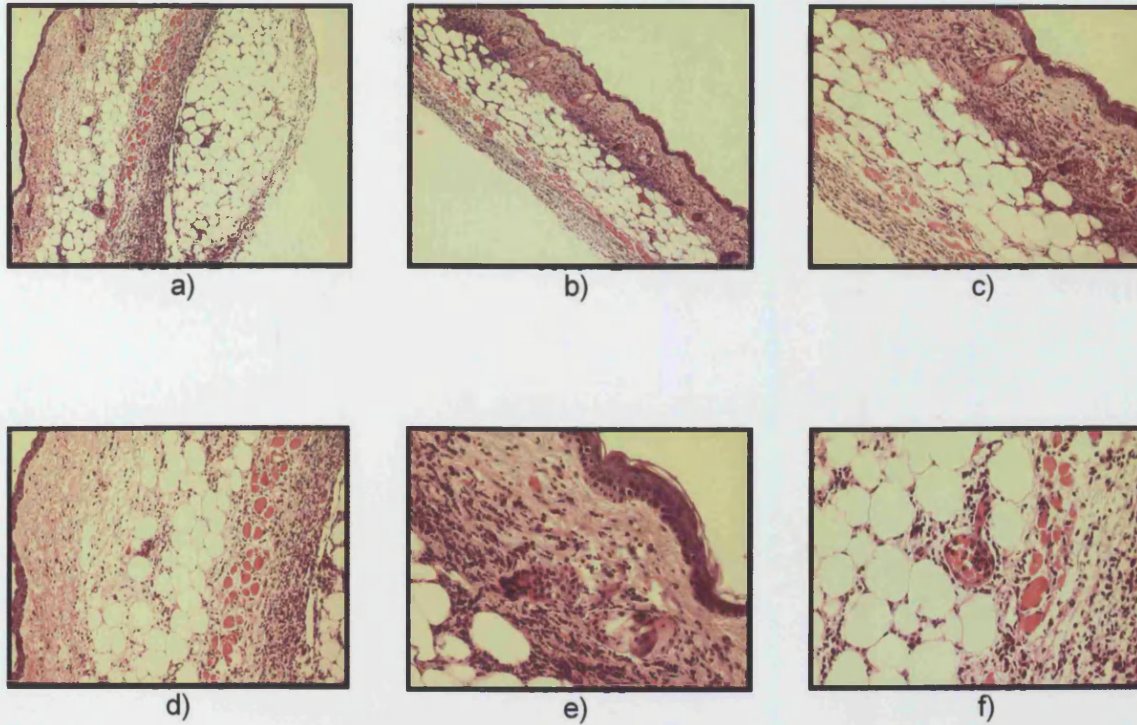


Figure 34 Demonstration of local Shwartzman phenomenon: polymorph infiltration, vasculitis and haemorrhage

- a) and b) x5, c) and d) x 10, e) and f) x 20 (Objective magnification; Haematoxylin and eosin).

11.5.1.1 No effect of passive CRP administration on the local Shwartzman reaction

The morning following intra-dermal LPS (10 µg), isolated human CRP (1 mg) or TC buffer was given intra-peritoneally to one group of age matched female mice and repeated 8 hours later. Approximately 36 hours after LPS administration, the mice were euthanised and the skins harvested and processed. A simple histology score for polymorph infiltration [mild (1), moderate (2) and severe (3)], was performed, blinded to treatment, by two observers in the laboratory (MBP and myself) and one external expert pathologist (Professor D. Evans). Figure 35 shows our findings.

The median human CRP concentration at bleed out was 22.97 mg/l (range 16.32). No consistent difference between groups was evident. Other histological findings of vasculitis were not sufficiently common to meaningfully quantify.

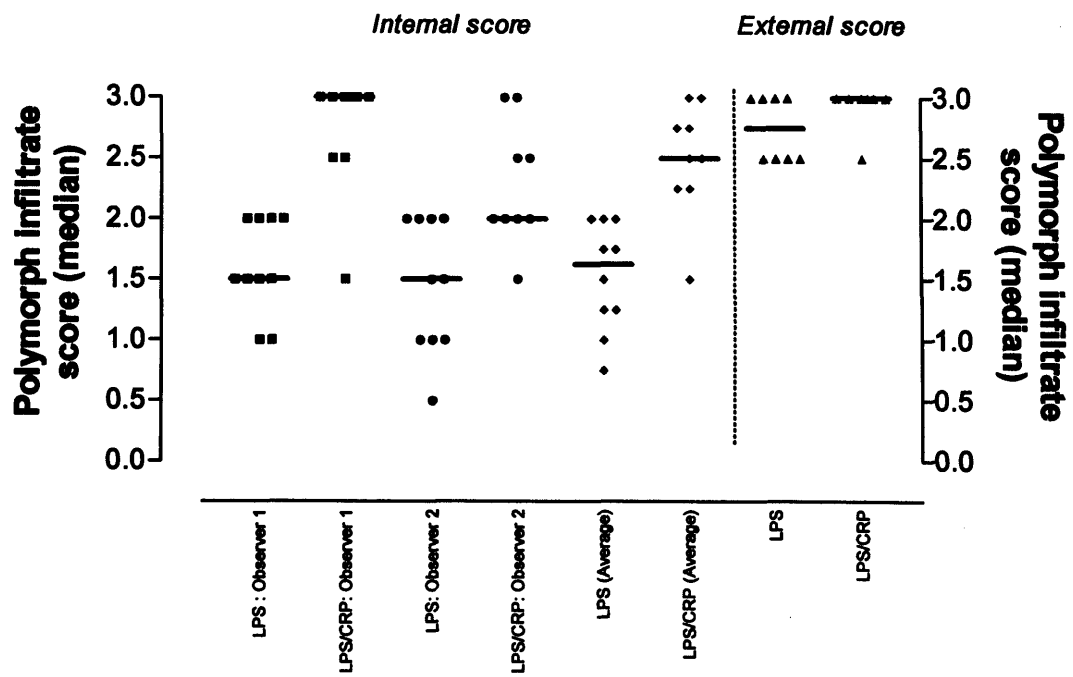


Figure 35 Passive CRP and polymorph infiltration in the Shwartzman reaction

- Mice treated with LPS +/- CRP; histology scores of three observers for each mouse

11.5.1.2 No effect of transgenic CRP production on the local Shwartzman reaction

The local Shwartzman reaction was also elicited in age matched male human CRP transgenic mice (control mice were negative for the transgene). Intradermal LPS (10 µg) was administered and 36 hours later the mice were killed and bled out. The median CRP at bleed out in transgenic mice was 18.90 mg/l (range 19.02). Again, there was no difference in the histological scoring (blinded to genotype) of polymorph infiltration (Figure 36). Haemorrhage and occasional vasculitis was noted but with no distinguishing pattern between groups and insufficiently frequently to score meaningfully.

11.5.2 Endotoxaemia and C-reactive protein

LPS lethality was generated by systemic administration of LPS to mice. Unless indicated the LPS originated from *Escherichia coli* O111:B4, and was administered at a dose of 10 mg/kg intra-peritoneally; sterile acute phase stimuli were generated 24 hours prior to endotoxin challenge by administration of either 0.5 ml 10% w/v casein or 0.25-0.5ml 2% w/v silver nitrate sub-cutaneously. All mice were bred on a pure C57BL/6 background, and were age matched. Analysis is presented separately for males and females and unless shown no significant difference in survival was detected by log Rank or Breslow testing.

11.5.2.1 Baseline and acute phase production of human CRP in transgenic mice

The known sexual dimorphism in baseline and acute phase serum concentrations of human CRP in the transgenic mice was confirmed before and after stimulation by injection of casein, silver nitrate or LPS (Figures 37 and 38). The males had dramatically higher values than the females, and mounted acute phase production of human CRP that corresponded well with the concentrations seen in human disease.

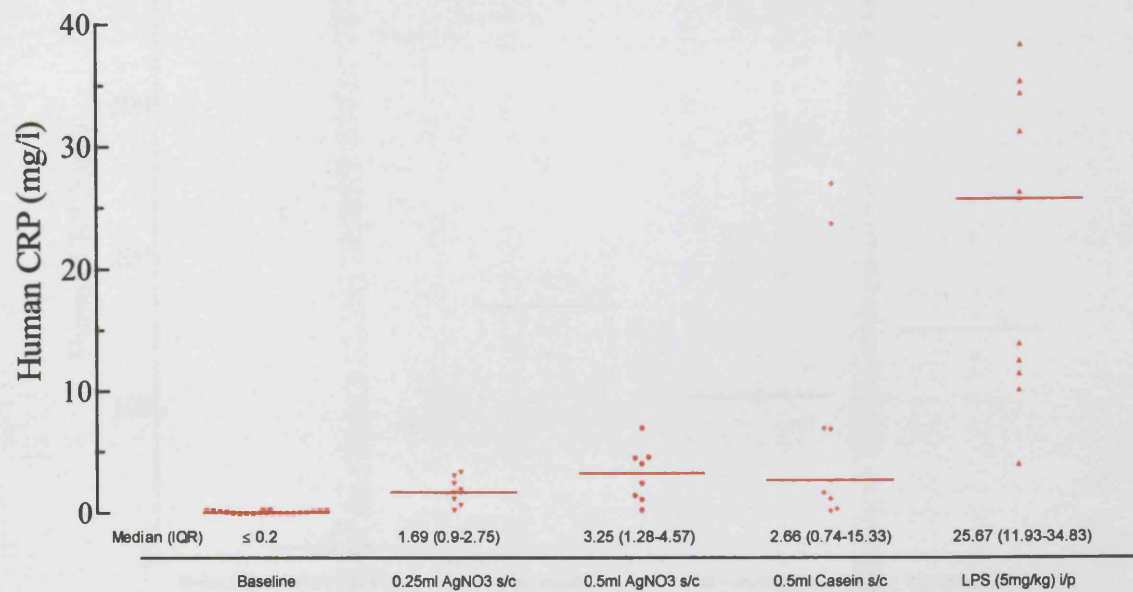


Figure 37 Production of human CRP in female transgenic mice after stimulation with sterile inflammatory stimuli

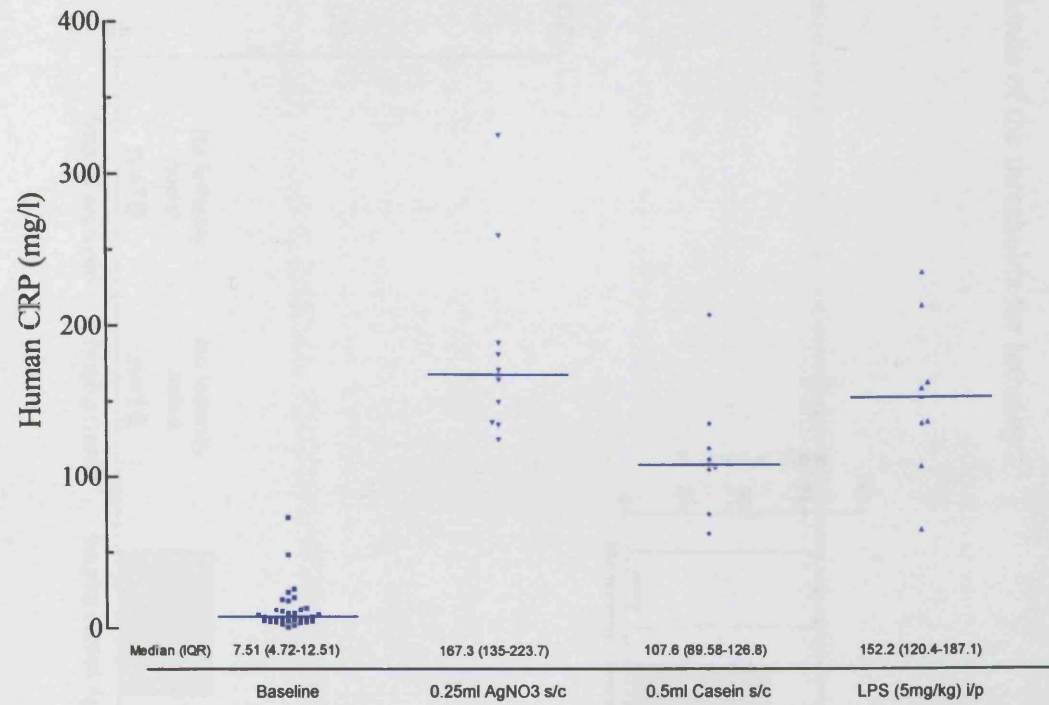


Figure 38 Production of human CRP in male transgenic mice after stimulation with sterile inflammatory stimuli

11.5.2.2 Systemic administration and effect of acute phase stimulation on LD80: On going acute phase responses do not protect against LPS lethality

We established that for female wild type (Wt) mice, the LD80 dose of LPS needed to be greater than 7.5 mg/kg (*E. coli* O111:B4). Figure 39 summarises our experience of the thresholds for lethality.

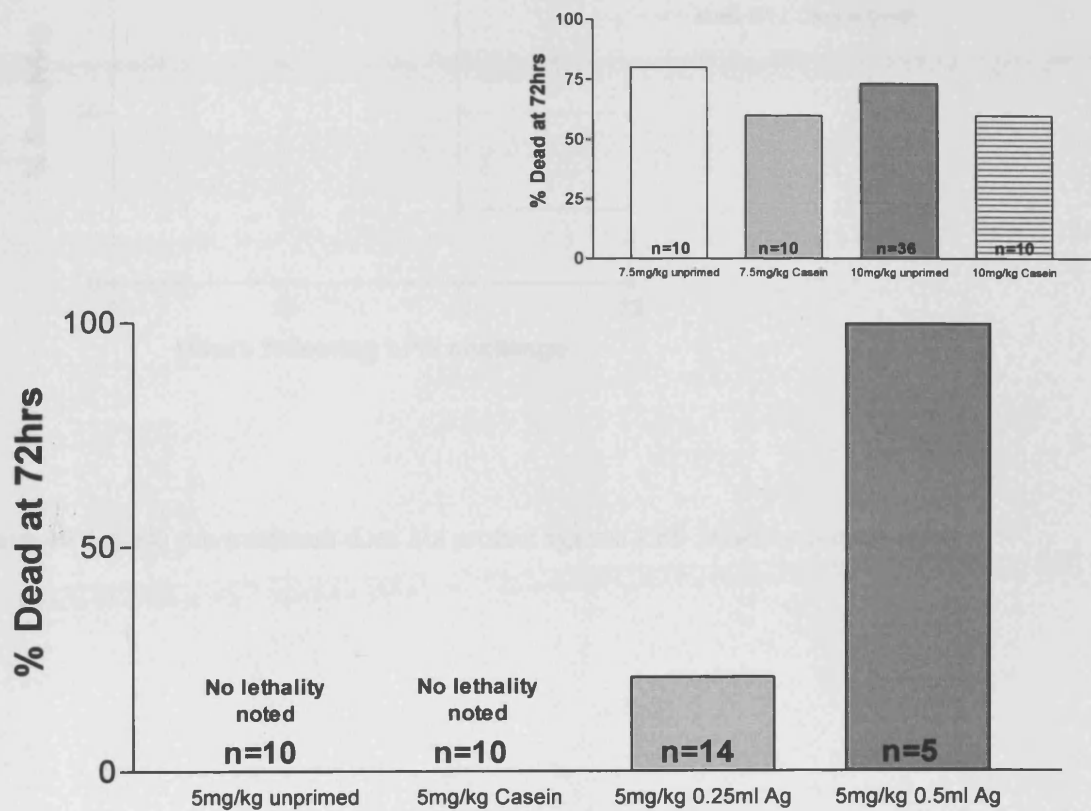


Figure 39 Lethality in female mice following endotoxin challenge

- priming by pre-existing sterile acute phase responses (s/c casein or silver nitrate; pooled results for lethality at 72 hours)

Mice in which major acute phase responses had been induced by sterile inflammation, by sub-cutaneous injection of casein solution (Figures 40 and 41), 24 hours beforehand, were not protected against LPS lethality. The harsher stimulus, silver nitrate did not protect either, and indeed lowers the threshold for LPS lethality (Figure 39).

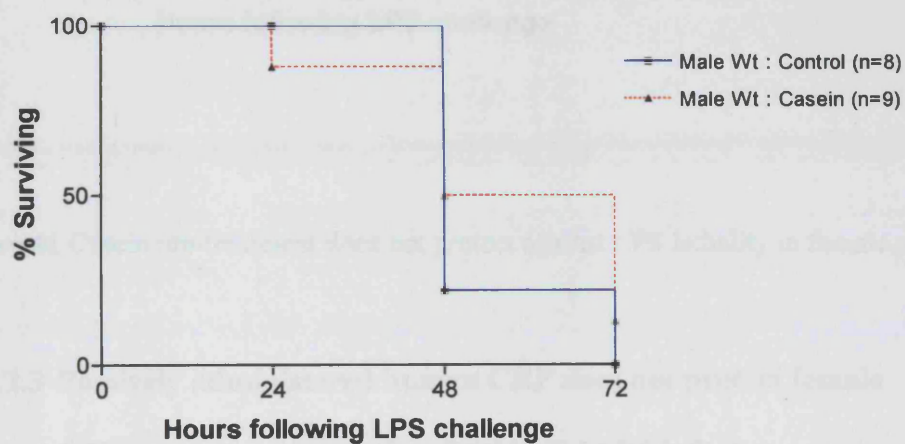


Figure 40 Casein pre-treatment does not protect against LPS lethality in male mice

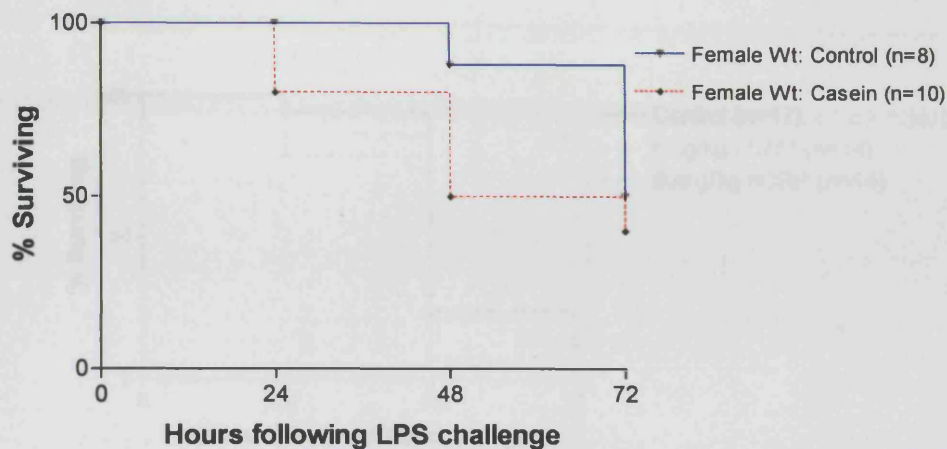


Figure 41 Casein pre-treatment does not protect against LPS lethality in female mice

11.5.2.3 Passively administered human CRP does not protect female

C57BL/6 mice against *E. coli* O111:B4 LPS lethality

In contrast to the previous observation in female Balb/c mice receiving *Salm. typhimurium* LPS [89], and the study of Mold *et al.* in male C57BL/6 mice receiving *E. coli* O55:B5 LPS [431], we observed no protection by human CRP injections in the female C57BL/6 mice challenged here with *E. coli* O111:B4 LPS (Figure 42).

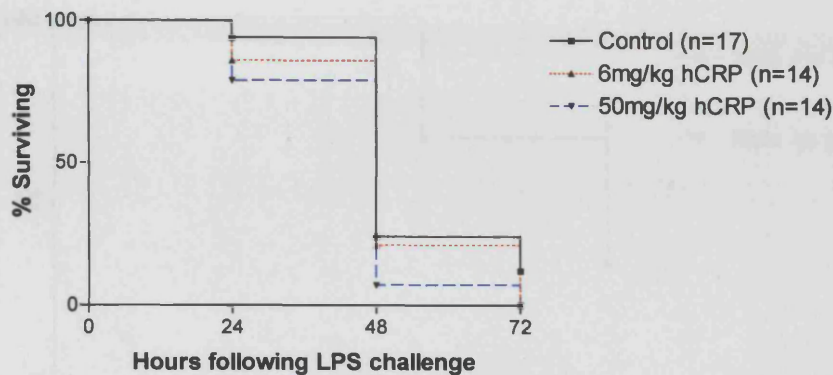


Figure 42 No effects of passive CRP on survival from lethal LPS challenge

- groups of female wild type C57BL/6 received an intra-peritoneal injection of isolated purified human CRP, either 6 or 50 mg/kg, or buffer alone in controls, 15 minutes before intra-peritoneal challenge with 10 mg/kg of *E. coli* O111:B4 LPS and again 4 hours later. The LPS content of the purified CRP used was extremely low, 0.1ng of LPS per mg of CRP; this means that with the highest dose of CRP administered there was about 0.2ng of LPS compared to the lethal challenge dose of ~200µg.

11.5.2.4 Human CRP transgenic mice are not protected against LPS

lethality

Both male and female transgenic mice mounted acute phase responses of human CRP to the stimulus of LPS injection. However, lethality following challenge with *E. coli* O111:B4 LPS did not differ significantly from that seen in wild type controls (Figures 43 and 44). Furthermore, the same absence of protection was observed in mice challenged respectively with LPS from *E. coli* O55:B5 or *Salm. typhimurium*, that were used in previously reported studies (Figures 45 and 46).

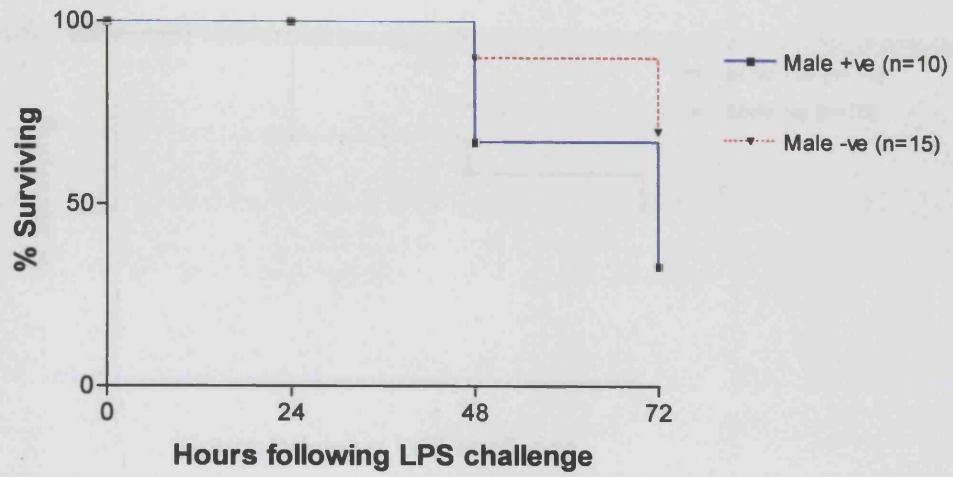


Figure 43 Male mice transgenic for human CRP are not protected against LPS (a)

- *E. coli* O111:B4; 10mg/kg, i/p.

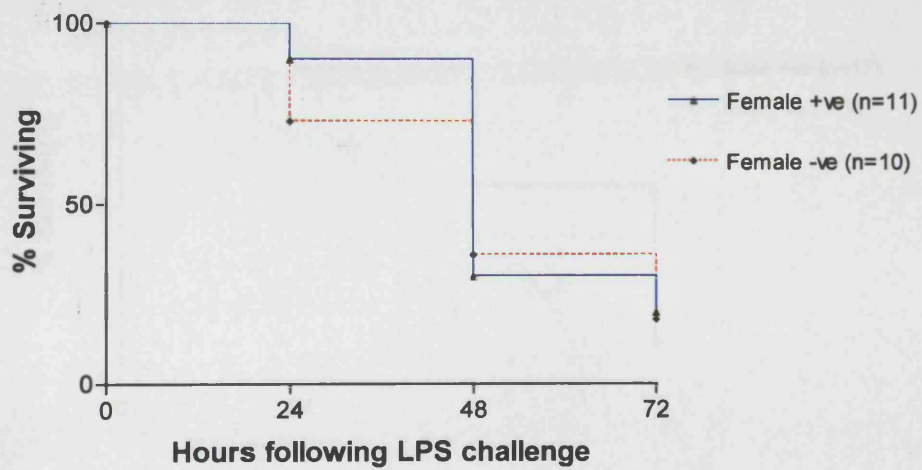


Figure 44 Female mice transgenic for human CRP are not protected against LPS

- *E. coli* O111:B4; 10mg/kg, i/p.

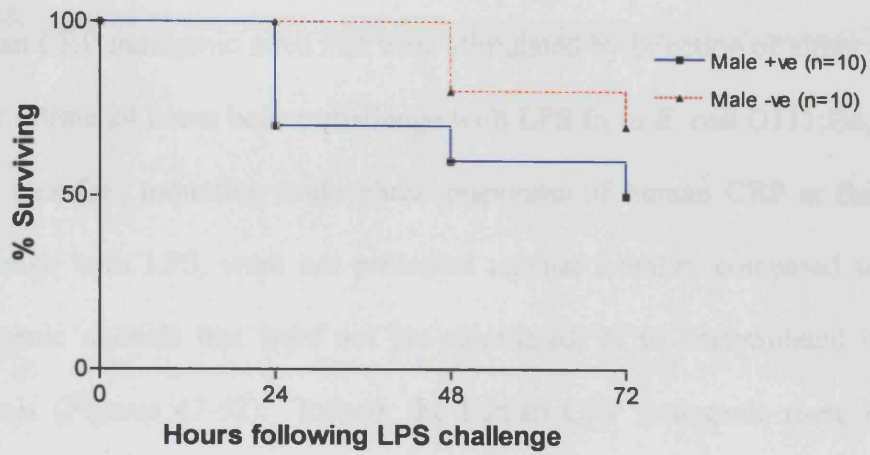


Figure 45 Male mice transgenic for human CRP are not protected against LPS (b)

- *E. coli* O55:B5; 10mg/kg, i/p.

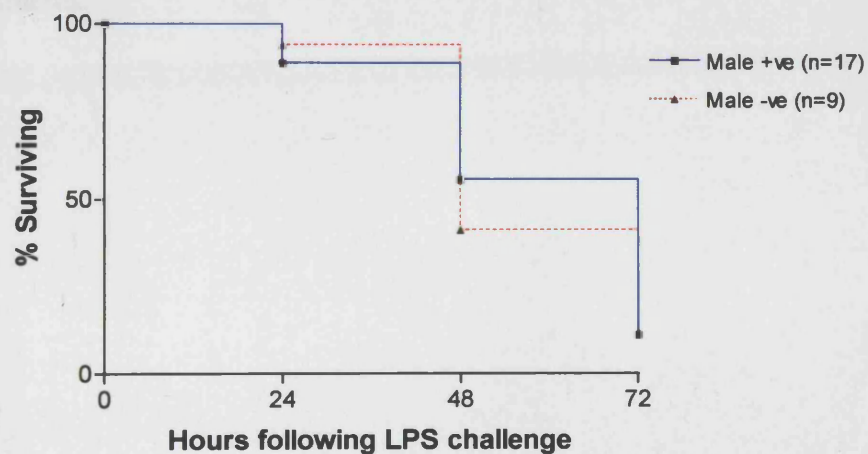


Figure 46 Male mice transgenic for human CRP are not protected against LPS (c)

- *S. typhimurium*; 10mg/kg, i/p.

11.5.2.5 On going acute phase responses in human CRP transgenic mice do not protect against LPS lethality

Human CRP transgenic mice that were stimulated by injection of either casein or silver nitrate 24 hours before challenge with LPS from *E. coli* O111:B4, and that were therefore mounting acute phase responses of human CRP at the time of challenge with LPS, were not protected against lethality compared to control transgenic animals that were not pre-stimulated, or to unstimulated wild type controls (Figures 47-52). Indeed, the human CRP transgenic male mice that were pre-treated with casein showed greater mortality than the control group (Figure 47). We observed the trend to greater mortality in the male not the female human CRP transgenic mice, consistent with the fact that the males produce abundantly more human CRP than the females. Even though the difference was statistically significant one must be very cautious not to over interpret this finding because we are very aware of the variability in these experiments.

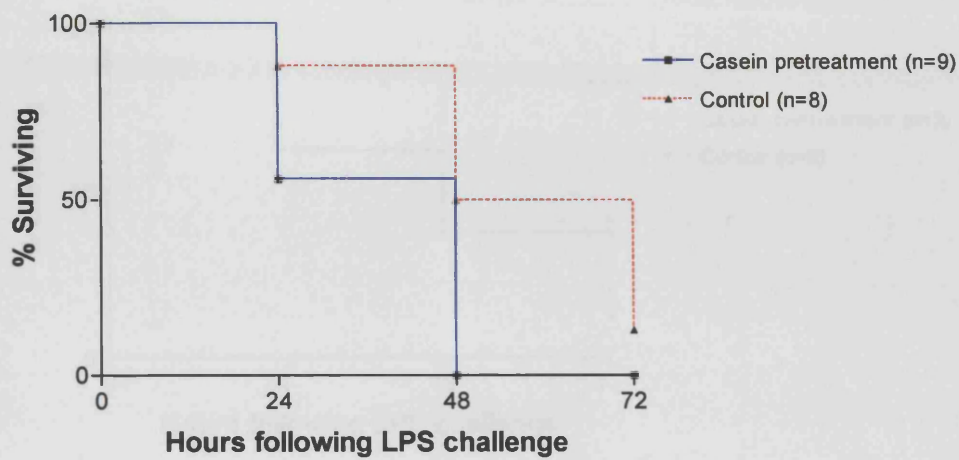


Figure 47 Male hCRPTg mice are not protected against LPS by casein pre-treatment - although the samples size is small, there was significantly greater mortality among males pre-treated with casein than in the corresponding control group, $P < 0.01$ (Log Rank test).

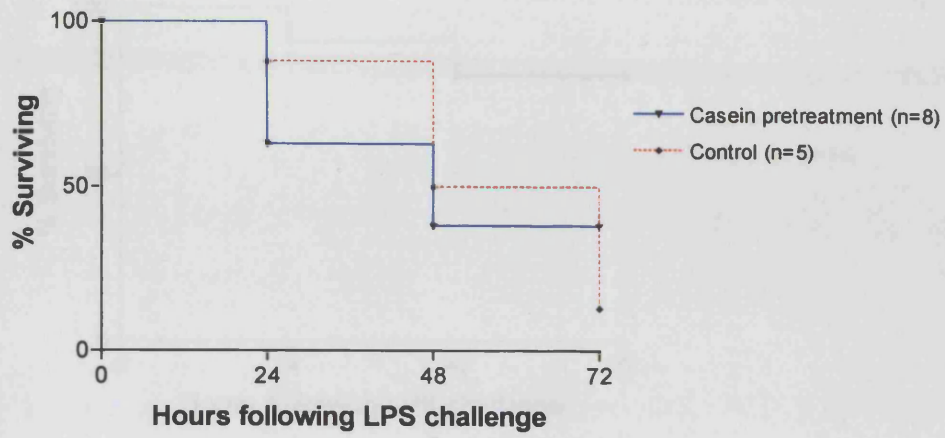


Figure 48 Female hCRPTg mice are not protected against LPS by casein pre-treatment

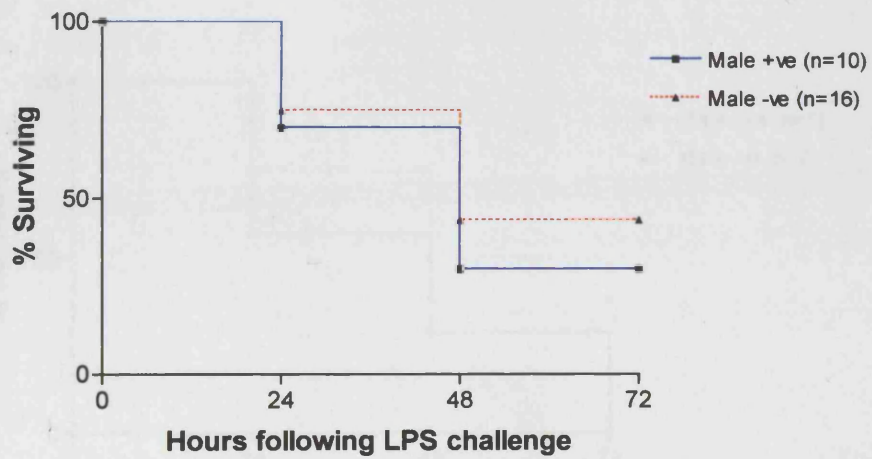


Figure 49 Silver nitrate (0.25ml) fails to protect male hCRPTg mice from LPS lethality

- 5mg/kg *E. coli* O111:B4 LPS

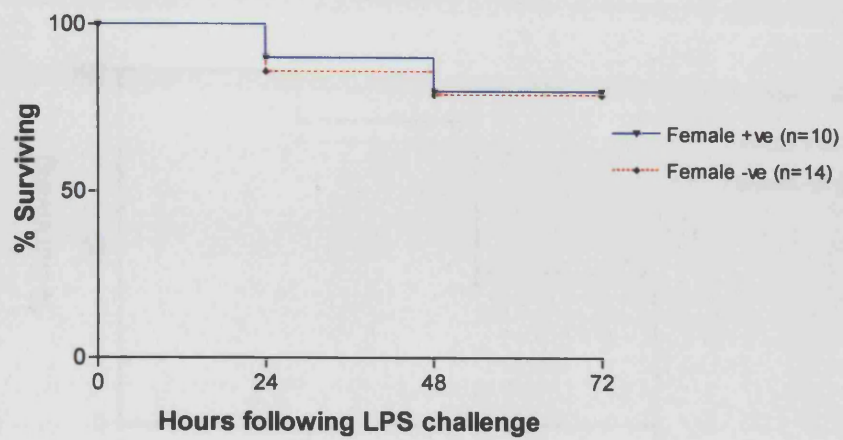


Figure 50 Silver nitrate (0.25ml) fails to protect female hCRPTg mice from LPS lethality
 - 5mg/kg *E. coli* O111:B4 LPS

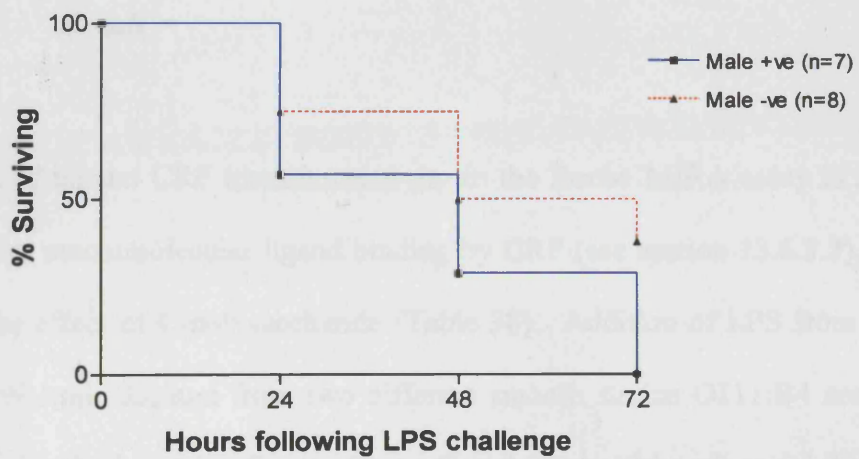


Figure 51 Silver nitrate (0.5ml) fails to protect male hCRPTg mice from LPS lethality
 - 5mg/kg *E. coli* O111:B4 LPS

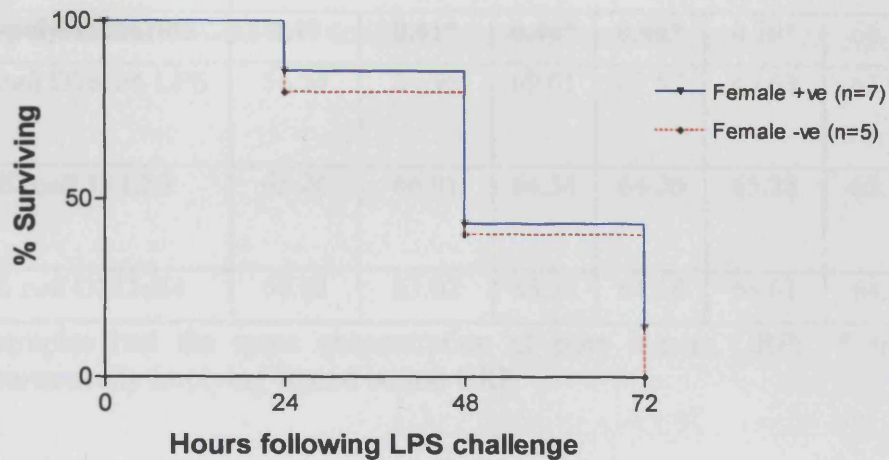


Figure 52 Silver nitrate (0.5ml) fails to protect female hCRPTg mice from LPS lethality

- 5mg/kg *E. coli* O111:B4 LPS

11.5.2.6 Human CRP does not bind to LPS from rough or smooth strains of *E. coli*

Loss of human CRP immunoreactivity in the Roche MIRA assay is a sensitive test for macromolecular ligand binding by CRP (see section 13.6.3.3), as shown by the effect of C-polysaccharide (Table 38). Addition of LPS from the rough *E. coli* strain J5, and from two different smooth strains O111:B4 and O26:B6, however, had no significant effect other than possibly minor inhibition. The failure of the LPS preparations to interfere in the assay is thus robust evidence that CRP does not bind significantly to LPS, at least via the calcium-dependent ligand binding B face of the CRP molecule.

Table 38 LPS does not block CRP reactivity in the MIRA assay

Ligand (mg/l)	2500	500	250	125	62.5	0
	<i>CRP Concentration measured (mg/l)</i>					
C-polysaccharide	0.4*	0.41*	0.46*	0.46*	0.39*	66.10
<i>E. coli</i> O26:B6 LPS	56.54	66.96	69.01	69.52	69.63	63.99
<i>E. coli</i> J5 LPS	65.26	66.01	64.34	64.26	65.28	65.30
<i>E. coli</i> O111:B4	60.03	63.02	63.33	64.18	65.01	64.20

- all samples had the same concentration of pure human CRP; * masked immunoreactivity implying ligand bound CRP

11.5.3 Collagen arthritis in human C-reactive protein transgenic mice

Human CRP transgenic C57BL/6 mice were immunised intra-dermally in the tail, with chick collagen (complete Freund's adjuvant, day 0), with an identical boost on day 21. They were studied clinically over a two month period as well as biochemically and histologically. This small pilot study included male CRP transgenic negative mice (wt), male CRP transgenic positive mice, and female CRP transgenic positive mice. It must be noted that the schedule employed to elicit collagen arthritis in the C57BL/6 strain is harsher than traditional methods [354] (which employ more genetically susceptible DBA/1 mice), and this was visible in some mice with important morbidity, particularly inflamed tails and occasional mortality. This concerned us, and is a critical reason for not repeating this study in this strain.

11.5.3.1 Acute phase response secondary to immunisation

Murine SAP

All animals mounted an acute phase response secondary to the immunisation schedule, regardless of the presence or absence of clinical arthritis. Figure 53 demonstrates the mouse SAP response in all mice, whilst Figures 54-56 demonstrate the mouse SAP response in the different subgroups. Human CRP genotype did not affect the global mouse SAP response, which acted in this case, as an independent marker of similar immunisation protocols.

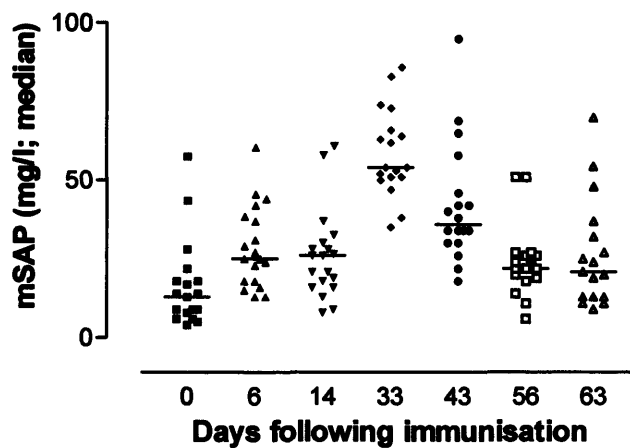


Figure 53 Murine SAP response to chick collagen immunisation (all animals)

- complete Freund's adjuvant Day 0 and 21

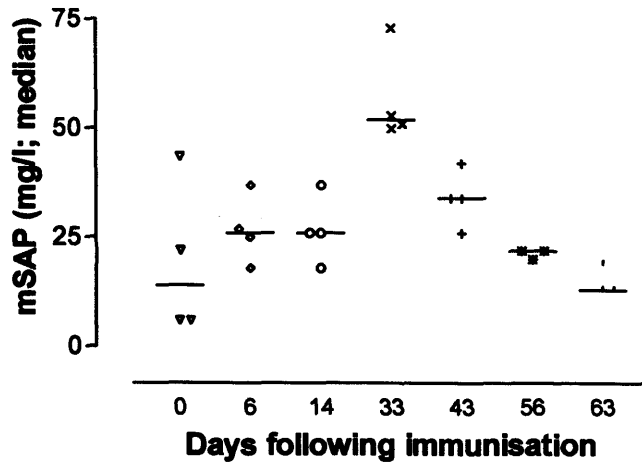


Figure 54 Murine SAP response to chick collagen immunisation: male hCRPtg +ve
 - complete Freund's adjuvant Day 0 and 21

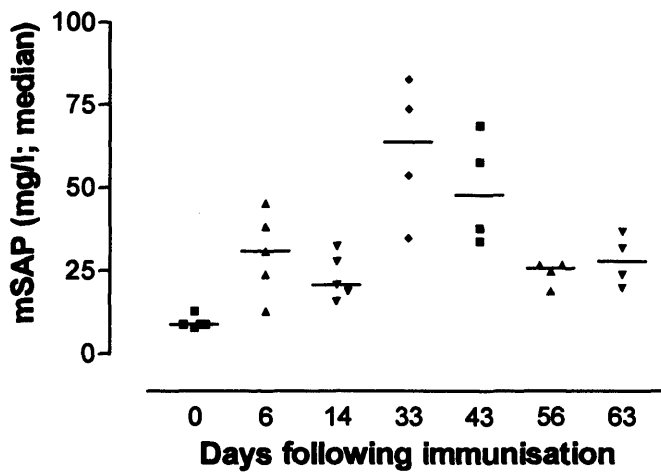


Figure 55 Murine SAP response to chick collagen immunisation: female hCRPtg +ve
 - complete Freund's adjuvant Day 0 and 21

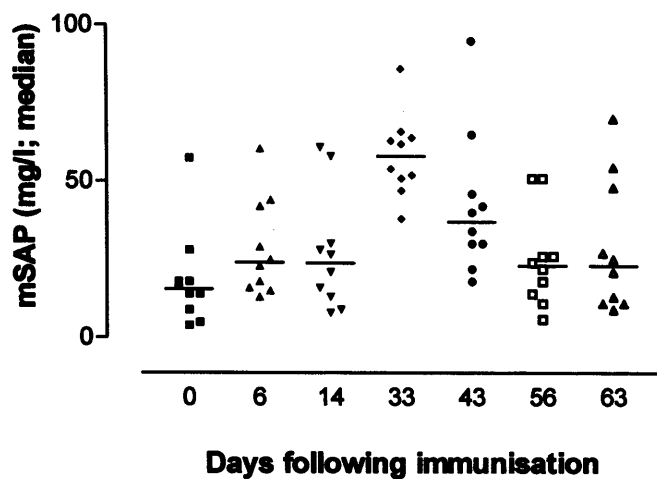


Figure 56 Murine SAP response to chick collagen immunisation: male wt mice

- complete Freund's adjuvant Day 0 and 21

Human CRP

When assayed, CRP was not detectable in female positive transgenic mice, despite the mSAP response to immunisation, and the use of a sensitive assay (MIRA). Interestingly the male mice demonstrated only a modest response (Figure 57). This was not consistent with the evident tissue damage in the tails of the mice immunised or the mSAP response.

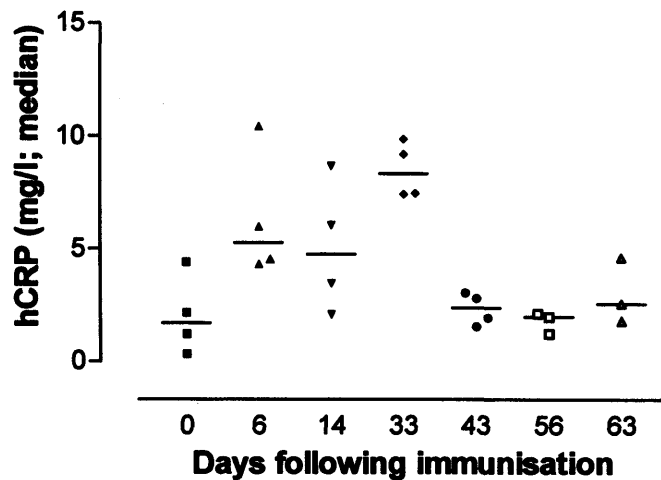


Figure 57 Human CRP values in male CRP transgenic mice immunised with collagen

11.5.3.2 Collagen antibody production, genotype, and clinical arthritis score

No reference serum was available for this assay. Therefore we used serum from a single animal with florid arthritis at the end of the experiment (arthritis score 11), that contained IgG anti-chick collagen antibodies. Pre-bleed sera did not contain any detectable antibody. There was a linear relationship across a wide range of dilutions of sera, and antibody production was evident prior to clinical signs of arthritis (Figure 58). Bleed out sera from the other animals were then

tested at a dilution of 1 in 1600 and compared to the reference sera (serial antibody titres were not measured).

No difference in antibody titre was seen between groups, and no relationship was seen between clinical arthritis and presence of antibody i.e. animals with no arthritis had detectable antibody and vice versa (Figures 59 and 60). This may simply represent a sampling error with regards the power of our study. Alternatively, and worthy of further investigation if this were found to be a robust and reproducible effect, it may represent changes in the handling or presentation of potential immunogens in CRP transgenic mice.

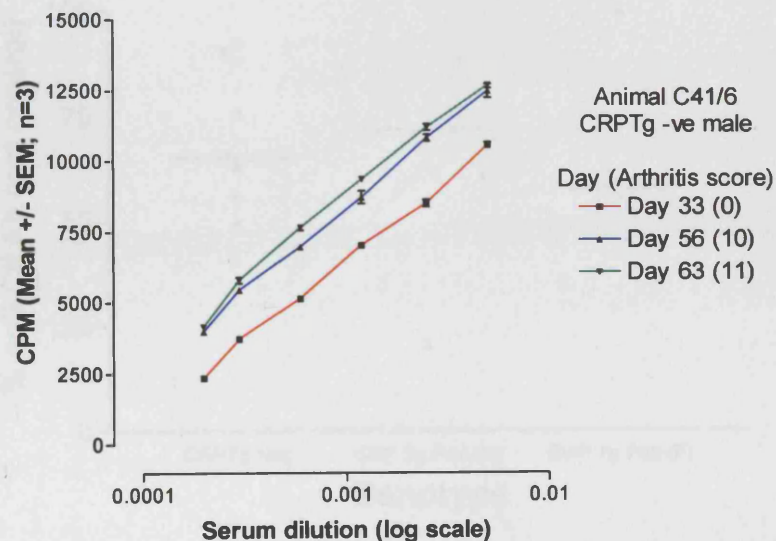


Figure 58 Linear relationship of anti chick collagen antibodies across a dilution series

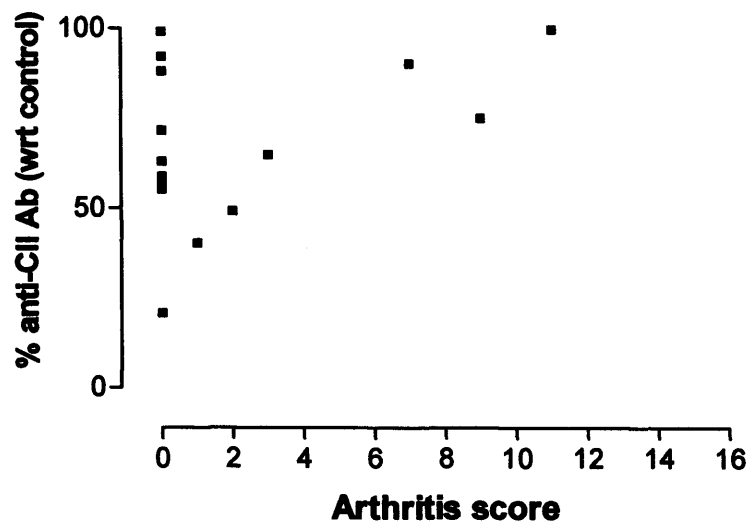


Figure 59 No relationship between clinical arthritis score and collagen antibody titre

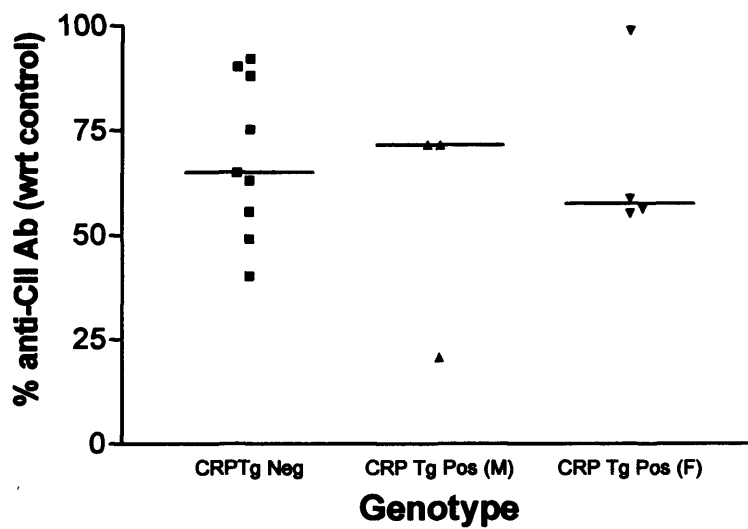


Figure 60 No relationship between genotype and collagen antibody titre

11.5.3.3 Clinical arthritis score and CRP genotype

Figure 61 demonstrates the clinical arthritis score for the study. None of the female CRP transgenic positive mice developed clinical evidence of arthritis. Indeed severe arthritis was only noticed in male CRP transgenic negative mice. There was however variability in the severity of the arthritis (Figure 61, inset), so further work is needed before any substantive conclusion can be drawn.

11.5.3.4 Histology

Professor D. Katz kindly reviewed the embedded sections (which had been decalcified). No immunohistochemistry could be performed on these sections. His findings, showed, where there was clinical evidence of arthritis, histological confirmation with synovitis, synovial hyperplasia and soft tissue inflammation as well as bone erosion. Where no evidence of arthritis was macroscopically seen, histology was normal. Representative examples of histology are shown in Figure 62.

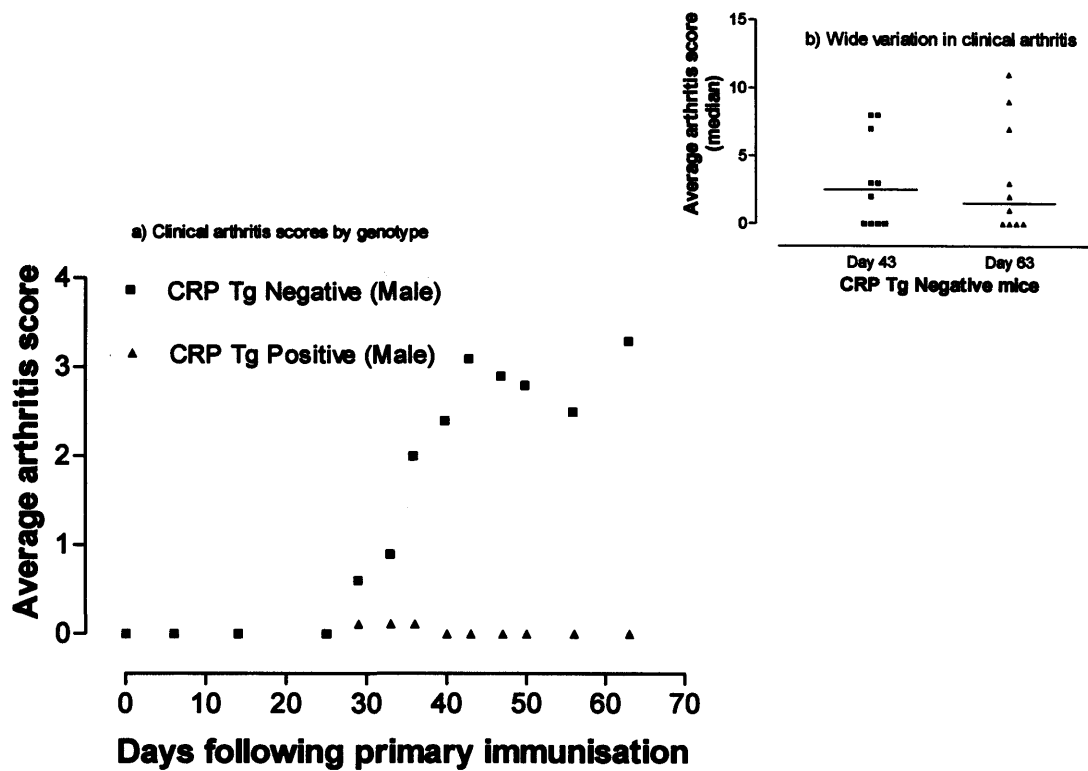


Figure 61 Clinical arthritis and CRP genotype; b) wide variation in severity of arthritis

- the average arthritis score is calculated from the score for each paw of every mouse, with the average for each genotype shown in a) and plotted individually in b).

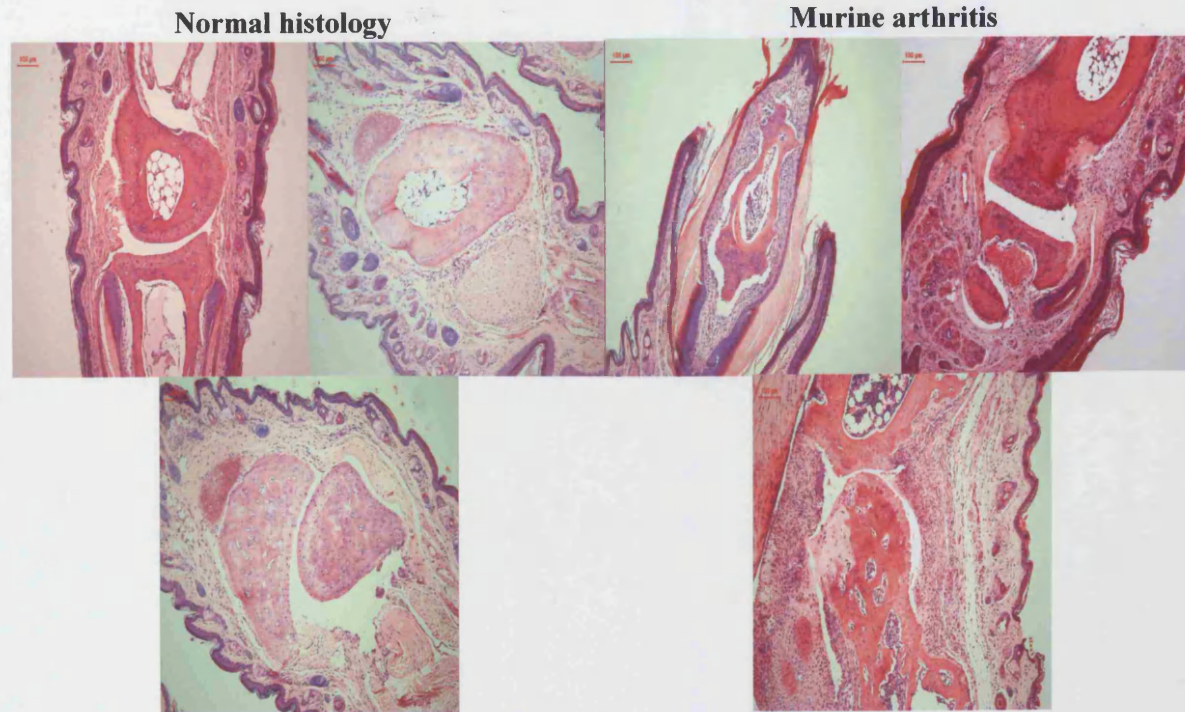


Figure 62 Typical histology from mice with and without clinical evidence of arthritis

- H&E staining; the left sided images show normal histology whilst those on the right demonstrate arthritis, synovitis, inflammation and bone erosion

11.6 Discussion

Despite many reports of properties of CRP, clear understanding of the actual biological functions of this phylogenetically ancient and highly conserved protein remains unclear. This reflects a number of important points pertaining to CRP, which are in addition to the caveats associated with any individual animal model of disease. Examples of the constraints and limitations that need to be considered for our work, as well as others before us, include [1, 12, 326]:

- No deficiency state or protein polymorphism of human CRP has been reported. There is thus no information from so called ‘experiments of Nature’;
- Mice, the most accessible model for *in vivo* investigation of the physiological and pathophysiological functions of proteins, produce CRP only as a trace protein that, although inducible in the acute phase response, never exceeds 1-2 mg/l, and no mouse CRP knockout has been produced;
- Although CRP is generally conserved, there are nonetheless very important differences between CRP in different species [75, 468] with respect to key structural and functional properties (demonstrated by simple experiments for rabbit CRP in Figure 63 and Tables 39 and 40) e.g. fine ligand-binding specificity, presence and nature of glycosylation,

protomer assembly, capacity to precipitate and aggregate ligands, baseline circulating concentrations, behaviour as acute-phase proteins, and capacity to activate autologous complement. Experiments in which heterologous CRP is administered to mice, or transgenically expressed, are not, therefore, necessarily physiological or representative of the actual functions of human CRP in man;

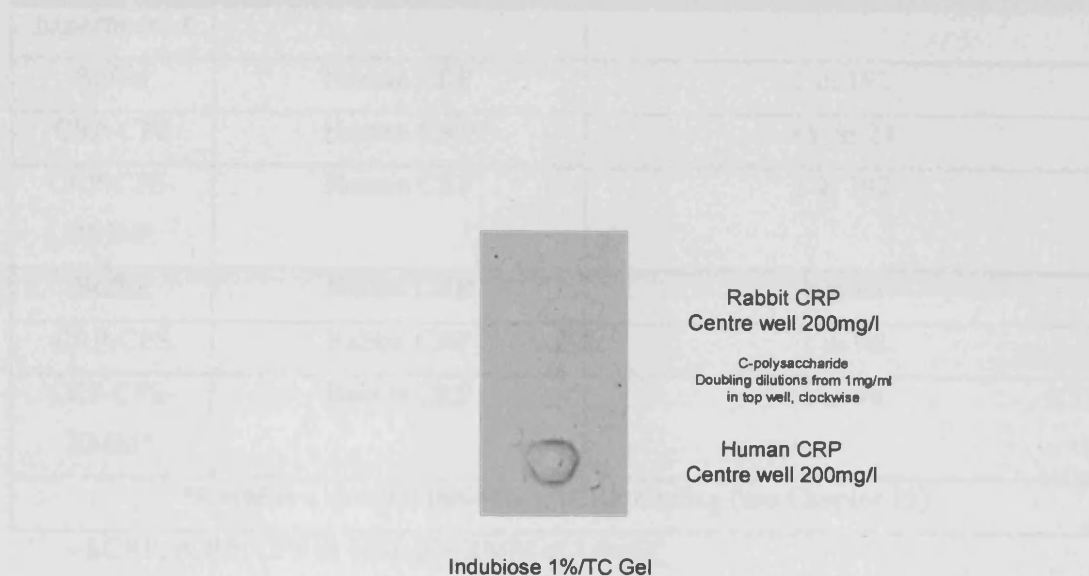


Figure 63 Rabbit CRP does not precipitate pneumococcal CPS

Table 39 Rabbit and human CRP bind pneumococcal CPS in the fluid phase

- demonstrated indirectly by changes in ability of CRP (100mg/l) in buffer to bind to Sepharose-PE (complexed CRP not being able to bind)

Concentration of CPS (mg/l)	% Human CRP bound to Seph-PE	% Rabbit CRP bound to Seph-PE
0	80	71
100	78	59
500	60	48
1000	44	37
5000	32	22

Table 40 Autologous activation of complement: rabbit and human CRP

- Despite both rabbit and human CRP binding commercial CPS there are differences in complement activation; a sheep red cell haemolytic assay in autologous serum demonstrates that rabbit CRP does not activate its autologous complement.

Condition	Protein	Autologous serum dilution at which no haemolysis seen
<i>Experiment 1</i>		
Buffer	Human CRP	1 in 192
CRP-CPS	Human CRP	<1 in 24
CRP-CPS-RMM*	Human CRP	1 in 192
Buffer	Rabbit CRP	1 in 96
CRP-CPS	Rabbit CRP	1 in 96
CRP-CPS-RMM*	Rabbit CRP	1 in 96
*RMM is a specific inhibitor of CRP binding (see Chapter 13)		

- hCRP, rCRP, CPS at 165mg/l; RMM at 3.6mM

- Many *in vitro* studies have been conducted with heterologous systems, and/or with commercially sourced CRP for which no adequate characterisation of purity or integrity is reported;
- Very few studies include rigorous controls that are essential for attribution of observed effects to CRP itself, such as inhibition by anti-CRP antibodies or by specific affinity chromatography absorption of the CRP preparations. The lack of a specific pharmacological inhibitor of CRP binding hampers *in vivo* studies in particular;

- Transgenic models may demonstrate aberrant protein expression not reflected in man e.g. the human CRP transgene is aberrantly expressed in our model, with abundant CRP production in the kidney and other tissues [435], whilst CRP in humans is produced in significant amounts only by the liver.

Chapter 14 discusses these concerns in greater detail.

Specifically we show here that protection of mice against LPS lethality by passive administration of human CRP is not a universally reproducible phenomenon. Furthermore, human CRP transgenic mice, that mount major acute phase responses of human CRP following acute phase stimulation, closely resembling events in man, were not protected against LPS toxicity. There are also conflicting reports about whether pre-existing acute phase responses protect mice against LPS lethality, and we confirm the previous finding that mice mounting an acute phase response to a sterile inflammatory stimulus remain as susceptible as control, non-pretreated, animals. It has been reported previously that human CRP does not bind to LPS, and we confirmed in this study, using a very sensitive indirect assay, that there was no evidence of a significant interaction between CRP and LPS from one rough and two smooth strains of *E. coli*.

Our present failure to find protection by exogenous human CRP suggests that this rather artificial phenomenon is unlikely to be of general significance. Apart from the heterologous nature of the human CRP in the present transgenic mice, they

represent a much more physiological model, either with or without an ongoing acute-phase response before LPS challenge. The previous observations of a protective role for CRP were in transgenic mice expressing rabbit CRP under a nutritional promoter (and moreover the control animals were not of the same strain) [429]. The whole gamut of acute-phase phenomena, including the cytokine cascade, acute-phase protein synthesis, and activation of all the molecular and cellular pro-inflammatory and defence systems, is engaged in our mice, so that any effects of human CRP can be mounted in a milieu that is as appropriate as possible. The absence of any protection in such mice therefore provides no evidence in favour of human CRP having an important function in host defence against LPS lethality. This is consistent with clinical experience, in that all patients with septicaemia and with septic shock have extremely high circulating CRP concentrations that persist until death, unless there is also concurrent severe liver failure. There is thus no evidence from clinical observation of a protective effect of CRP in this situation.

With respect to the local Shwartzman reaction we found no effect on the histological lesion of either exogenously administered or transgenically produced human CRP. Further study in this area might nevertheless be valuable as more quantitative histology along with immunohistochemistry looking for CRP and/or complement deposition in the skin, might demonstrate subtle differences.

Finally our pilot study in arthritis did not produce any significant conclusions but does suggest avenues for further work. Although it is reported that C57BL/6 mice can be used for collagen arthritis [354], we found that initiation of arthritis

in this strain was associated with an unacceptable morbidity for the mice (in view of the immunisation schedule involving two injections with complete Freund's adjuvant), and occasional mortality. Intriguingly in our hands, in the very small numbers studied, all animals had similar acute phase responses and antibody titres, but male CRP transgenic positive mice didn't develop any clinical arthritis whilst the male CRP transgenic negative mice did, albeit variably. Larger and more appropriate experiments are necessary to take this study further. Importantly back crossing the CRP transgene on to a more susceptible strain (DBA/1) is necessary followed by a study adequately powered to look for differences between genotype as well as gender.

11.7 Summary

The biology of CRP *in vivo* remains to be clarified. Our attempts to reproduce protection against endotoxaemia in the most physiologically relevant model available, shows no significant effect of CRP, in either local or systemic administration of endotoxin. A pilot study of collagen arthritis in transgenic human CRP mice suggested a possible protective role for human CRP but the study size and limitations are such that this is not a substantive finding, but one that should generate further interesting work.

12 Transgenic expression of human C-reactive protein and atherosclerosis

12.2 Introduction

Atherosclerosis is not just the consequence of focal arterial lipid accumulation. Fundamental processes include endothelial injury, intimal cholesterol accumulation, foam cell formation, migration and proliferation of smooth muscle cells, expression of an extracellular matrix and local thrombus formation, secondary organisation, calcification and/or plaque rupture [362]. Although high grade arterial stenoses are important, more so are qualitative aspects of plaques themselves, as critical factors associated with acute complications. Vessel occlusion occurs due to acute plaque rupture and thrombus formation and atherothrombosis supervening on unstable atherosclerotic plaques causes most coronary events and stroke. Among the functional features of plaques associated with vulnerability, inflammation has emerged as a leading pathophysiological mechanism [363, 364]. The association between circulating concentrations of CRP, an extremely sensitive systemic marker of inflammation, and future atherothrombotic events has provoked speculation about possible direct pathogenetic roles for this lipid binding, complement activating, molecule. This chapter describes the effects of transgenic expression of human CRP on atherosclerosis in apoE knockout mice.

12.3 Background

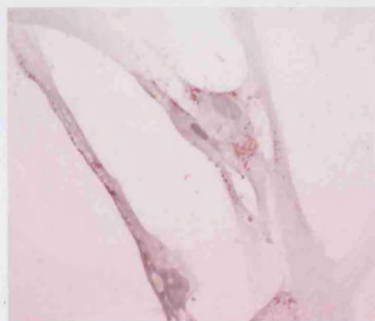
Systemic inflammation alters the probability of the occurrence of a cardiovascular event and markers of inflammation such as raised white cell counts [469], elevated values of CRP and SAA [168, 179], as well as raised erythrocyte sedimentation rates [168] are all consistently associated with future atherothrombotic disease. Indeed immediately preceding clinical episodes of infection and/or inflammation elsewhere in the body are also associated with cardiac events [470, 471].

The exquisitely sensitive and non-specific nature of the CRP response to inflammation, along with its robustness and ease of measurement analytically, has focussed much of the recent attention on it rather than other inflammatory markers. Its use as a prognostic marker has been investigated in detail since the 1990s, although the association had been studied as long as ago as the 1950s, particularly with regards myocardial infarction [472-474]. Notably the European Concerted Action on Thrombosis and Disabilities study (ECAT) looked at coagulation factors that might predict coronary events in out patients with stable and unstable angina, and CRP was included to correct for possible confounding by the fact that many clotting proteins are acute phase reactants [475]. Even with an insufficiently sensitive assay, CRP values predicted coronary events better, statistically, than any of the other markers in the study [173]. With a sensitive immunoassay [144] the ECAT investigators demonstrated clearly that raised circulating concentrations of CRP were predictors of coronary events in patients

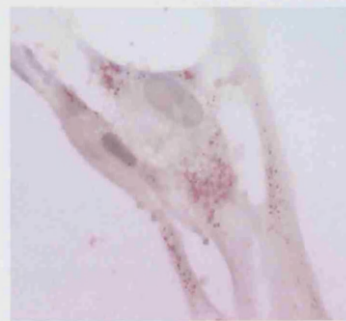
with stable or unstable angina [176]. When measured in patients with severe unstable angina being admitted to hospital, a striking prognostic role for CRP was later demonstrated; elevation of CRP (and SAA) at the time of hospital admission predicted a poor outcome in patients with unstable angina [172]. The Multiple Risk Factor Intervention Trial (MRFIT) then became the first prospective study in "healthy but high risk individuals" to document the relation between CRP and coronary heart disease mortality (1996) [174]. There then followed an explosion of interest in this area. Subsequent studies have demonstrated, both for men and women, that elevated values of CRP, albeit in the previously thought of as normal range, predict future cardiovascular events in patients with and without pre-existing cardiovascular disease. Hence the best estimates to date are now of an excess 50% risk of a major cardiac event in an otherwise healthy individual whose CRP concentration falls in the top, as opposed to bottom tertile (See chapter 10) [168].

The proposal that CRP may not only be a marker of low grade systemic inflammation associated with the processes underlying atherosclerosis was originally made in the 1980s [110, 111]. As already reviewed in detail in Chapter 8, complexed CRP activates complement, and damaged lipoprotein particles, including those typically found within atheroma, are potential ligands for CRP. Immunohistochemical studies have demonstrated that CRP is found deposited in diseased coronary arteries, in association with both lipoproteins and complement components. CRP is seen in a diffuse rather than a focal deposition in the deep fibroelastic layer and in the fibromuscular layer of the intima adjacent

to the media and additionally most foam cells below the endothelium stain positively for CRP. Furthermore in cell culture studies co-incubation of CRP with LDL stimulates foam cell formation, a key process associated with atherogenesis (Figure 64). Other *in vitro* observations, amongst which include the demonstration that CRP can upregulate tissue factor production by monocytes, have contributed to the suggested role for CRP in atherothrombosis, although such *in vitro* observations are increasingly being questioned.



x20



x40

Figure 64 Foam cell formation: co-incubation of native LDL with human CRP

- in collaboration with Dr X. Ruan, UCL, primary human vascular smooth muscle cells (TCS CellWorks) were incubated for 24 hours at 37°C with LDL (200 µg/ml) and CRP (100mg/l); Foam cell formation was demonstrated by oil red O staining; Objective magnification x 20 and x 40.

CRP may thus be a direct mediator of atherogenesis and in particular atherothrombotic events. *In vitro* studies have inherent limitations, particularly with regards the applicability of using isolated human CRP in non-physiological settings, and plasma proteins are known to enter the arterial wall by transudation and to be detectable in plaques [476-478]. *In vivo* studies are therefore needed, but as discussed in Chapters 11 and 14, with regards CRP, conventional animal work is limited because although the evolutionary conservation of sequence, subunit organisation and protein fold seen with the pentraxins proteins is strong, there are major structural and functional differences between CRP's of different species.

12.4 ApoE Knockout mice

Mice were traditionally viewed as an atherosclerosis-resistant species until the studies of Paigen *et al.* demonstrated that certain strains of mice, such as the C57BL/6, could be induced to form fatty streaks in the aortic sinus with addition of fat, cholesterol, and cholate to the diet [479, 480]. However, since the lesions are largely restricted to the aortic sinus and don't progress much past the fatty streak stage this approach cannot contribute to modelling advanced stages of the disease. In the early 1990's the development, by homologous recombination in embryonic stem cells, of apolipoprotein (apo) E-deficient mice revolutionised the use of murine models in the study of cardiovascular disease [481, 482].

ApoE, a glycoprotein [483], is a structural component of VLDL synthesised by the liver and intestinally synthesised chylomicrons. It is also a constituent of a

subclass of HDLs involved in cholesterol transport activity among cells. One of the most important roles of apoE is to mediate high affinity binding of chylomicrons and VLDL particles that contain apoE to the LDL receptor and a hepatic chylomicron remnant receptor. This allows for the specific uptake of these particles by the liver which is necessary for preventing the accumulation in plasma of cholesterol rich remnants. The homozygous inactivation of the *apoE* gene results in animals that are devoid of apoE in their sera [484]. The mice appear to develop normally, however they exhibit five times the normal serum plasma cholesterol. The elevation of plasma lipid values seen in apoE^{-/-} mice reflects a marked increase in the plasma concentration of remnant chylomicron particles along with a modest elevation of VLDL and LDL [481]. There is a subsequent predilection for spontaneous development of atherosclerotic lesions particularly in the aortic root, at the lesser curvature of the aortic arch, the principal branches of the aorta, and in the pulmonary and carotid arteries (Figure 65). Monocyte attachment to endothelial cells has been observed by 6 weeks of age and foam cell lesions develop as early as 8 weeks, and after 15 weeks advanced lesions (fibrous plaques) are observed. The latter consist of a fibrous cap containing smooth muscle cells surrounded by connective tissue matrix that cover a necrotic core with numerous foamy macrophages. These spontaneous lesions progress and can cause severe occlusion of the coronary artery ostium by 8 months, although without functional consequence (Figures 66 and 67) [485, 486].

In order to directly investigate the possible role of human CRP in atherogenesis *in vivo*, we have studied normal diet fed apoE knockout mice with and without

transgenic expression of human CRP as they spontaneously develop hypercholesterolemia and atherosclerosis.



Figure 65 View of the heart and proximal vessels in an apoE^{-/-} mouse (1 year old)

- this demonstrates the regions of interest in the mouse heart, aorta, and proximal branches (Courtesy of Dr M. Kahan, UCL)

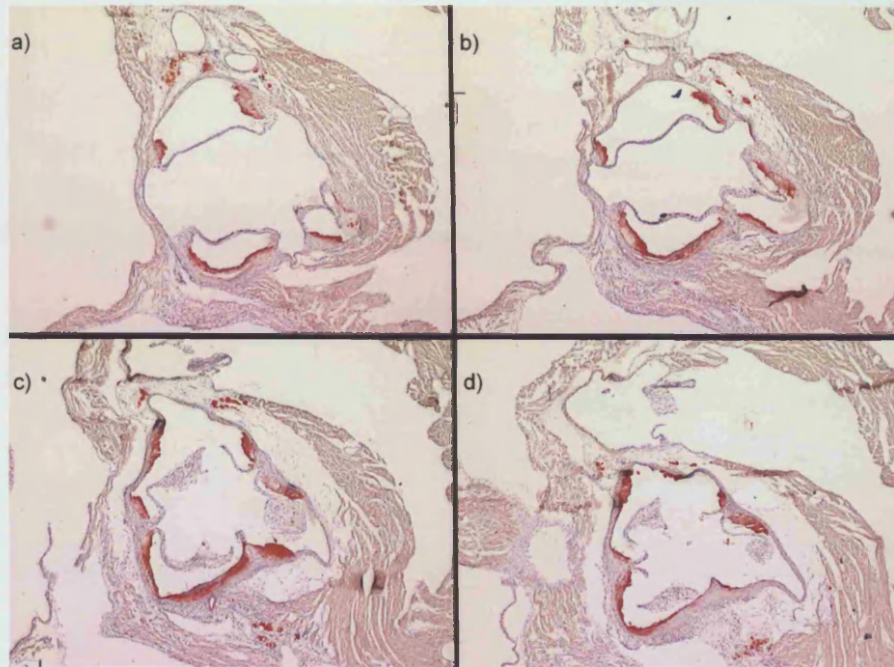


Figure 66 Typical Oil Red O stained lipid lesions in a 20 week old apoE^{-/-} mouse
 - 10 μM sections through the aortic sinus (a-d) as quantified in this study; Objective magnification x2.5

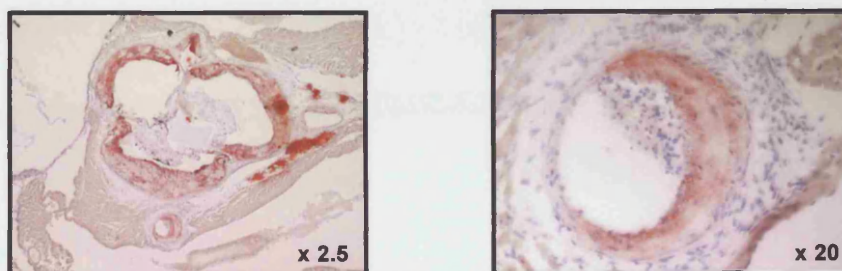


Figure 67 Advanced lesions of an apoE^{-/-} mouse at one year of age
 - note coronary artery involvement (right hand image); Oil Red O staining for lipid deposition;
 Objective magnification x 2.5 and x 20

12.5 Results

12.5.1 Measurement of human CRP in hyperlipidaemic serum

Matrix effects on CRP assay and recovery of isolated pure human CRP spiked into apoE^{+/+} and apoE^{-/-} sera were studied in parallel using the Dade-Behring BNII microparticle-enhanced turbidimetric immunoassay and the Roche MIRA method. The BNII and Roche MIRA assays are both specific for human CRP; neither detect anything significant in sera of mice that are not transgenic for human CRP.

The Spearman rank correlation coefficient for CRP determination of identical hyperlipidaemic murine serum samples (20-30 mg/l hCRP) on different occasions on the MIRA was 0.943 (P=0.005; n=6), with the absolute values of the second reading being 18.2% (SD 5.3%) higher than the first. When the same samples were measured both on the MIRA and the BNII the Spearman rank correlation between the two assays was 1.00 (P<0.0001; n=4), with the BNII giving absolute values 19.5% (SD 5.2%) lower than those of the MIRA. The two different CRP assays, therefore give consistently comparable results.

Moreover there was identical recovery of CRP when apoE^{-/-}-hCRP⁺ serum was diluted 1:4 in either lipaemic apoE^{-/-} serum or normal apoE^{+/+} mouse serum: the Spearman rank correlation coefficient (comparing dilution in lipaemic as opposed to normal serum) for both assays was 0.9 (P=0.037; n=5). Furthermore,

both assays showed similar recovery when isolated pure human CRP was spiked into normal human serum, typically hyperlipidaemic apoE^{-/-} mouse serum, or Tris buffered saline containing 4 g/l bovine serum albumin (Table 41).

Table 41 Analytical recovery of pure human CRP

- comparison of different assays (MIRA and BNII) and diluents

Spiked pure hCRP (mg/l)	Diluent	MIRA (mg/l)	BNII (mg/l)
0	Normal human serum*	1.44	1.01
5		5.66	6.04
10		10.47	11.5
0	apoE ^{-/-} serum	0.34	<0.16
5		5.2	4.5
10		10.24	10.4
0	4% TCB	0	<0.16
5		4.86	5.08
10		10.53	9.4

*CRP concentration 0.88mg/l

12.5.2 Circulating human CRP is not complexed and is cleared

normally in apoE^{-/-} mice

CRP can bind to phospholipids by virtue of its specific, calcium-dependent reactivity with phosphorylcholine residues. However, prior studies of acute-phase human serum by gel filtration and density gradient ultracentrifugation showed that human CRP is in a free, uncomplexed form, despite the coexistent

presence of the various classes of serum lipoproteins, all of which contain phospholipids [78, 111]. In contrast, when isolated CRP is aggregated by immobilisation at a sufficient density on a solid phase and then exposed to normal human serum, it selectively binds LDL and traces of VLDL [111]. Native human CRP in solution also forms complexes with the abnormal lipoprotein β -VLDL in serum of patients with type III hyperlipoproteinaemia as well as complexes in sera from individuals with type IV and type V hyperlipoproteinaemia [109].

Importantly when sera from apoE^{-/-}-hCRP⁺ mice and from apoE^{-/-} or apoE^{+/+} mice spiked with pure human CRP, were analyzed by size exclusion chromatography, the elution volume (12.65 ml) and profile of the human CRP were constant and completely distinct from the fractions containing cholesterol (elution volume 7.75 ml). There was thus no evidence for complex formation between human CRP and any lipoprotein or other macromolecular ligand in the lipaemic apoE knockout serum (Figures 68 and 69).

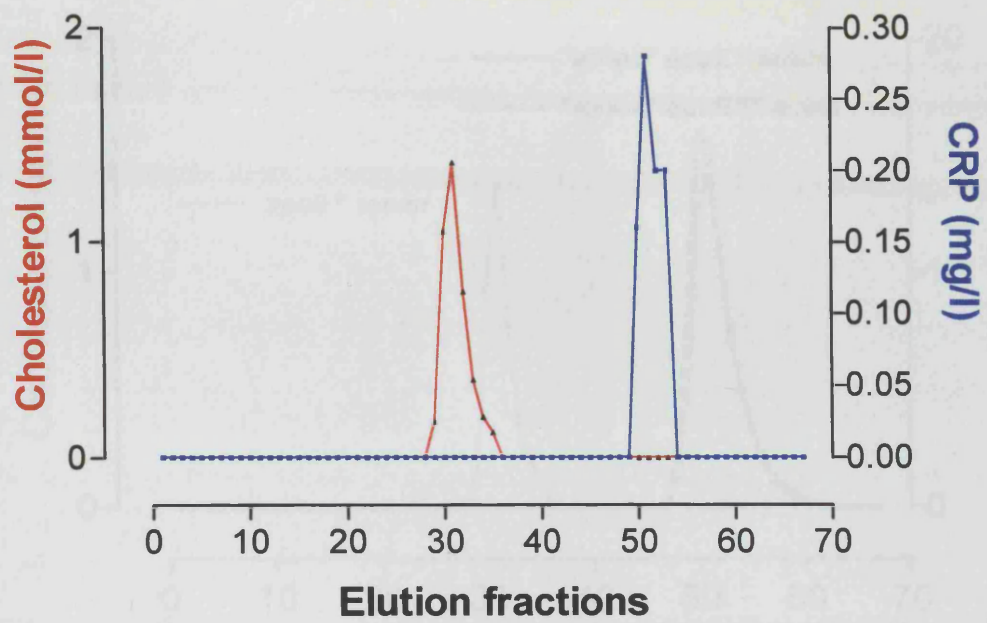


Figure 68 Gel filtration profile of *ex-vivo* apoE^{-/-}-hCRP⁺ serum

- demonstrating non-overlapping elution profiles for cholesterol and human CRP

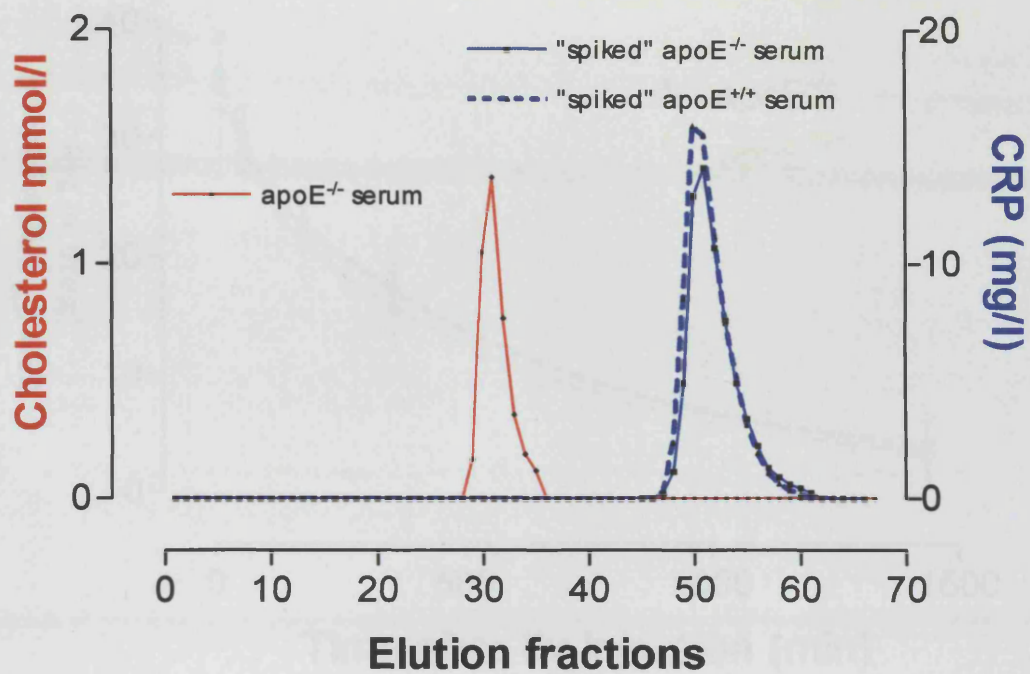


Figure 69 Gel filtration profiles of human CRP spiked apoE^{-/-} serum

- hCRP spiked to 200mg/l; demonstration of non-overlapping elution profiles for cholesterol and human CRP

In addition the plasma clearance of intra-venously injected trace radiolabelled human CRP was identical in apoE^{-/-}-hCRP⁺ and apoE^{+/+}-hCRP⁺ mice (Figure 70).

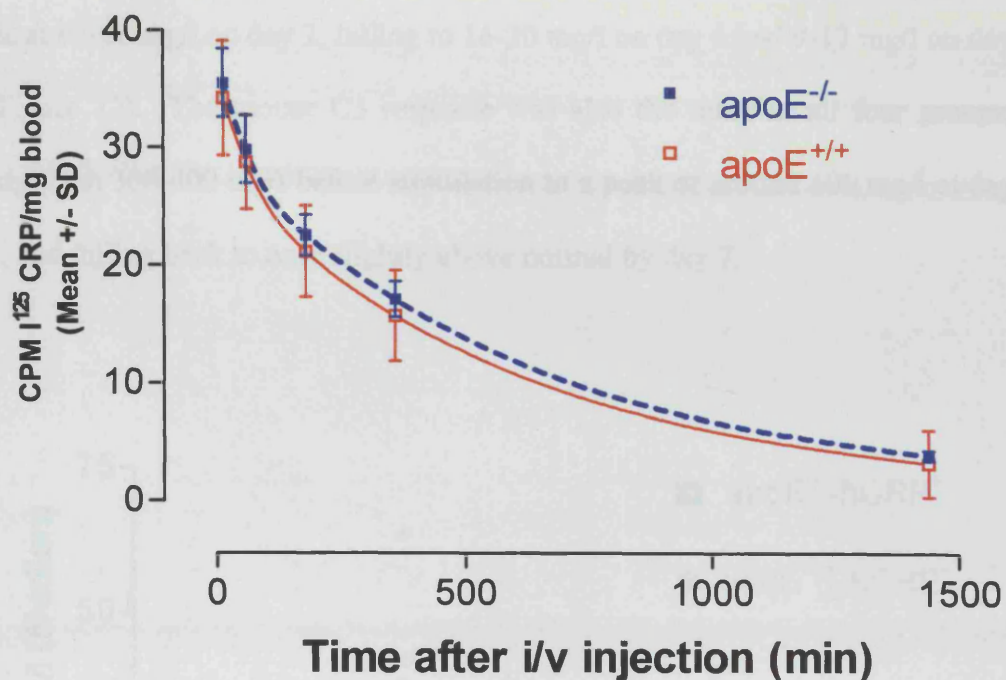


Figure 70 Plasma clearance of ^{125}I -hCRP in apoE^{-/-}-hCRP⁺ and apoE^{+/+}-hCRP⁺ mice

- each point represent the mean (SD), in 4 mice per group, of radioactivity remaining in the blood after intra-venous injection of 1 μg tracer at time 0

12.5.3 Acute phase response to inflammation in apoE^{-/-} and apoE^{+/+} mice

Following sub-cutaneous injection of casein, the peak acute phase concentration of transgenic human CRP at day 2 was significantly greater in apoE^{-/-} than apoE^{+/+} mice: median (range) 42 (41-63) mg/l, n=5, compared to 19 (7-24) mg/l, n=9, ($P < 0.01$). Values fell thereafter with no difference between the groups on days 4 and 7 (Figure 71). In contrast the acute phase response of mouse SAP was the same in these two groups as well as in apoE^{-/-} and apoE^{+/+} mice without

transgenic human CRP. Median SAP values rose from a baseline of 3-7 mg/l to peak at 67-86 mg/l on day 2, falling to 16-20 mg/l on day 4 and 9-13 mg/l on day 7 (Figure 72). The mouse C3 response was also the same in all four groups, rising from 300-400 mg/l before stimulation to a peak of around 600 mg/l at day 2-4, and falling back to only slightly above normal by day 7.

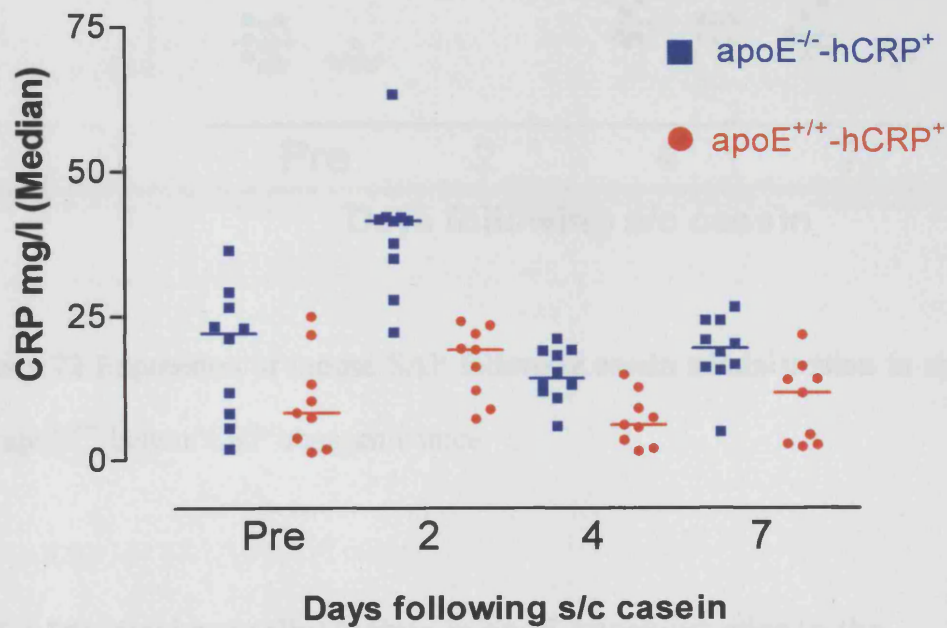


Figure 71 Expression of human CRP following casein administration in apoE^{-/-} and apoE^{+/+} human CRP transgenic mice

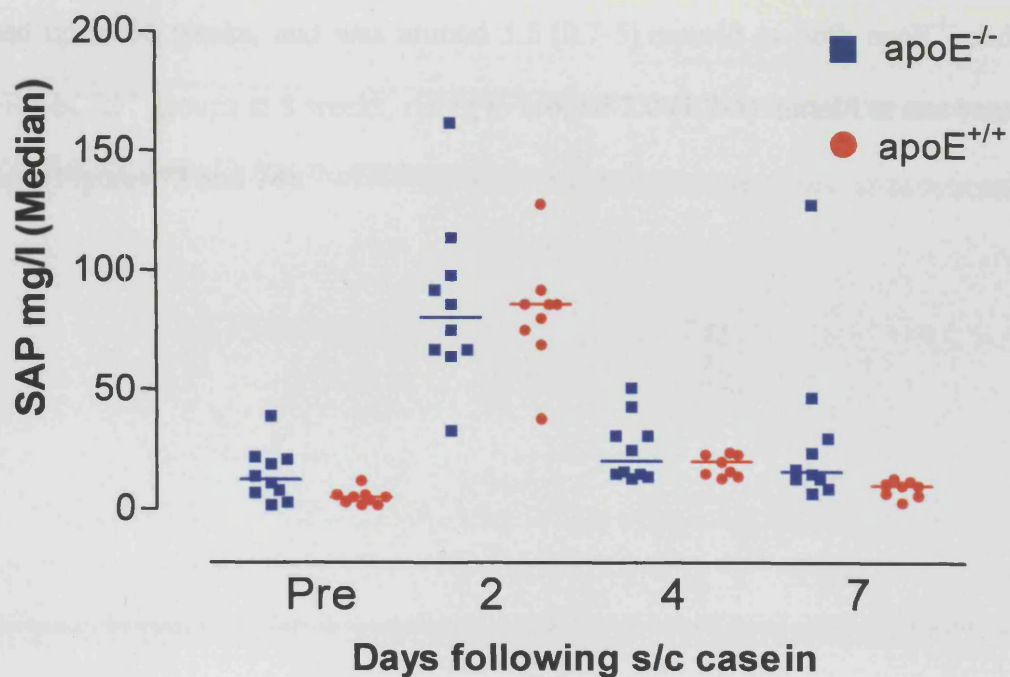


Figure 72 Expression of mouse SAP following casein administration in apoE^{-/-} and apoE^{+/+} human CRP transgenic mice

12.5.4 Identical hyperlipidaemia in apoE knockout mice in the presence and absence of transgenic human CRP expression

The apoE deficient mice developed the same characteristic progressive hypercholesterolemia and hypertriglyceridaemia, irrespective of whether they were transgenic for human CRP or not. The median (range) serum cholesterol concentration was around 2.7 (1.9-3.5) mmol/l in the apoE^{+/+} and apoE^{+/+}-hCRP⁻ mice at all ages tested up to 56 weeks, and was around 11 (7-17) mmol/l in both apoE^{-/-} and apoE^{-/-}-hCRP⁺ groups at 8 weeks, rising to around 17 (9-21) mmol/l at one year of age. The median (range) serum triglyceride concentration was

around 1.0 (0.5-1.5) mmol/l in the apoE^{+/+} and apoE^{+/+}-hCRP⁺ mice at all ages tested up to 56 weeks, and was around 1.5 (0.7-5) mmol/l in both apoE^{-/-} and apoE^{-/-}-hCRP⁺ groups at 8 weeks, rising to around 2.0 (1.2-5) mmol/l at one year of age (Figures 73 and 74).

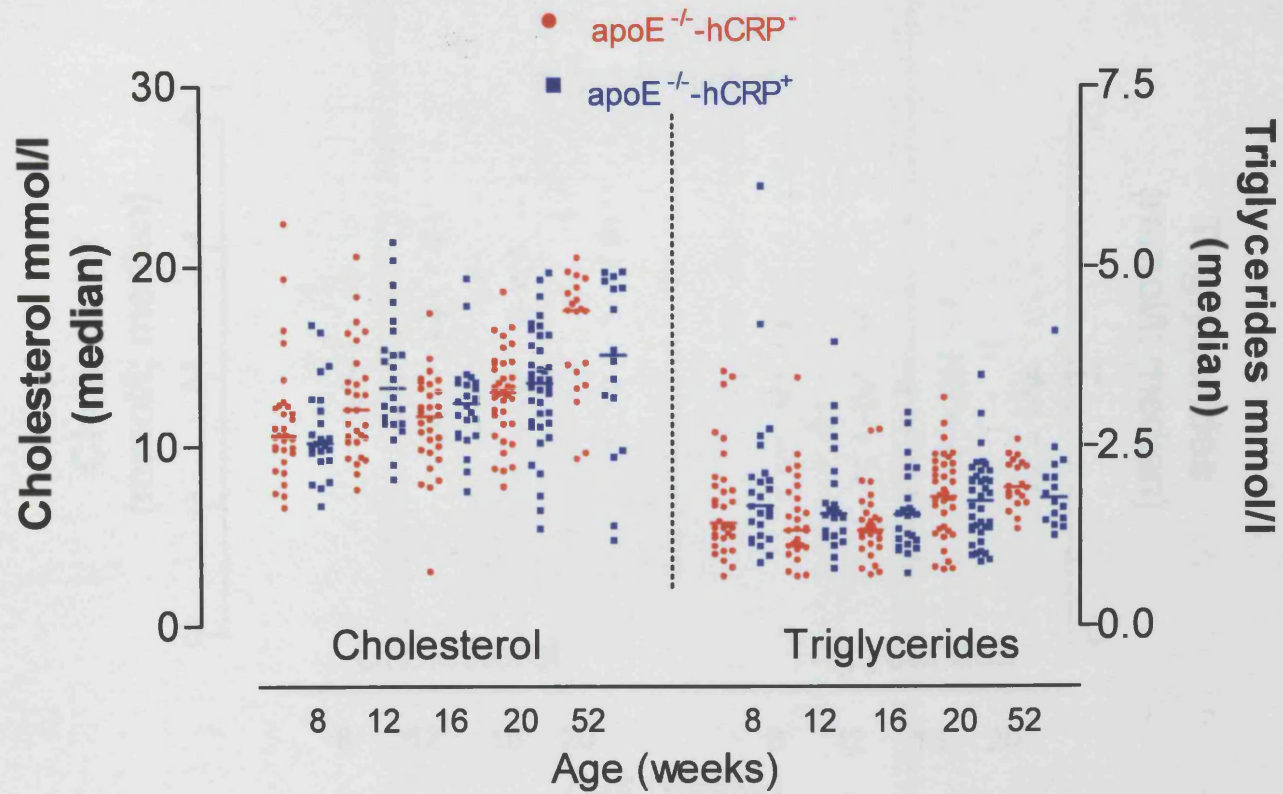


Figure 73 Changes in serum cholesterol and triglycerides in apoE^{-/-} mice with and without the human CRP transgene

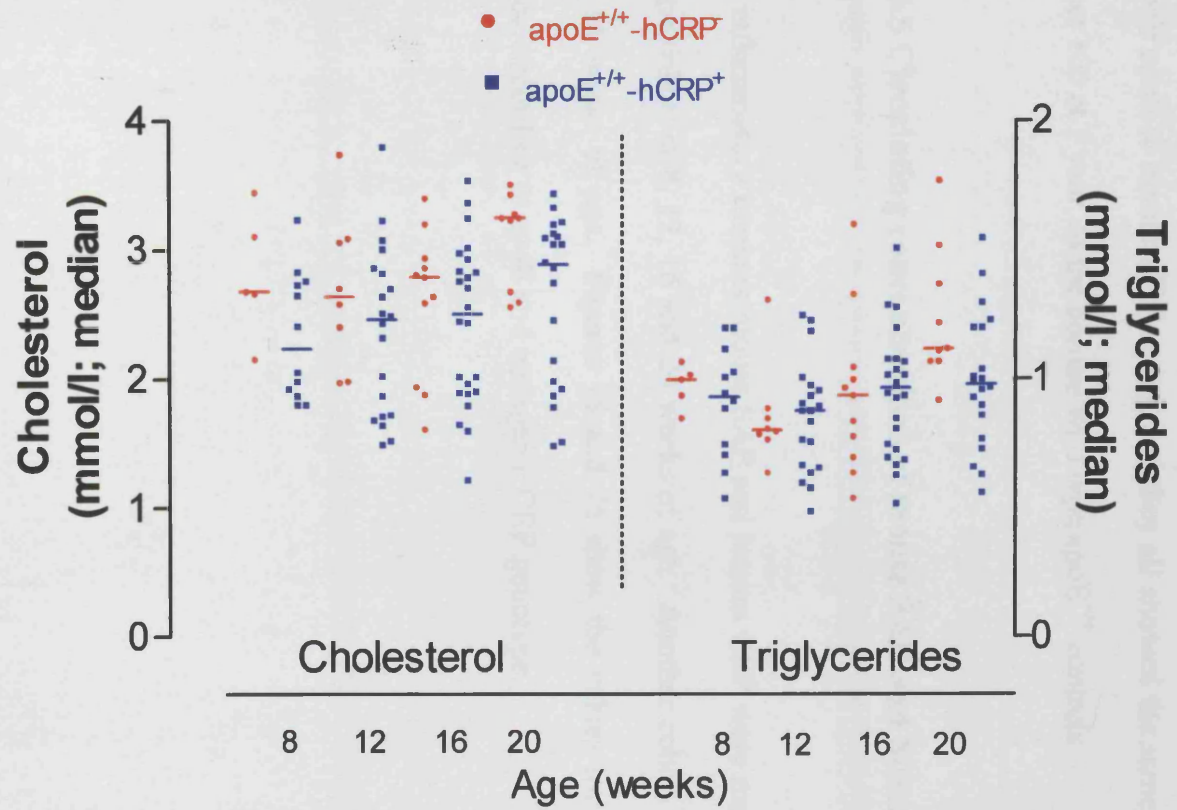


Figure 74 Changes in serum cholesterol and triglycerides in apoE^{+/+} mice with and without the human CRP transgene

There was no difference in weight between groups with and without transgenic human CRP, for example at one year the mean (SD) weights were: apoE^{-/-}-hCRP⁺, 35.2 (1.8) g and apoE^{-/-}, 34.9 (3.0) g, n=16 in each group, while all apoE^{+/+}-hCRP⁺ (n=3) and apoE^{+/+} mice (n=6) weighed 33.0 g at this age. There was also no difference in morbidity between any of the groups, regardless of their apoE or human CRP status, and they all showed the same low mortality, <5 per 100 at 1 year, as the normal wild type apoE^{+/+} controls.

12.5.5 Circulating concentrations of mouse SAP and human CRP

The inflammatory markers mouse SAP and human CRP were measured serially in one cohort at 8, 12, 16 and 20 weeks of age. Another cohort was bled at 52 and 56 weeks of age. Figures 75 and 76 show the values for SAP for these bleeds, according to apoE and transgenic CRP genotype.

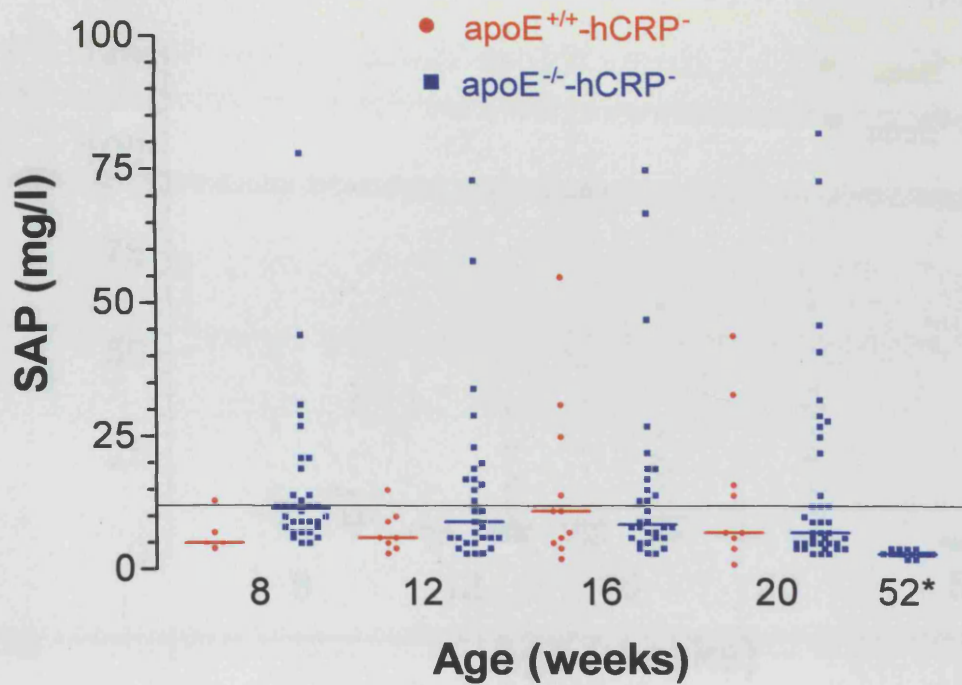


Figure 75 Mouse SAP concentrations in apoE^{+/+} and apoE^{-/-} mice, wildtype for transgenic human CRP

- median values indicated; broken line represents values >12mg/l; * separate cohort

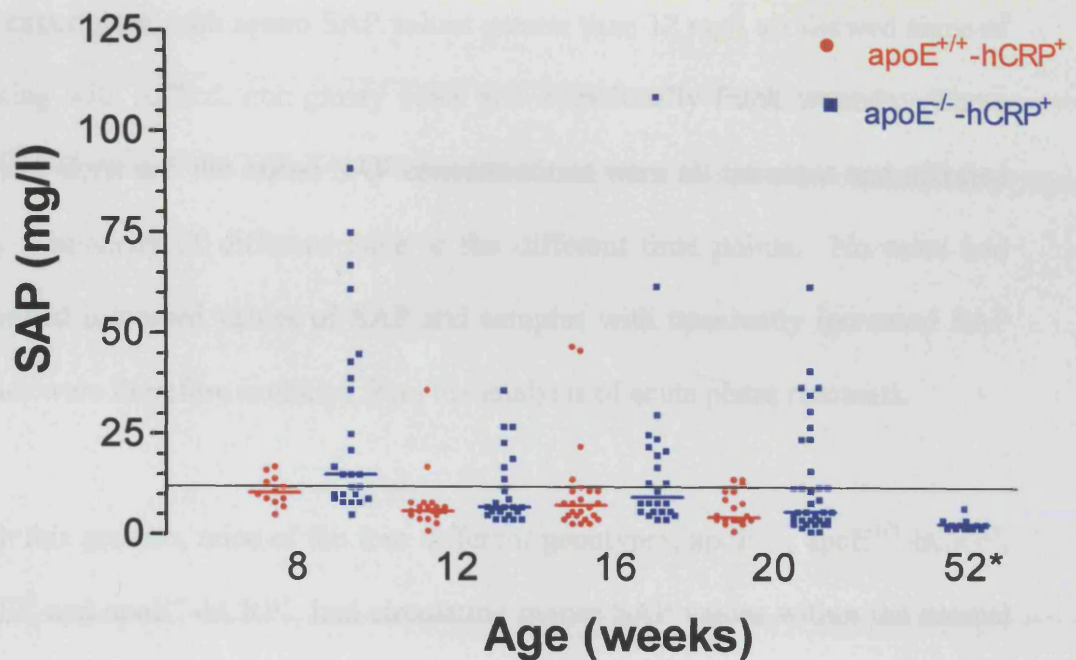


Figure 76 Mouse SAP concentrations in apoE^{+/+} and apoE^{-/-} mice expressing transgenic human CRP

- median values indicated; broken line represents values >12mg/l; * separate cohort

Male C57BL/6 mice have an unavoidable tendency to fight with each other [487], leading to minor injuries and inflammation and thus to acute phase responses that may complicate assessment of inflammation related to development of atherosclerosis in the apoE^{-/-} mice. However, in contrast to its human homologue, which is a stable constitutive plasma protein, mouse SAP is an exquisitely sensitive acute phase reactant, as responsive to bacterial lipopolysaccharides as the pyrogen test [488]. In this regard we have used mouse SAP to control for systemic evidence of inflammation.

Baseline values in healthy normal C57BL/6 animals are 2-10 mg/l, and mice, in this experiment, with serum SAP values greater than 12 mg/l all showed signs of fighting with ruffled, non-glossy coats and occasionally frank wounds. These clinical signs and the raised SAP concentrations were all transient and affected only a minority of different mice at the different time points. No mice had sustained increased values of SAP and samples with transiently increased SAP values were therefore excluded from the analysis of acute phase reactants.

With this proviso, mice of the four different genotypes, apoE^{+/+}, apoE^{+/+}-hCRP⁺, apoE^{-/-} and apoE^{-/-}-hCRP⁺, had circulating mouse SAP values within the normal range at all time points between 8 and 52 weeks (Table 42).

Table 42 No increase in mouse SAP production in apoE^{-/-} mice

- intercurrent inflammation is accounted for by exclusion of mice in whom mouse SAP values were greater than 12mg/l

Genotype	apoE ^{+/+}	apoE ^{+/+} -hCRP ⁺	apoE ^{-/-}	apoE ^{-/-} -hCRP ⁺
Age	mSAP mg/l (median; range)			
8 wk	5 (4-7) n=4	9 (5-12) n=8	9 (5-12) n=21	9 (5-12) n=11
12 wk	6 (3-10) n=7	6 (1-8) n=18	6(3-11) n=17	6 (4-11) n=17
16 wk	6 (2-11) n=7	6 (3-12) n=20	6.5 (3-11) n=18	6 (4-11) n=13
20 wk	6 (1-7) n=5	4 (1-11) n=17	5 (3-12) n=27	4.5 (1-12) n=28
52 wk *	ND	ND	3 (2-4) n=16	3 (2-7) n=13

* Separate cohort

None of the 16 apoE^{-/-}-hCRP⁺ animals that were killed at 56 weeks had SAP concentrations outside the normal range (Median 3 mg/l, range 2-7; n=16). Mouse SAP assays thus provided no evidence of a systemic inflammatory response associated with either hyperlipidaemia or the development and progression of atherosclerosis in apoE^{-/-} mice, regardless of whether they expressed transgenic human CRP or not.

12.5.6 Increased circulating concentrations of human CRP in apoE^{-/-} mice

The known widely variable baseline circulating CRP concentration among male human CRP transgenic mice, was observed here in both the apoE^{-/-} and apoE^{+/+} background, even after exclusion from the analysis of samples containing acute phase values of mouse SAP. However, from 12 weeks onwards, the CRP values were consistently higher in the apoE^{-/-}-hCRP⁺ mice compared to the apoE^{+/+}-hCRP⁺ controls, but did not change significantly after that time (Figure 77 and Table 43). CRP values were not surprisingly correlated reasonably closely with mouse SAP values (Figure 78; r² values consistently between 0.4 and 0.8) and there was a suggestion of a correlation with increasing hypercholesterolaemia and age (Figures 79 and 80; r² values of 0.5-0.9). However the absolute number of mice studied, the difficulty of controlling for extraneous sources of inflammation, as well as the likelihood of numerous confounders mean that further statistical analysis of any such interaction cannot yield rigorous information.

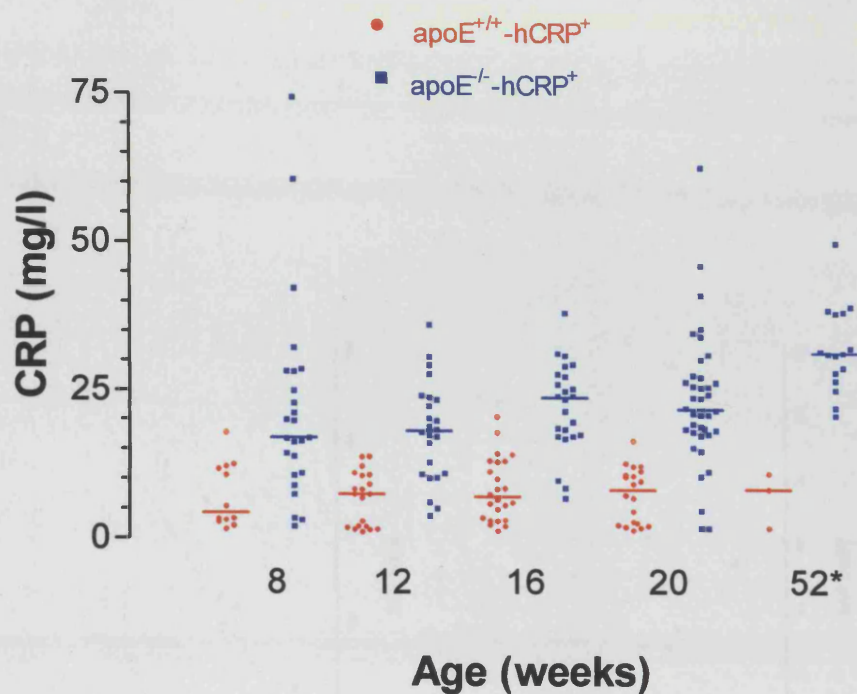


Figure 77 Transgenic human CRP expression is increased in apoE^{-/-} mice
 - * separate cohort

Table 43 Transgenic expression of human CRP is increased in apoE^{-/-} mice
 - even when intercurrent inflammation is accounted for by exclusion of mice in whom mouse SAP values were greater than 12mg/l

Genotype	apoE ^{+/+} -hCRP ⁺	apoE ^{-/-} -hCRP ⁺
Age	hCRP mg/l (median; range)	
8 wk	11.1 (3.0-17.7) n=8	10.9 (2.0-22.8) n=11
12 wk	7.8 (1.1-13.7) n=18	17.1 (3.8-36.1) n=17, <i>P</i> <0.005
16 wk	6.2 (1.2-14.2) n=20	24.0 (9.8-31.2) n=13, <i>P</i> <0.0001
20 wk	9.1 (1.3-12.6) n=17	20.9 (1.8-41.1) n=28, <i>P</i> <0.0001
52 wk *	8.27 (1.7-10.9) n=3	31.4 (20.8-50.0) n=16, <i>P</i> <0.0001

* separate cohort

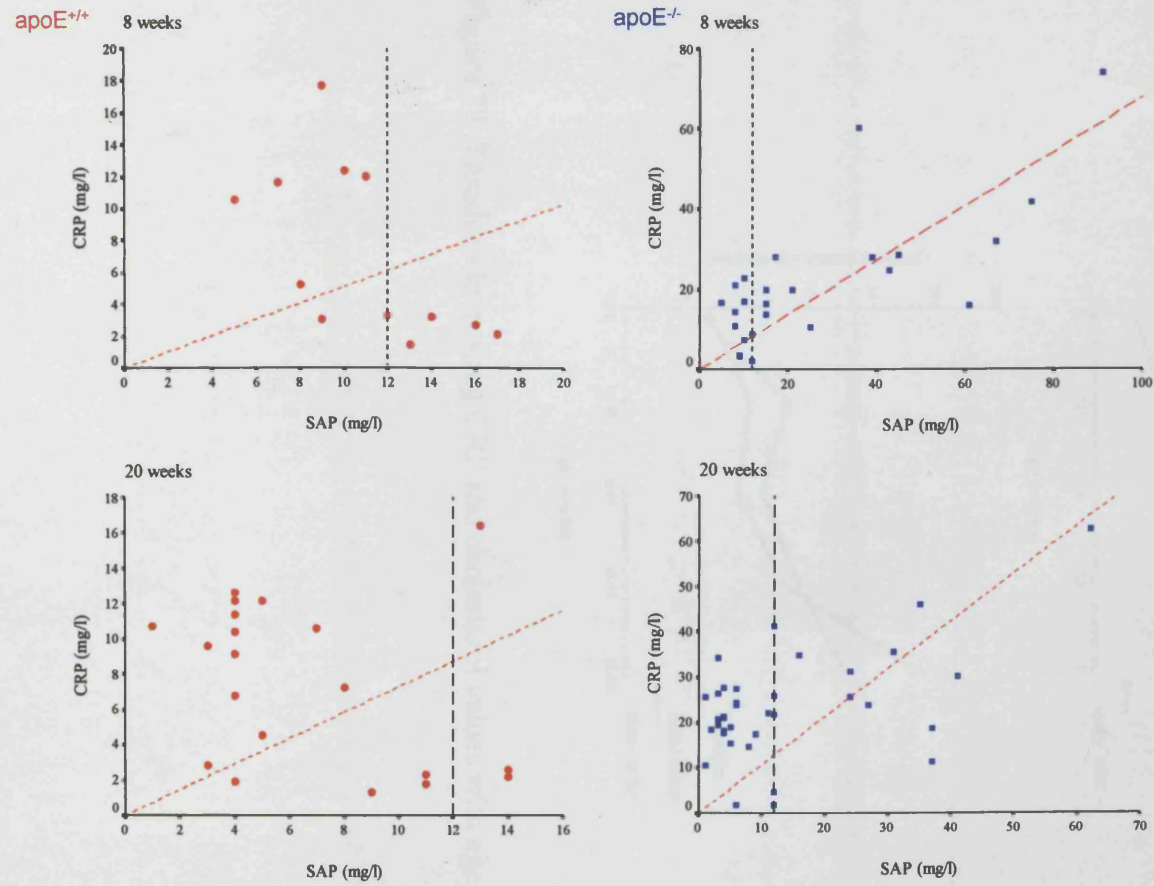


Figure 78 Correlation between human CRP and mouse SAP production
 - red line represents linear regression fit; black line indicates 12mg/l mouse SAP

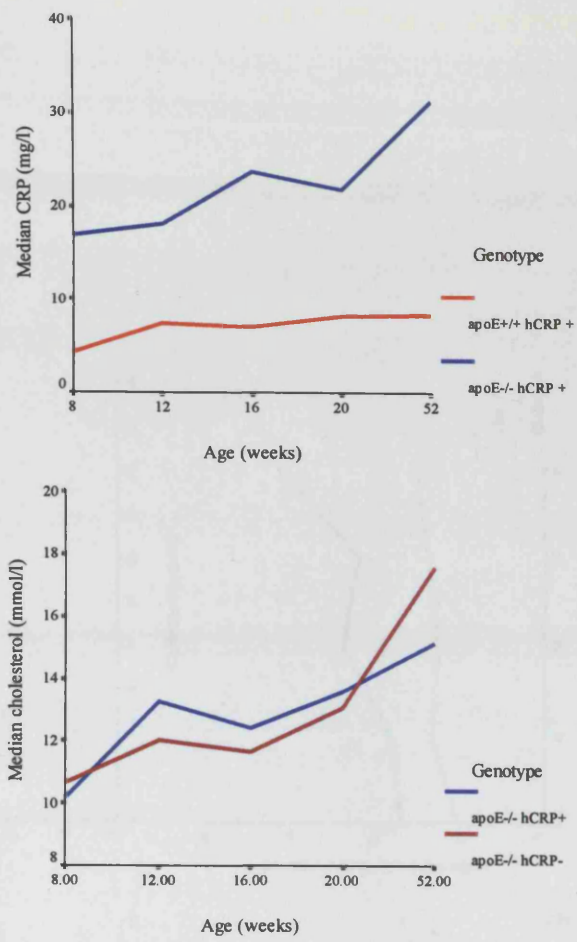


Figure 79 Trends to increasing CRP and cholesterol values with age

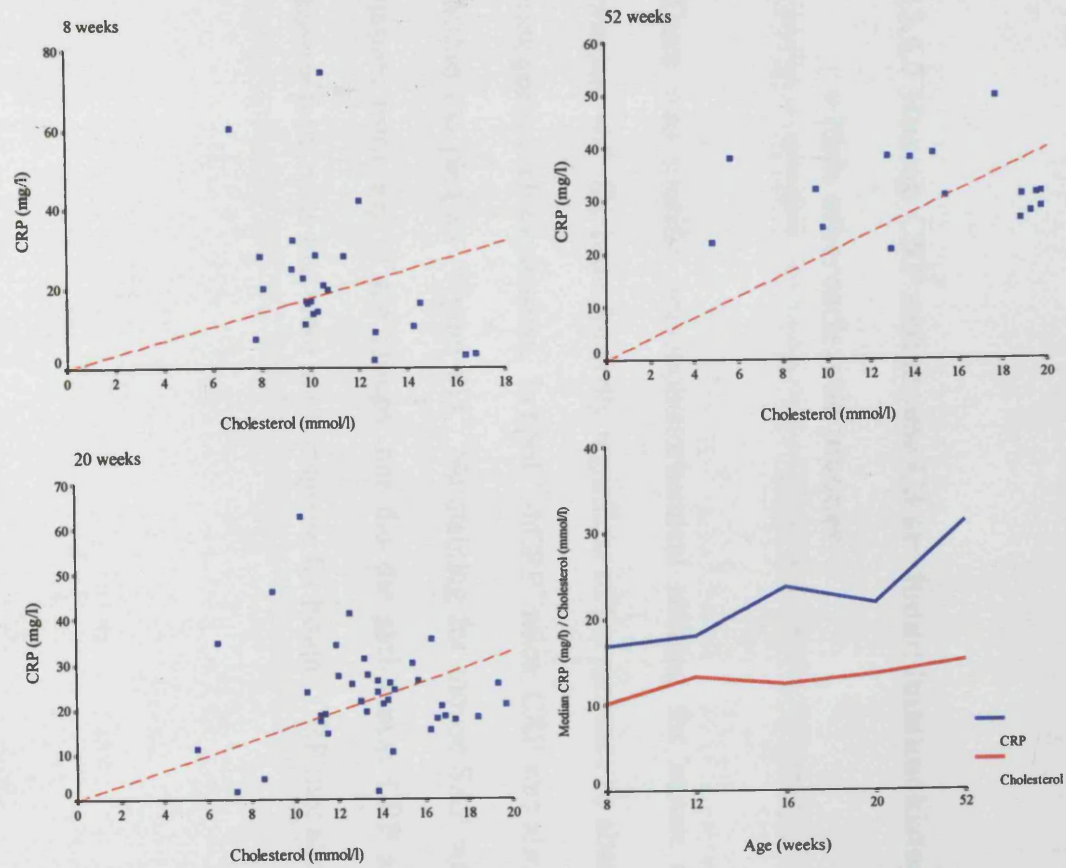


Figure 80 Crude correlation between CRP expression and hypercholesterolaemia in $apoE^{-/-}hCRP^{+}$ mice

- broken red line represents linear regression fit

These differences in CRP values between the apoE^{-/-} and apoE^{+/+} groups were not due to interference in the CRP immunoassay by the progressive hypercholesterolemia of the apoE^{-/-} mice, as shown above and similarly circulating human CRP is not complexed and is cleared normally in apoE^{-/-} mice. Thus the higher CRP concentrations in the apoE^{-/-}-hCRP⁺ mice appear to reflect increased CRP synthesis.

12.5.7 Human CRP and mouse C3 are found immunohistochemically within atherosclerotic plaques

There was specific immunohistochemical staining for mouse C3 in all the plaques, with the same intensity regardless of the presence or absence of human transgenic CRP expression. In apoE^{-/-}-hCRP⁺ mice, CRP was also demonstrable within the plaques (Figure 81). No staining for mouse SAP was observed in tissues from any of the groups, nor did the anti-human CRP antiserum stain tissues from mice that were not transgenic for human CRP (not shown).

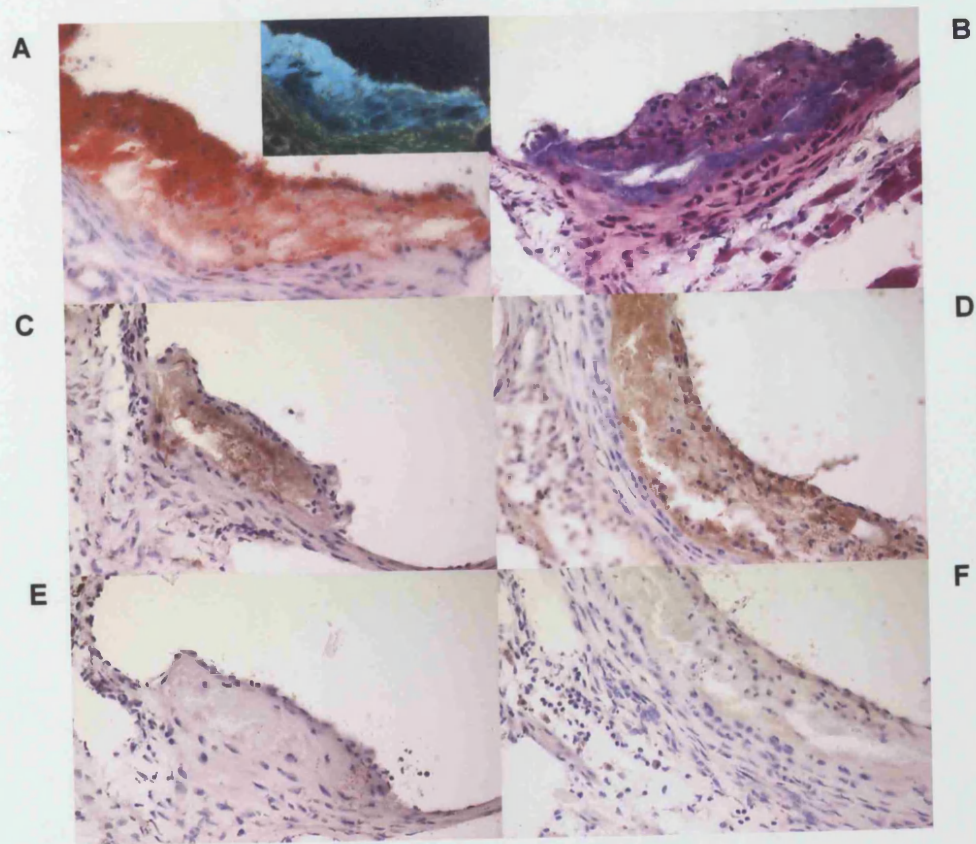


Figure 81 Typical atherosclerotic plaques in the aortic sinus at 20 weeks

A, oil red O stain (inset, polarised light); *B*, haematoxylin and eosin stain; *C*, immunostain with anti-human CRP antibody; *D*, immunostain with anti-mouse C3 antibody; *E*, immunostain with anti-human CRP antibody preabsorbed with immobilised pure human CRP, showing complete absence of staining observed in *C*; *F*, anti-mouse C3 antibody preabsorbed with immobilised mouse C3, showing complete absence of staining observed in *D*. Original magnification x320.

There was also no difference seen morphologically in the lesions between animals expressing human CRP and those wildtype for the transgene, with typically advanced lesions comprising a necrotic core (with cholesterol clefts) surrounded by proliferating smooth muscle cells and varying amounts of extracellular matrix (fibrous cap), including collagen and elastin (Figures 82 and 83).

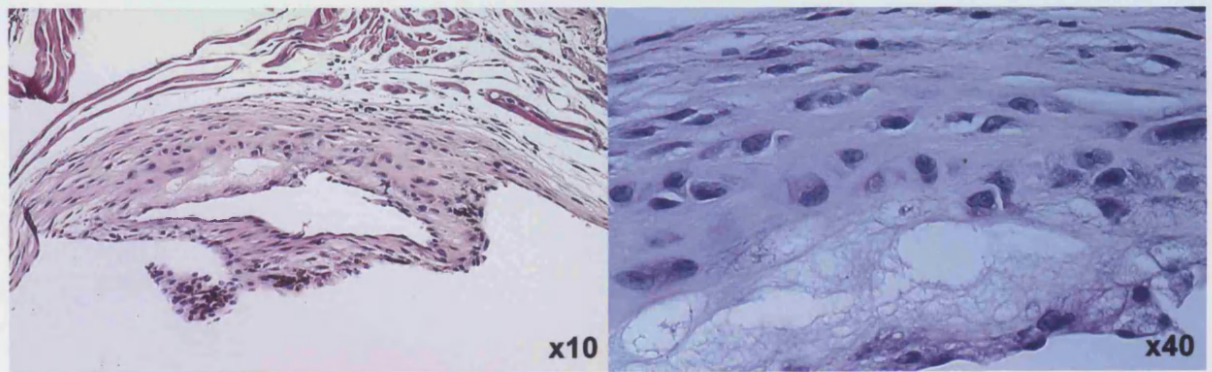


Figure 82 Low and high power views of a typical lipid rich lesion

- Haematoxylin and eosin; Objective magnification x 10 and x 40

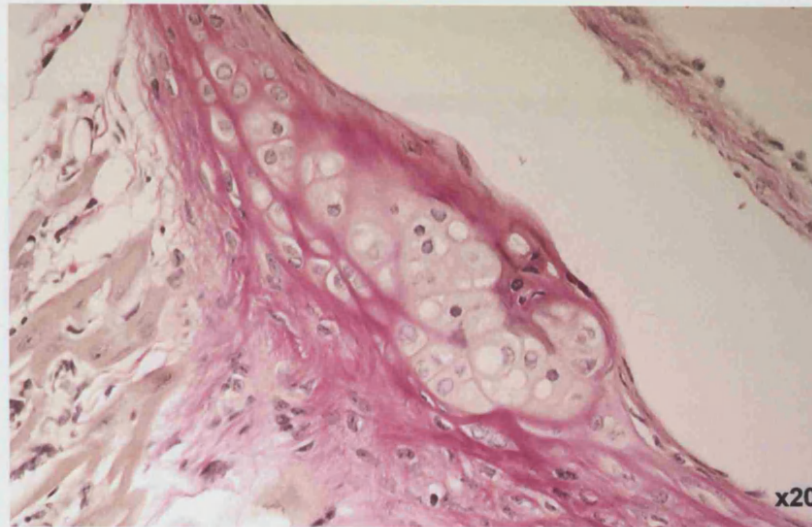


Figure 83 Verhoeff Van-Gieson staining of elastin in a typical lesion

- Objective magnification x 20

12.5.8 Identical aortic atherosclerotic plaque size in apoE knockout mice in the presence and absence of transgenic human CRP expression

Three separate cohorts of mice were killed at 12, 20 and 56 weeks of age respectively, for estimation of the extent of atherosclerosis in the aortic sinus. Wild type animals had no atheroma, regardless of the presence or absence of transgenic human CRP (not shown). All the apoE deficient mice had substantial atherosclerotic plaques which increased in size with time, but there was no

difference at any time point between the groups with and without transgenic human CRP (Figure 84). All animals in each group are included in this analysis, but exclusion of those that were mounting an acute phase response of SAP, indicative of intercurrent pathology as discussed, did not affect the result at any of the time points tested.

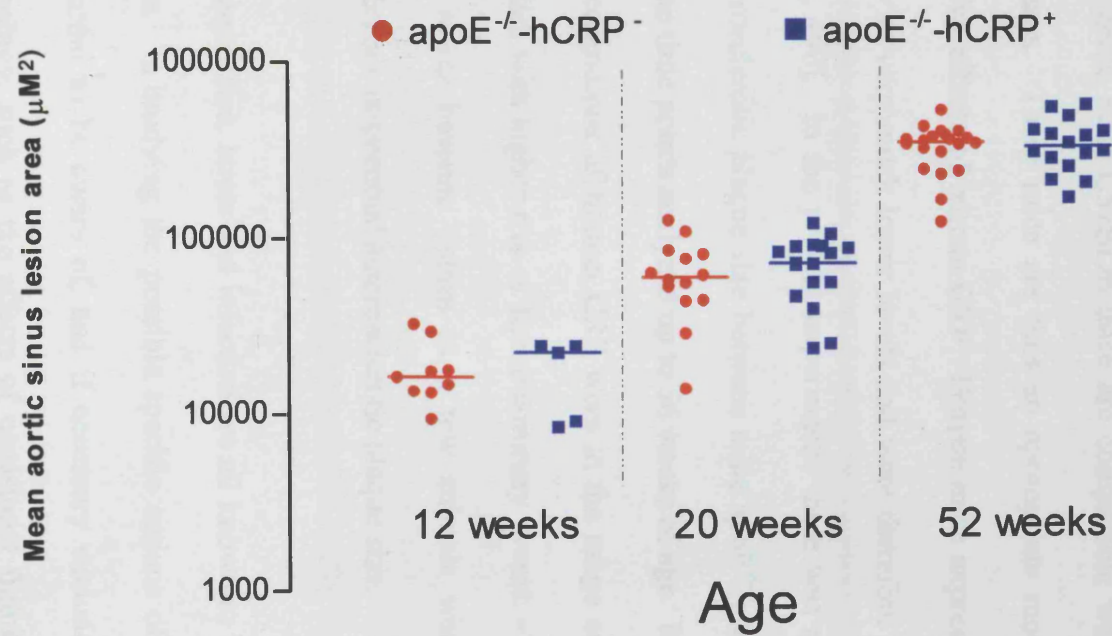


Figure 84 Atherosclerotic lesion area within the aortic sinus of apoE^{-/-}-hCRP⁻ and apoE^{-/-}-hCRP⁺ mice

- lesion size (mean area plotted for each mouse studied) increased progressively with age but there was no significant difference between the animals with and without human CRP at any point. Horizontal lines represent medians.

12.6 Discussion

Mouse CRP is a trace plasma protein, the concentration of which does not exceed 1-2 mg/l even at the peak of the acute phase response [72, 489], whereas the plasma concentration and acute phase behaviour of human CRP in human CRP transgenic male C57BL/6 mice are comparable with these features of CRP in humans. These mice are thus an appropriate model for investigation of the *in vivo* effects of human CRP. Female mice express the human CRP transgene only at very much lower levels and were therefore not studied here [350, 351, 427, 490]. In the present experiments there was no difference in aortic sinus atherosclerotic plaque size between male apoE^{-/-} and apoE^{-/-}-hCRP⁺ mice at any of the time points analyzed up to 56 weeks of age. In most mice the initial serum concentrations of human CRP were in the range associated in epidemiological studies with higher risk of future coronary events, ~2.5 mg/l or more (Table 43), but lower baseline values in a few animals were not associated with any difference in eventual atherosclerotic plaque size.

Inflammation, stress and infection are all known to be pro-atherogenic in apoE^{-/-} mice. In studying the possible specific actions of human CRP it is therefore essential to be aware of, and if necessary exclude, intercurrent inflammatory pathology, such as the effects of occasional fighting that is inevitable among male C57BL/6 mice, or subclinical intercurrent infections that may occur in mouse facilities. Measurements of mouse SAP, which is an exquisitely sensitive acute phase reactant, are uniquely valuable in this respect. However, the minor transient acute phase responses caused by fighting in some mice in the present

study had no effect on atherogenesis. Also, very notably, the development of atherosclerosis in the apoE^{-/-} mice proceeded without sufficient inflammation to be reflected systemically, even by mouse SAP.

Nevertheless at 12 weeks of age the concentrations of human CRP were generally higher in apoE^{-/-}-hCRP⁺ than in apoE^{+/+}-hCRP⁺ mice, although they did not change significantly thereafter. We excluded the possibility that the higher CRP values were an assay artefact related to measurement by optical techniques in lipaemic serum. We also found no evidence for significant binding of human CRP to the abnormal murine lipoproteins in apoE^{-/-} serum. The plasma clearance of intravenously injected human CRP was identical in apoE^{-/-} and apoE^{+/+} animals, so the higher serum CRP values must reflect increased transgenic human CRP production in the apoE^{-/-} mice. Expression of this human CRP transgene in the mouse is under strong sex hormone control, and it was therefore notable that when testosterone and oestradiol were measured later for us by Dr S.J. Whiting and Dr G.L. Jones (Clinical Chemistry Department, Royal Free Hospital), there was a marked reduction in circulating oestradiol concentration in apoE^{-/-} mice compared to apoE^{+/+} controls at around one year of age. Insufficient serum was available from earlier time points to assay these hormones, but oestrogen/testosterone imbalance related to apoE deficiency and/or its metabolic consequences, such as hypercholesterolaemia, is a possible explanation of the raised human CRP values (Table 44). Measurement of murine SAA, not performed in these experiments, might also help in further understanding the differences noted for CRP and SAP.

Table 44 Serum concentrations of oestradiol and testosterone

- at one year in apoE^{-/-} and apoE^{+/+} mice with and without transgenic human CRP [491]

	apoE ^{+/+} (n=13)	apoE ^{+/+} -hCRP ⁺ (n=18)	apoE ^{-/-} (n=19)	apoE ^{-/-} -hCRP ⁺ (n=15)	<i>P</i> *
Testosterone (nmol/l)					
Median (range)	1.6 (0.6-151.2)	2.4 (0.6-116.8)	1.6 (0.2-79.2)	2.6 (0.2-49.2)	0.34
Oestradiol [‡] (pmol/l)					
Median (range)	486 (324-664)	471 (344-720)	102 (49-300)	99 (99-268)	0.0001
n (%) undetectable values	0	0	9 (47.4)	11 (73.3)	0.0001

*Kruskal-Wallis test

[‡]Oestradiol assay had sensitivity of 50 pmol/l. Serum samples were of limited volume and had to be diluted 1:1 or 1:2 for measurement. Values for samples that were undetectable at these dilutions are replaced by the cut-off multiplied by the dilution minus 1 (e.g. <50 when diluted at 1:2 is replaced by 99, when diluted at 1:1 it is replaced by 49). The *P* values obtained by non-parametric analysis are not significantly changed by using a different replacement value.

In addition, there may be different regulation of the human transgene compared to autologous murine SAP and C3, which showed no increased production. It is known, for example, that the transgene is aberrantly expressed in this model, with abundant CRP production in the kidney and other tissues, whilst CRP in humans is produced in significant amounts only by the liver. Baseline CRP concentration in humans shows a strong positive relationship with obesity, and although at one year the weights of the apoE^{-/-} mice were only about 6% greater than those of the apoE^{+/+} animals, a difference that was not statistically significant, there may perhaps be differences in their adipose tissue. In particular

macrophage accumulation and pro-inflammatory cytokine production are more marked in obese adipose tissue, which might be more extensive in apoE^{-/-} mice, and may conceivably be reflected more sensitively by human CRP than mouse SAP.

The presence of human CRP within the atherosclerotic plaques of the human CRP transgenic apoE^{-/-} mice, and of mouse C3 in the plaques in all apoE^{-/-} animals, is not informative about whether these proteins are having either beneficial or harmful local effects. Although our findings provide no evidence for a pro-atherogenic role of human CRP *in vivo*, it is essential to recognise that the transgenic human CRP is in a murine rather than human environment of lipoproteins, complement, extracellular matrix, tissue and inflammatory cells, cytokines, mediators, and cellular receptors. For example, the lipoproteins that are pivotal in pathogenesis of atherosclerosis differ greatly between mouse and human, as do their respective complement systems. We have confirmed that human CRP complexed with its highest affinity ligand, C-polysaccharide, does activate C3 in mouse serum (see Figure 108, Chapter 13), but the effects of human CRP binding to potentially important endogenous murine ligands, such as lipoproteins, apoptotic cells, and cellular debris, are not known. Also mouse complement C5-9 has much lower activity than the human terminal sequence, and is not required for atherogenesis in apoE knockout mice. Therefore any extrapolation to human pathophysiology from this or similar models must be guarded.

Using frozen sections specifically cut and stored for future work, Dr G. Tennent and Professor A. Dhillon (UCL) have gone on to immunostain for Mac-3 (a mouse macrophage differentiation antigen) and VCAM-1 (vascular cell adhesion molecule-1) within the atherosclerotic lesions (Figure 85). The staining increased progressively in intensity from 12 to 20 to 56 weeks in both groups of apoE^{-/-} mice, but was not different between apoE^{-/-} and apoE^{-/-}-hCRP⁺ animals, confirming the initial observation of no difference in lesion morphology secondary to transgenic human CRP production.

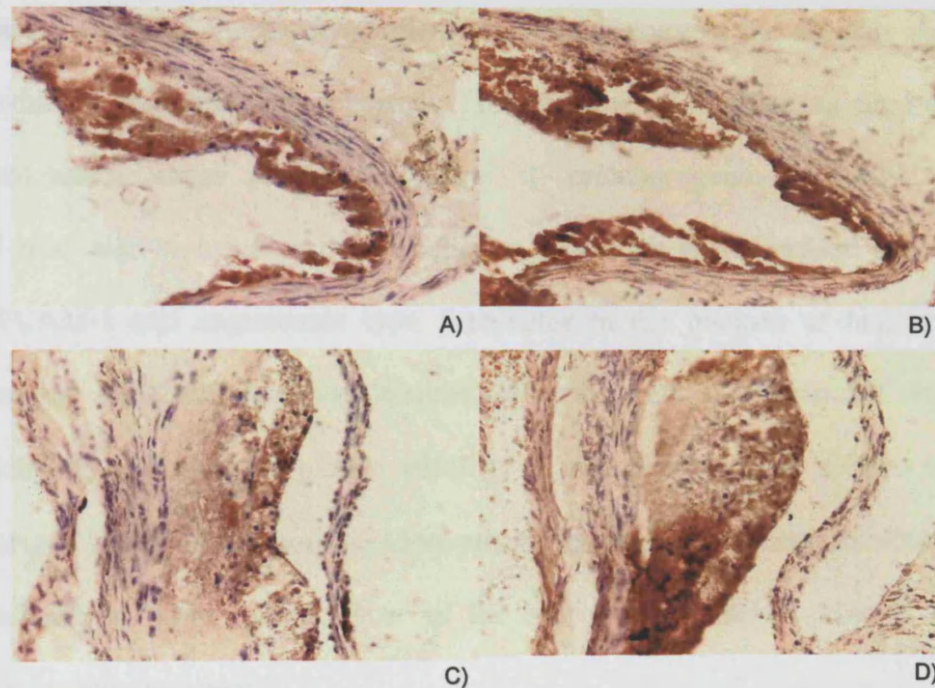


Figure 85 Intimal macrophages (Mac-3) and expression of VCAM-1

- aortic atheromatous lesions in 20 week old apoE^{-/-} mice with (C/D) or without (A/B) transgenic human CRP; x20 Objective; A) and C) Mac-3; B and D) VCAM-1 (Courtesy of Dr G. Tennent).

A recent study, using essentially the same model as here, concluded that human CRP accelerated progression of atherosclerosis in apoE^{-/-} mice [436]. Female

and male animals were used and some received turpentine injections to induce inflammation and acute phase responses. Difference in lesion size was observed only in males, and only at the end of the study, at 29 weeks. In the unstimulated mice the difference was of marginal statistical significance that could be abolished by elimination of a single outlier. The difference in the turpentine treated group cannot be ascribed specifically to human CRP as turpentine is a major non-specific inflammatory stimulus. Also the baseline and acute phase human CRP concentrations reported in male mice by Paul *et al.* are extraordinarily high (100-500 mg/l), suggesting, if the values are correct, the presence of active intercurrent inflammatory pathology in the animals studied, for which no controls were reported. This is important because, as cited above, inflammation, stress and infection are all proatherogenic in apoE^{-/-} mice. Paul *et al.* also reported increased collagen deposition and increased abundance of VCAM-1 and angiotensin type 1 receptor in the plaques of human CRP transgenic mice, and decreased plasma C3 concentration. In contrast we have detected no differences in plaque histology or morphology in our cohorts and no difference in C3 values; nor was there any difference in presence, distribution or abundance of either VCAM-1 or of the macrophage marker, Mac-3, in the lesions at any time point.

In contrast to the above study, two other independent groups have found results similar to ours. Reifenberg *et al.* have studied transgenic rabbit CRP production in apoE knockout mice and show no effect on lesion development [492] whilst Trion *et al.* [493] found no effect of human CRP on early lesion development in apoE*3-Leiden/hCRP transgenic mice.

One group has now reported the effects of passively administered human CRP in apoE knockout mice [127]. They compared native and modified CRP administration, using saline controls and claim that modified CRP and native CRP have opposite effects on atherosclerosis in apoE^{-/-} mice: native CRP increased whereas modified CRP reduced atherosclerosis. The study lacks appropriate controls, administers CRP weekly (the half life of human CRP in the mouse is 4 hours [422]) and attempts to study modified CRP, which as already discussed, is not established widely as having a physiological role. Regular administration of a foreign protein in this way may also of course generate an antibody response that could interfere significantly with any findings. The results reported cannot therefore, as claimed by the authors, explain the conflicting reports for CRP in models of atherogenesis.

Clinical evidence about an association between CRP values and the necessarily indirect surrogate indices of overall burden of atherosclerosis in humans is conflicting. The significant epidemiological association of increased baseline circulating CRP concentration is with atherothrombotic events, rather than with the underlying atherosclerosis. Also, CRP values are not increased, even in patients with severe coronary atherosclerosis, when they experience variant angina caused by coronary spasm rather than acute coronary syndromes that presage occlusive atherothrombotic events. Thus, since normal diet fed apoE^{-/-} mice do not develop unstable coronary plaques or suffer from spontaneous atherothrombotic events, despite their severe atherosclerosis, the convincing negative result of the present study does not preclude a role for CRP in man. More complex plaques have been reported in the innominate artery of aged fat

fed apoE^{-/-} mice, with evidence of intra-plaque haemorrhage and atherothrombosis [494]. These lesions merit further investigation in the human CRP transgenic model, especially in view of the initial, but yet to be repeated, report that arterial thrombosis is increased after vascular injury in human CRP transgenic mice [434].

This new transgenic mouse strain may also offer insights to other related biological processes. It is known that statins for example reduce CRP production in humans but the precise mechanism is not clear. One *in vivo* study in apoE^{+/-}-hCRP⁺ mice found evidence of a direct suppressive effect of statins and PPARα activators on hCRP expression independent of cholesterol lowering [271], although a further study looking at low dose atorvastatin use in hyperlipidaemic mice found no effect on transgenic human CRP production [495]. If the effects of statins in this transgenic strain can be further clarified, not only might it unravel the stimuli generating the increased CRP production, but it may also suggest that this *in vivo* model could be used to investigate changes in a potentially clinically relevant surrogate marker during the development of novel therapies.

Recently it has been shown that LDL receptor^{-/-} mice immunised with *S. pneumoniae* have high circulating titres of oxLDL-specific IgM that are cross-reactive with pneumococcal determinants and that immunisation decreased the extent of atherosclerosis [496]. Studies dating back to the 1980s showed that pretreatment of mice with human CRP prior to immunisation with *S. pneumoniae* resulted in a dose-dependent inhibition of the antibody response to PC [421], and it is known that the spectrum of ligand recognition by CRP closely resembles

that of E06, an autoantibody to oxidised LDL, which is a T15 clone specific anti-PC antibody with specific binding to PC on oxidised but not on native phosphatidylcholine [112]. Clinically circulating levels of oxidised LDL (measured using the monoclonal antibody E06) are strongly associated with angiographically documented coronary artery disease [497]. There is thus an intriguing association between CRP and the innate immune response to atherosclerosis [498] that this strain of mice might have the ability to shed further light on. For example measuring the titre of antibodies against oxidised LDL would ascertain whether or not the transgenic production of human CRP impacts on antibody production. Similarly does the presence of transgenic human CRP alter the level of oxidised LDL particles in this model?

12.7 Summary

We show that transgenic expression of human CRP had no effect on development, progression or severity of spontaneous atherosclerosis, or on morbidity or mortality, in male apoE deficient C57BL/6 mice up to 56 weeks, despite deposition of human CRP and mouse complement component 3 in the plaques. Extrapolation to human pathology, from this xenogeneic combination of human CRP with apoE deficiency-mediated mouse atherosclerosis, must be guarded. Nevertheless the present results do not suggest that human CRP is either proatherogenic or atheroprotective *in vivo*. In the context of CRP which predicts atherothrombotic rather than atherosclerosis burden, a variety of models will be required to robustly and conclusively address its role in cardiovascular biology.

13 Novel inhibitors of pentraxin binding

13.2 Introduction

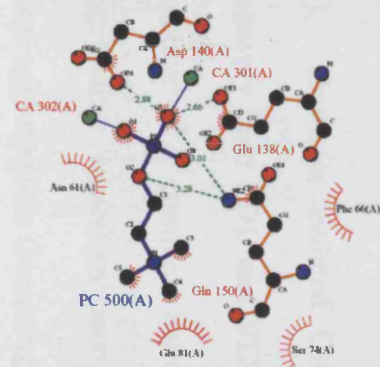
Whilst of course the correlation of circulating CRP concentrations with the severity, extent and progression of many different pathologies, and the prognostic significance of these associations, are consistent with CRP being a marker of disease, it is an intriguing concept that it may also contribute to pathogenesis. A definitive way to test this concept will be the development and use of novel drugs that specifically block CRP binding and its potential pro-inflammatory effects *in vivo* [34, 326]. Suitable compounds may be bound by CRP and thereby block the site of interaction between CRP and the target ligand, may bind to CRP to alter its structure thereby to inhibit or prevent binding to the ligand, or may bind to the ligand and in so doing mask it, preventing its recognition by CRP. Alternative strategies affecting upstream hepatic production, or downstream clearance of CRP, are also conceivable.

Our approach, inhibition of ligand binding at the phosphocholine binding site, is first put into context by briefly discussing the details of the ligand binding properties of CRP and the consequent, complement activation that occurs, before expanding on our specific strategy for rationally designing a CRP inhibitor.

In the context of drug design, structural understanding can help rational synthesis of inhibitors. Each of the five identical noncovalently bound subunits of human CRP has the same orientation in the pentamer, with a PC binding site located on one face of each subunit (the binding, B, face). The PC binding site consists of a hydrophobic pocket formed by residues Leu⁶⁴, Phe⁶⁶, and Thr⁷⁶, and two calcium ions which are bound to CRP by interactions with the side chains and main chain carbonyls of amino acids from different parts of the primary structure [32, 118]. Crystallographic analysis of CRP-PC complexes (Figure 87) has demonstrated that the phosphate group of PC directly coordinates with the two calcium ions. The choline moiety of PC lies within the hydrophobic pocket. The exposed face of Phe⁶⁶ provides hydrophobic interactions with the methyl groups of choline, while the side chain of Glu⁸¹, which is located on the other side of the pocket, interacts with the positively charged quaternary nitrogen of choline. Mutational analyses of Thr⁷⁶ in CRP has confirmed the significance of the hydrophobic pocket for PC binding [119, 120].

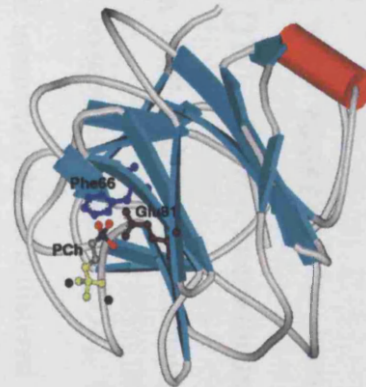
CRP also binds a variety of other ligands that include both autologous (e.g. native and modified plasma lipoproteins, damaged cell membranes, a number of different phospholipids and related compounds, and small nuclear ribonucleoprotein particles) and extrinsic structures (e.g. many glycan, phospholipid and other components of micro-organisms, such as capsular and somatic components of bacteria, fungi and parasites, as well as plant products) [38, 44, 84, 106, 110, 111, 461, 499-503].

Adapted from <http://www.ebi.ac.uk/>



a) LIGPLOT of phosphocholine interactions with CRP

Adapted from *The Journal of Immunology*, 2002, 169: 3217-3222



b) Ribbon diagram of the x-ray crystal structure of CRP-Phosphocholine complex

Only one of the five subunits bound to PC is shown. The side chains of Phe66 (blue) and Glu81 (brown) are highlighted. The two calcium ions are in black. Yellow, the phosphate group of PC; red, the three methyl groups of choline; green, the choline nitrogen.

Figure 87 Structural analysis of CRP-Phosphocholine binding

- the major interactions between CRP and phosphocholine occur at either end of the phosphocholine molecule and involve an interaction between the calcium ions and two of the phosphate oxygens at one end and an interaction between the positively charged quaternary nitrogen of phosphocholine and Glu 81 at the other end.

There is of course clear evidence suggesting a very important role for the abnormal deposition of LDL, a major class of plasma lipoprotein, in arterial walls in the pathogenesis of atherosclerosis. Oxidation of LDL, entrapped in the arterial intima is believed to be of critical importance, because the lipoprotein is converted to a ligand of scavenger receptors [362, 364, 504]. LDL is derived by metabolism of very low density lipoproteins, and both these classes of lipoprotein particles, as well as high density lipoprotein, consist of apoproteins together with varying proportions of phospholipid, triglyceride, esterified, and nonesterified cholesterol [505]. Although CRP binds phospholipids by virtue of its specific, calcium-dependent reactivity with PC residues, analysis of acute-phase serum by gel filtration and by density gradient ultracentrifugation shows that human CRP is in a free, uncomplexed form, despite the coexistent presence of the various classes of serum lipoproteins, all of which contain phospholipids [78]. However isolated CRP, when aggregated by immobilisation, does selectively bind LDL and VLDL [110]. *In vitro* interestingly, Ca^{2+} -dependent formation of a complex between CRP and VLDL accounts for a 'biphasic transmittance waveform' seen in coagulation assays [358], whilst CRP, also causes agglutination (creaming) of intravenously administered lipid suspensions [506].

All of the constituents of LDL, including phospholipids, cholesterol, fatty acids, and apolipoprotein, can be subject to oxidation and might contribute to the effects observed with oxidised LDL. Non-oxidative modifications of LDL can also generate potentially atherogenic molecules, and enzyme modified LDL (ELDL)

shares characteristics with lipoproteins isolated from atherosclerotic lesions, including particle size, an important and relevant capacity to be bound by CRP and to activate complement [360]. E-LDL consists of heterogeneously sized fused LDL particles with diameters of 25 to 500 nm, formed after treatment with trypsin and cholesterol esterase (with or without neuraminidase treatment). The apolipoprotein B is extensively degraded, with a ratio of free to esterified cholesterol of ~1:1. Tissue proteases degrade the lipoprotein B "shell", rendering underlying lipids accessible to cholesterol esterase. Release of fatty acids increases the relative content of free cholesterol, and neuraminidase removes an important complement inhibitor from the particles. Potentially relevant ligand binding sites for CRP are subsequently also more accessible than in the native lipoprotein particle [113, 187, 505, 507]. The interactions between CRP and lipoproteins may therefore play a part in the normal function of CRP as well as a role in lipoprotein pathophysiology [56, 508]. In particular, because of the long speculation as to the role of CRP in atherogenesis, inhibition of the interaction between CRP and lipoproteins represents a specific patho-physiologically relevant target for our studies.

13.4 Complement activation by ligand bound C-reactive protein

The interaction between CRP and the complement cascade is robust and well established. When CRP is ligand bound it becomes capable of activating the classical pathway of complement via C1q and achieving activation and fixation of C3, the main adhesion molecule of the complement system, as well as

engagement of the terminal lytic phase, C5-C9 [47-49, 51, 53, 55]. Much of the published work (see Table 37) that directly examines the role of CRP in experimental models of disease indicates that CRP may have an anti-inflammatory role that down-regulates infiltration of inflammatory cells and reduces tissue damage. This would be consistent with the finding that complexed CRP is relatively inefficient at generating the terminal phase of complement activation [55, 56] and that involvement of CRP down-regulates other potentially inflammatory aspects of complement activation (Figure 88). Recent work in different models involving handling of apoptotic cells also indicates that CRP has anti-inflammatory properties [59].

The association of increased CRP production with disease conditions has generally been interpreted on the basis that CRP production reflects the severity of the underlying disease and/or the presence of intercurrent complications. This was broadly seen in the context of the predominant, although not necessarily consensual, view that the role of CRP *in vivo* was anti-inflammatory. However activation of the complement cascade can no doubt be a pro-inflammatory process and CRP could under certain circumstances have significant pro-inflammatory effects [191]. By binding to ligands exposed on cells or other autologous structures as a result of infection, inflammation, or ischaemia, and triggering complement activation, it may exacerbate tissue damage leading to more severe disease. Indeed the enhancement seen in myocardial infarction size in rats treated with human CRP was complement dependent [201]. Therefore an important aspect of our strategy was the need to specifically demonstrate inhibition of complement activation as a parameter in drug development.

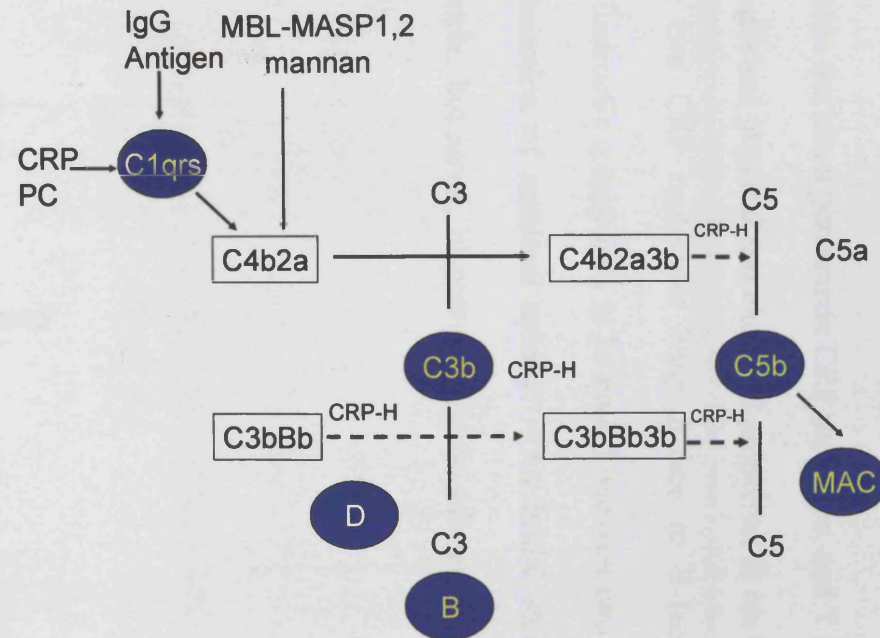


Figure 88 CRP and the complement cascade

- ligand bound CRP binds C1q which is most efficient at early classical pathway activation (C1, C4, C2). There is however evidence that CRP inhibits the alternative pathway/Lectin pathway; it seems to decrease alternative complement pathway C3 and C5 convertase activities. The inhibitory effects are mediated by CRP binding to factor H; factor H binds C3b to promote degradation by factor I and binds to the alternative C3 convertase and both C5 convertases to accelerate their decay. *Abbreviations:* MBL – Mannose binding lectin and MASP – MBL associated serum protease [55]

13.5 Strategies for developing a C-reactive protein inhibitor

We have focussed our efforts on inhibitors that are specifically bound in the calcium dependent ligand binding site of CRP, in particular those that comprise palindromic molecules of the general formula X-Y-X. In this formula X is a structure specifically recognised and bound by the calcium dependent ligand binding site present on the so-called “B”, or binding, face of each protomer within the intact pentameric CRP molecule, and Y is a linker structure of suitable length and physical and chemical properties to enable the compound to be bound by two CRP molecules lying B-face to B-face, and for up to five such palindromic compounds to be bound between two CRP molecules to provide an interaction of maximal avidity. Our initial studies have revolved around a simple, but novel, bisphosphocholine substance, we have called RMM (Figure 89).

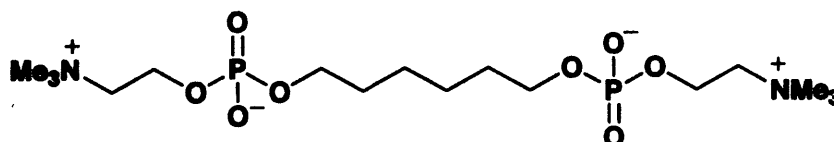


Figure 89 Structure of RMM

The rationale for compounds that are capable of cross-linking two native pentameric CRP molecules to create a face-to-face, "B-B" dodecamer, is derived from previous work on the very closely related protein, SAP [94]. SAP is the other member of the pentraxin family of plasma proteins and it shares extensive amino acid sequence homology, secondary, tertiary and quaternary structure with CRP. Thus it has a closely similar assembly of identical protomers, arranged with cyclic pentameric symmetry in a disc-like configuration, with a calcium dependent ligand binding site located on the B face of each protomer. The ligand specificity of SAP overlaps but is clearly distinct from that of CRP, but is as important for the pathophysiological functions of SAP as it is for those of CRP. SAP is a therapeutic target in amyloidosis and Alzheimer's disease, and a large collaborative effort has shown that compounds with the general structure X-Y-X, but specific for binding by SAP, are highly potent inhibitors of SAP binding and very effective in blocking SAP function *in vivo*. Such compounds, specific for SAP, for example, (R)-1-[6-(R)-2-Carboxy-pyrrolidin-1-yl]-6-oxo-hexanoyl]pyrrolidine-2-carboxylic acid (CPHPC), form complexes with SAP in which SAP is held as a dodecamer crosslinked by 5 drug molecules and the structure has been solved at atomic resolution by X-ray crystallography (Figures 90 and 91). Furthermore, in the presence of such SAP-specific compounds that cause dodecamerisation of SAP, SAP molecules are very rapidly cleared from the plasma *in vivo* and catabolised [94].

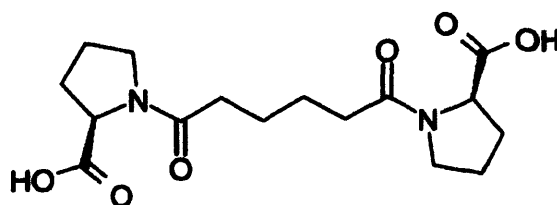


Figure 90 Structure of the D-Proline palindromic inhibitor of SAP, CPHPC

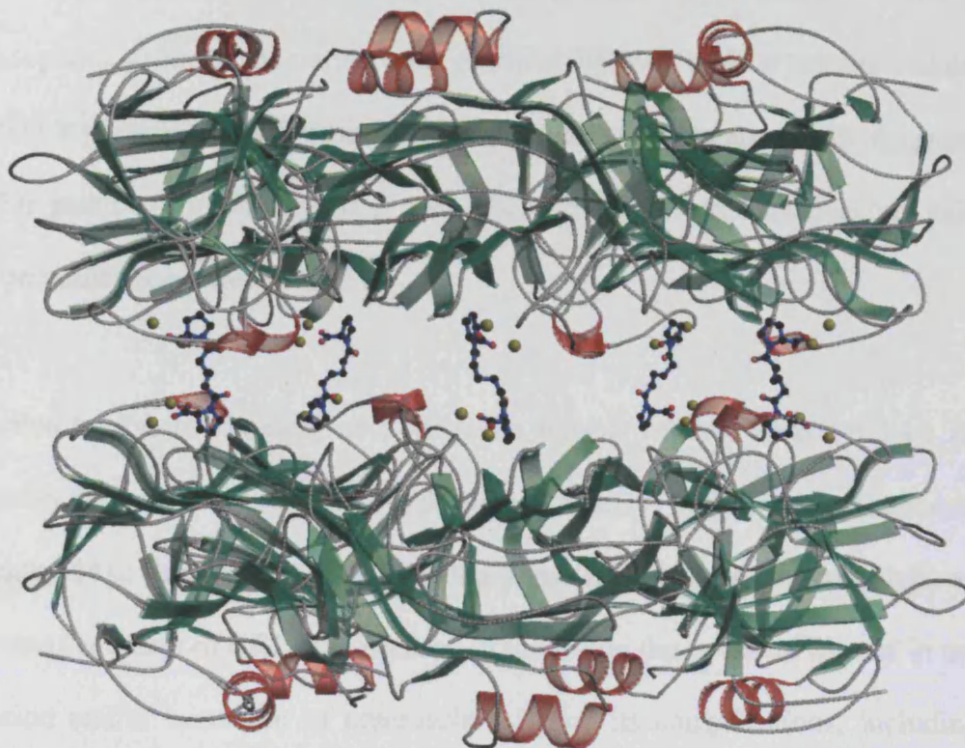


Figure 91 Decamerisation of human SAP by CPHPC

- two SAP pentamers cross-linked via their B faces by five molecules of CPHPC (blue) viewed perpendicular to the five-fold axis. A face helices are shown in red. The two calcium ions bound to each SAP subunit are yellow [94].

On the basis of the previous structural studies by Professor S. Wood [94] it was known that in the CRP phosphocholine complex, a phosphocholine molecule is positioned parallel to the pentamer surface on each of the five CRP subunits and oriented with the choline moieties, pinned between Glu⁸¹ and Phe⁶⁶, towards the five-fold axis. Two oxygen atoms of the phosphate group of phosphocholine directly co-ordinate the two bound calcium ions of CRP but the third is oriented away from the protein surface, providing a suitable exit point to direct a cross

linking chain towards a putative 2-fold axis related subunit of an adjacent CRP pentamer. On this basis it was decided to select a six carbon linker to connect the phosphate groups, generating two phosphocholines with a hexane linker, expecting a greater separation between binding sites of a putative CRP decamer caused in part by rotation of $\sim 20^\circ$ of each CRP subunit towards the five-fold axis of the pentamer compared to SAP.

Drugs that inhibit the binding of CRP to its ligands *in vivo*, and/or reduce its availability for such binding, may block the contribution of CRP to pathogenesis of disease [34]. Such compounds have the potential to shed significant light on the *in vivo* functions of CRP, and perhaps in the future the potential for use in the prevention and/or treatment of atherosclerosis and its complications, including cardioprotective effects in acute myocardial infarction, stroke and peripheral vascular disease in particular. Moreover they may provide a powerful tool for determining whether increased CRP production merely reflects atherosclerosis or does indeed participate in its pathogenesis and complications. It is conceivable that other diverse inflammatory conditions could also be targets for such compounds.

The present chapter therefore describes our studies, from screening assays onwards, of compounds capable of inhibiting the binding of CRP and in particular the innovative work on RMM *in vitro* and *in vivo*.

13.6 Results

13.6.1 C3 cleavage and CRP ligands

As discussed complement activation is one major final pathway through which pro-inflammatory effects of human CRP may be important. The effects of CRP-ligand binding *in vitro* on C3 cleavage were therefore assessed by crossed-immunoelectrophoresis, including where appropriate its inhibition.

13.6.1.1 Cobra venom factor and normal serum

Isolated cobra venom factor, which functions as an unregulated C3b molecule, activating and depleting C3 and C5 via the alternative pathway, was used to demonstrate the standard changes seen in migration of C3 cleavage products in human and mouse serum (Figures 92 and 93).

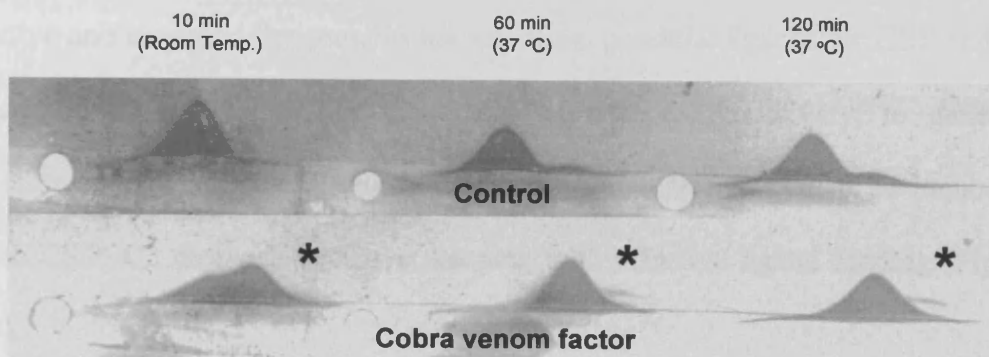


Figure 92 Demonstration of C3 cleavage products in human serum

- generated by incubation with Cobra venom factor at 37°C for 120 minutes; note the change in electrophoretic mobility with C3 activation (*).

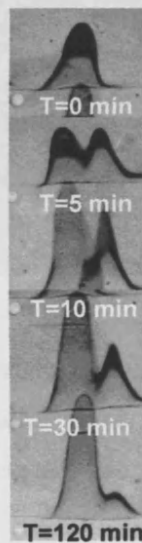


Figure 93 Demonstration of C3 cleavage products in mouse serum

- generated by incubation with Cobra venom factor at 37°C

13.6.1.2 Modified lipoproteins and C3 cleavage when ligand bound to CRP

Native and modified lipoproteins are important potential ligands for CRP and we therefore modified LDL enzymatically as well as oxidatively, to generate potentially physiologically relevant ligands. When fresh ELDL is co-incubated with CRP, C3 cleavage occurs in keeping with effective ligand binding (Figure 94).

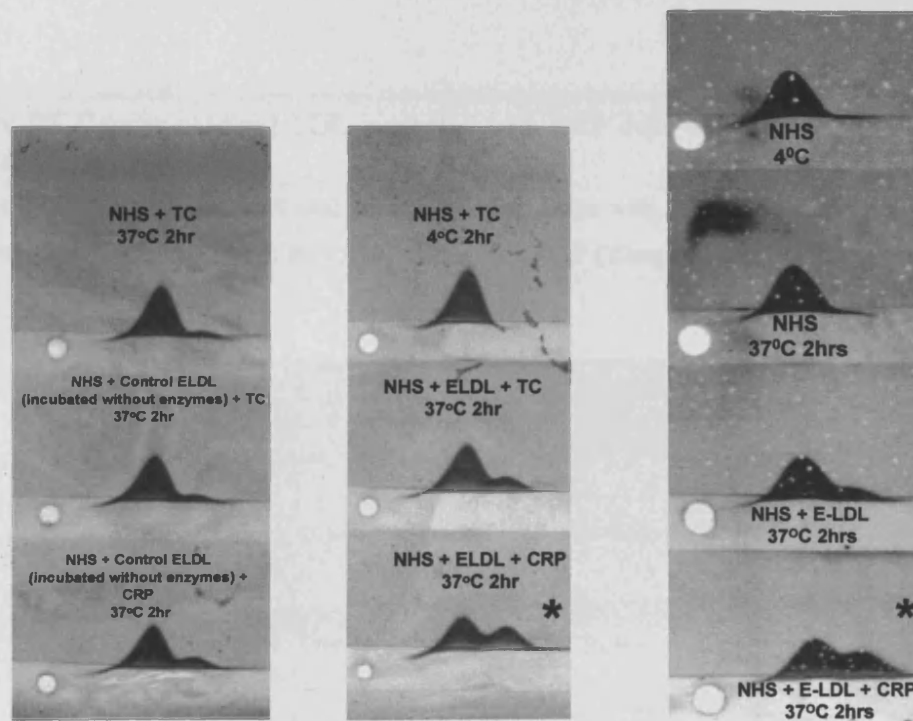


Figure 94 Activated human C3 cleavage profiles

- enzyme modified LDL (~40mg/l total protein) and human CRP (20mg/l) were coincubated in human serum bled for complement; * indicates activated C3 profiles

However this effect is not seen to any great extent with either oxidised LDL or VLDL (*cf.* positive control *; Figures 95 and 96). VLDL was provided by Miss N. Marshall (University of Liverpool).

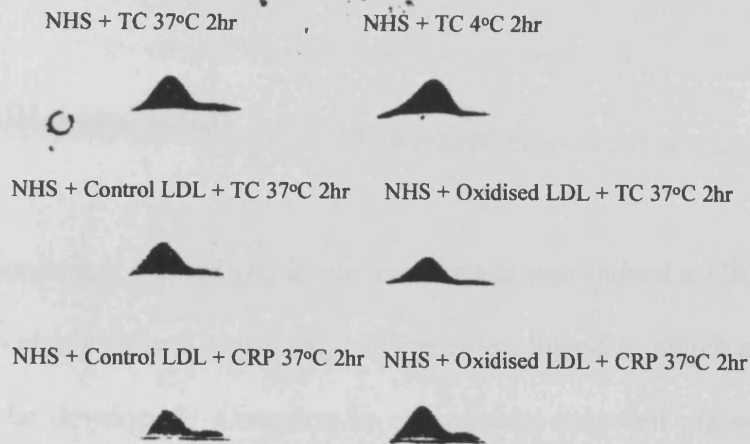


Figure 95 Copper oxidised LDL incubated with CRP does not generate C3 cleavage products

- Copper oxidized LDL (40 mg/l total protein; prior oxidation with 100 μ M CuSO₄) or native LDL (40 mg/l total protein) was incubated with human CRP (20mg/l)

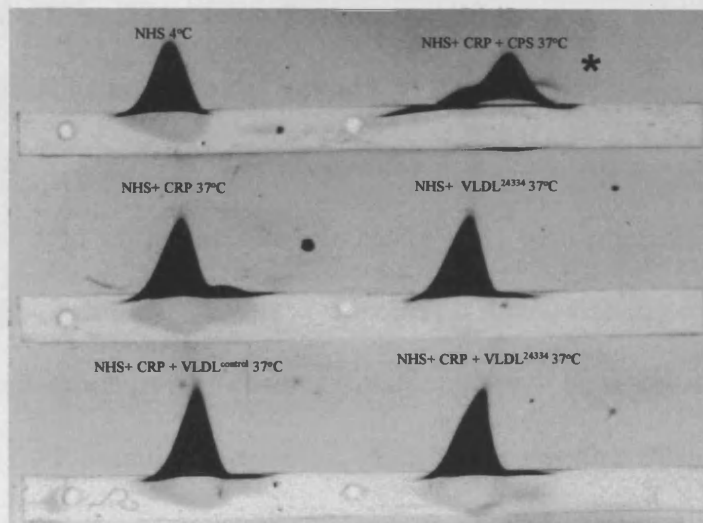


Figure 96 Co-incubation of VLDL with human CRP does not activate complement

- VLDL (normal and acute phase serum [VLDL²⁴³³⁴]) was co-incubated with human CRP (20mg/l) in normal human serum, for 2 hours at 37°C. CPS-CRP (CPS at 400mg/l) complexes acted as a positive control for C3 activation (*).

13.6.2 Plate assays for CRP binding and its inhibition

13.6.2.1 ELDL based assays

Having demonstrated that ELDL in our own hands was indeed a CRP ligand we proceeded to choose it as a physiologically relevant ligand to which a plate based assay could be developed. Coupling to commercial stripwell plates of ligands, was initially based on hydrophobic interaction, but then subsequently we used covalent association.

The binding properties, under varying conditions were studied using purified human CRP iodinated with I¹²⁵ (~125 ng human CRP offered per well unless otherwise stated), as this allows for very sensitive assays, without recourse to secondary means to measure the amount of CRP bound to immobilised ligand. After gel filtration, >95% of the radioactivity was precipitable with 10% w/v Tri-Chloro-Acetic acid and functional integrity was confirmed in each case by demonstrating retained intact specific ligand-binding activity for phosphoethanolamine immobilised on carboxyhexyl Sepharose. On average radiolabelled CRP bound to Sepharose-PE with greater than 90% efficiency and the final specific activity was 0.75-1 microcuries/microgram protein.

Table 45 summarises for hydrophobic plates the properties of immobilised ELDL (in carbonate buffer pH 9.0). There was an increased absolute amount of CRP bound as the concentration of ELDL (as estimated on the basis of the initial protein concentration of LDL) offered to the hydrophobic plate was increased.

Background binding was very low and calcium chelation clearly inhibited CRP binding back to background levels. Similarly PC and competition by “cold” CRP returned binding to background rates. There is therefore clear demonstration of specific binding through the calcium dependent ligand binding sites of human CRP to immobilised ELDL.

Table 45 Summary of binding properties of hydrophobically immobilised ELDL

ELDL (approx conc.)	Buffer (with 1% BSA)	hCRP (ng) bound mean +/- SD [N]
300 µg/ml	Tris-Ca ²⁺	4.0 +/- 0.9 [3]
100 µg/ml	Tris-Ca ²⁺	2.2 +/- 1.1 [16]
10 µg/ml	Tris-Ca ²⁺	1.9 +/- 0.8 [6]
0 µg/ml (2% BSA)	Tris-Ca ²⁺	0.2 +/- 0.0 [8]
100 µg/ml	Tris-EDTA	0.3 +/- 0.0 [3]
0 µg/ml (2% BSA)	Tris-EDTA	0.2 +/- 0.1 [3]
100 µg/ml	40mM PC (Tris-Ca ²⁺)	0.4 +/- 0.1 [5]
100 µg/ml	1mg/ml cold CRP (Tris-Ca ²⁺)	0.4 +/- 0.0 [2]
Control LDL (~170 µg/ml)	Tris-Ca ²⁺	0.5 +/- 0.0 [4]

In modifying the assay, the relative amount of offered protein to that bound was assessed, and Figure 97, demonstrates how as expected, the lower the total

concentration of CRP offered, the greater the relative proportion bound. This is relevant to optimising the assay properties.

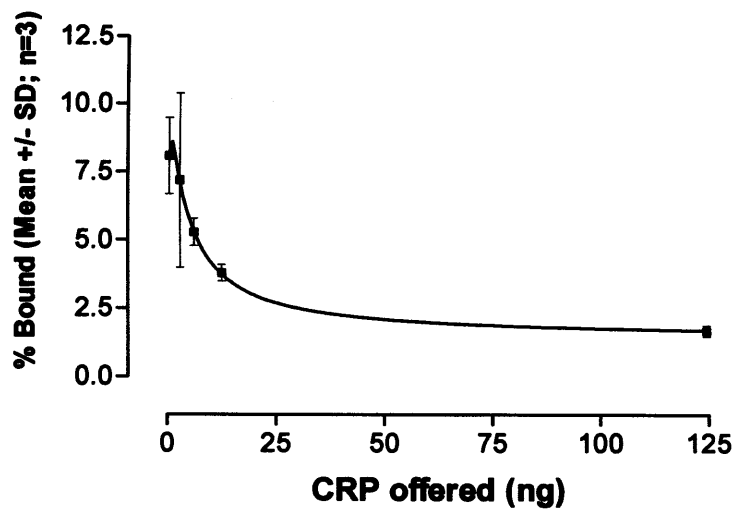


Figure 97 Binding of CRP to hydrophobically immobilised ELDL

Figure 98 demonstrates how there is a clear dose dependent relationship between PC and inhibition of CRP binding. In this experiment the IC_{50} was calculated (one site competition model) to be approximately 17 μ M. This is within expectations [36, 39, 45, 509-511] as CRP is reported to have a binding affinity (K) to phosphocholine around 1-10 μ mol/l.

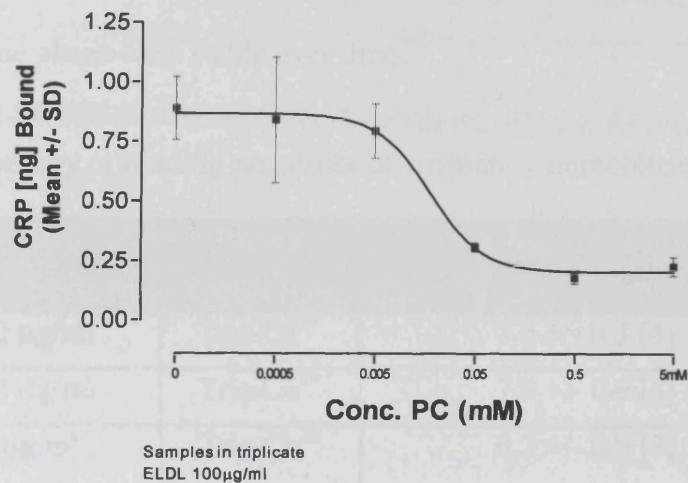


Figure 98 Inhibition by PC of CRP binding to hydrophobically immobilised ELDL

Although effective, hydrophobic interaction was not very efficient. We therefore assessed the effects of covalently coupling to N-oxysuccinimide surface amine binding plates (Figure 99).

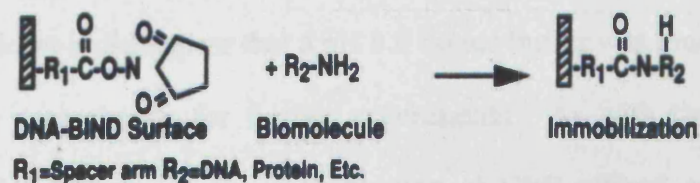


Figure 99 Biochemistry of the covalent attachment of ligands to strip-well plates

Table 46 summarises the binding properties of the now covalently immobilised ELDL. Once again calcium dependence is clear as is inhibition by free PC. Importantly the plates were stable over time.

Table 46 Summary of binding properties of covalently immobilised ELDL

ELDL (approx conc. offered)	Buffer (with 1% BSA)	hCRP (ng) bound mean +/- SD [N]
300 µg/ml	Tris-Ca ²⁺	7.0 +/- 0.3 [4]
150 µg/ml	Tris-Ca ²⁺	7.9 +/- 0.0 [3]
0 µg/ml (PBS)	Tris-Ca ²⁺	0.2 +/- 0.0 [3]
300 µg/ml	Tris-EDTA	0.2 +/- 0.0 [3]
150 µg/ml	10mM PC (Tris-Ca ²⁺)	0.1 +/- 0.0 [3]
150 µg/ml one week storage +4°C	Tris-Ca ²⁺	12.5 +/- 0.0 [4]
All with ELDL in PBS pH 9.0		

For reference we assessed whether or not changes in the buffer used for ELDL coupling affected the properties of the assay (Table 47). Although perhaps not greatly significant it did appear that a pH 8.0 borate buffer was most optimal, and therefore this was chosen for further experiments. As with the hydrophobic plates the effect of varying the concentration of CRP offered, under different conditions, was also documented (Figures 100 and 101).

Table 47 Effects of variation in ELDL buffer and concentration on CRP binding

ELD	Buffer	hCRP (ng) bound mean \pm SD [N = 3]
150 μ g/ml	PBS pH 7.3	6.1 \pm 0.6
75 μ g/ml		6.4 \pm 0.2
37.5 μ g/ml		5.6 \pm 0.2
18.8 μ g/ml		5.2 \pm 0.1
9.4 μ g/ml		4.1 \pm 0.5
4.7 μ g/ml		2.0 \pm 0.1
150 μ g/ml	PBS pH 8.0	5.4 \pm 0.3
75 μ g/ml		5.2 \pm 0.0
37.5 μ g/ml		5.0 \pm 0.1
18.8 μ g/ml		4.9 \pm 0.8
9.4 μ g/ml		3.2 \pm 0.1
4.7 μ g/ml		1.7 \pm 0.1
150 μ g/ml	PBS pH 9.0	5.0 \pm 0.1
75 μ g/ml		4.9 \pm 0.4
37.5 μ g/ml		4.3 \pm 0.3
18.8 μ g/ml		3.6 \pm 0.5
9.4 μ g/ml		2.5 \pm 0.2
4.7 μ g/ml		1.4 \pm 0.1
150 μ g/ml	Boric acid pH 8.0	8.8 \pm 0.5
75 μ g/ml		7.7 \pm 0.3
37.5 μ g/ml		7.3 \pm 0.3
18.8 μ g/ml		6.6 \pm 0.7
9.4 μ g/ml		4.4 \pm 0.4
4.7 μ g/ml		2.3 \pm 0.1
150 μ g/ml	Carbonate pH 9.0	6.3 \pm 0.7
75 μ g/ml		6.0 \pm 0.5
37.5 μ g/ml		5.1 \pm 0.3
18.8 μ g/ml		4.5 \pm 0.3
9.4 μ g/ml		3.3 \pm 0.2
4.7 μ g/ml		2.1 \pm 0.1

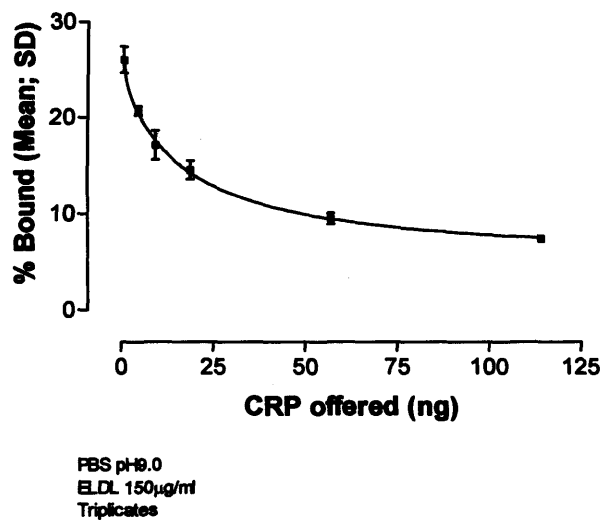


Figure 100 Binding of CRP to covalently immobilised ELDL: PBS buffer

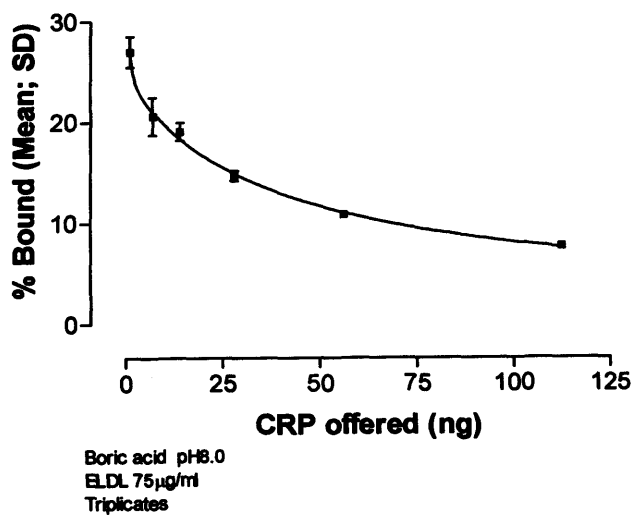


Figure 101 Binding of CRP to covalently immobilised ELDL: Borate buffer

Specific chemical substances were then shown to inhibit ligand binding. The compounds chosen were phosphocholine, which is known to be the best natural ligand for CRP, phosphoethanolamine (PE), to which CRP is also well known to bind, and phosphoserine (PS), a related phosphorylated molecule the binding to which of CRP has not previously been reported. These were compared with hexadecyl-phosphocholine (HDPC), which we initially investigated as a proprietary candidate inhibitor of CRP binding; it being more widely known as Miltefosine, and used in the treatment of visceral leishmaniasis [512, 513]. CPHPC and phosphorylcholine oleoyloxyethyl ester (oleoyl) along with acetyl-L-carnitine hydrochloride (carnitine) were also assayed.

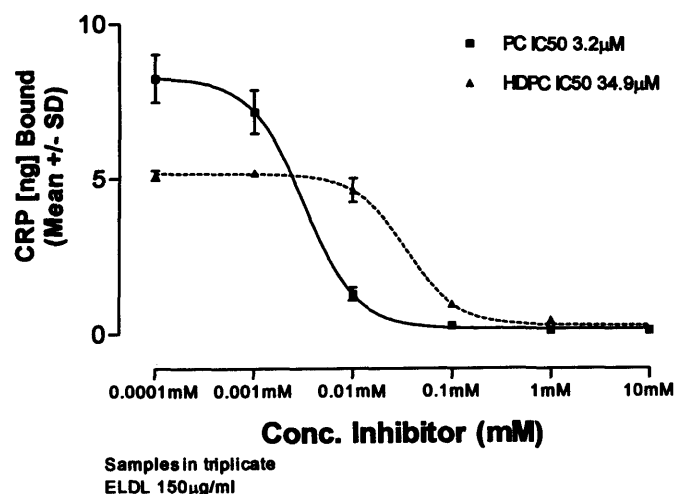


Figure 102 Inhibition of CRP binding to covalently immobilised ELDL by PC and HDPC

- borate buffer pH 8.0

Figure 103 demonstrates the structure of the potential ligands whilst figures 104 and 105 show how in this assay PC had an IC_{50} of between 3-10 μ M whilst HDPC was less efficient with an estimated IC_{50} of around 20-35 μ M. Phosphoethanolamine had an IC_{50} of approximately 100 μ M. The other ligands were not very efficient, if at all, inhibitors of CRP binding. Of relevance for experiments discussed later, CPHPC inhibited binding with an IC_{50} of nearly 1mM.

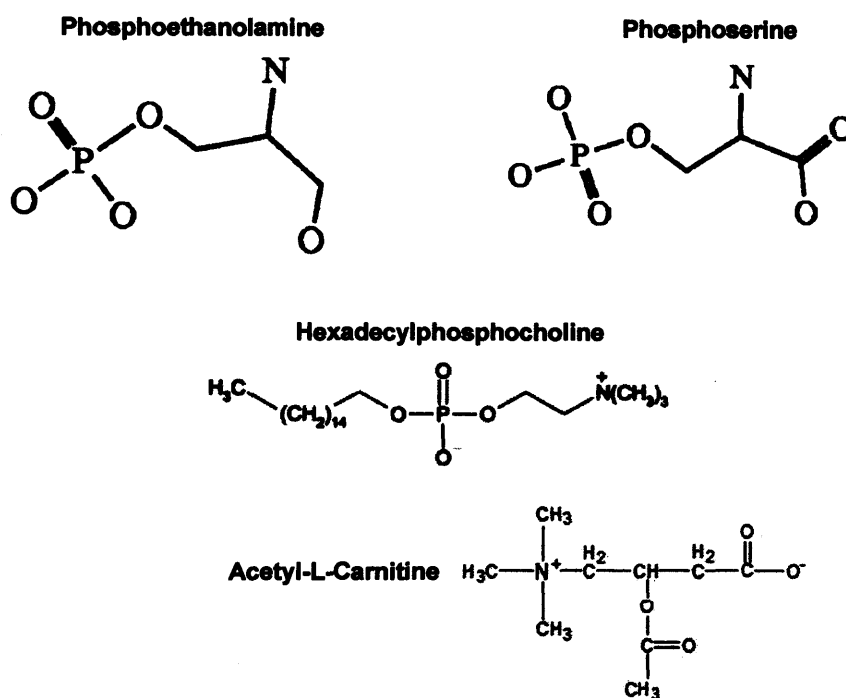


Figure 103 Structure of relevant ligands

The IC_{50} s quoted are approximations, and are included as markers of efficacy. Different methods, such as calorimetry or equilibrium dialysis, not appropriate in a screening setting, would be needed to study the binding interactions further.

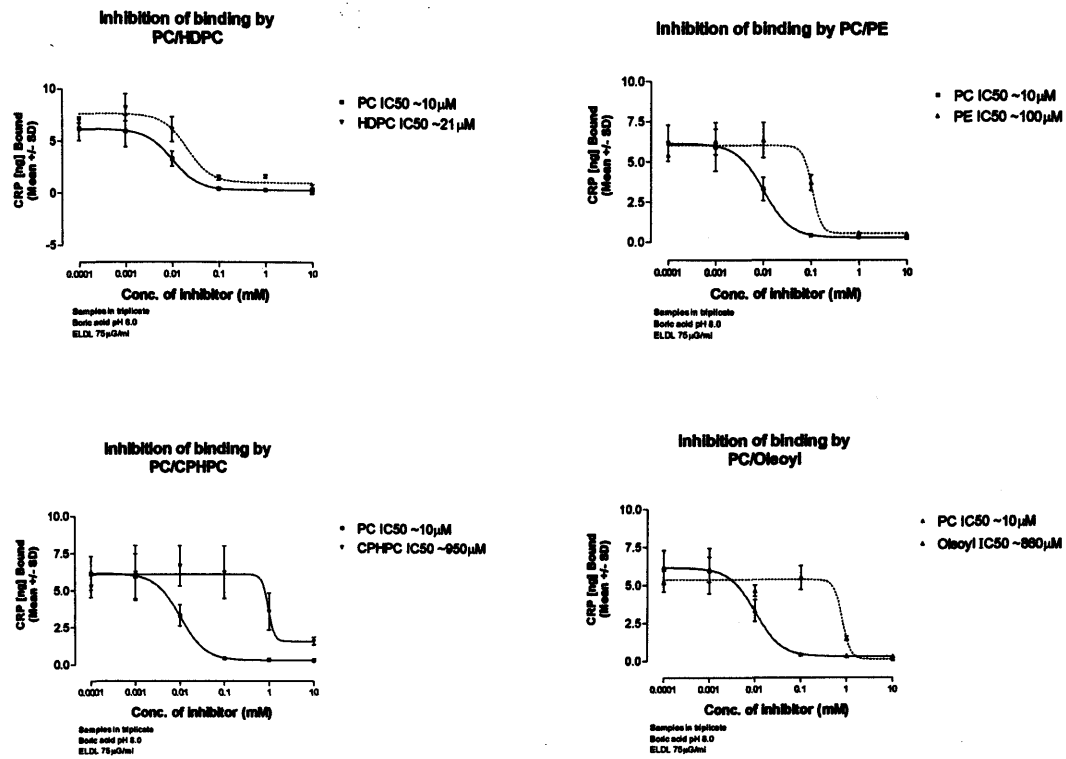


Figure 104 Inhibition of binding of CRP to covalently immobilised ELDL

- the potential inhibitors HDPC, PE, CPHPC and Oleoyl are compared with PC

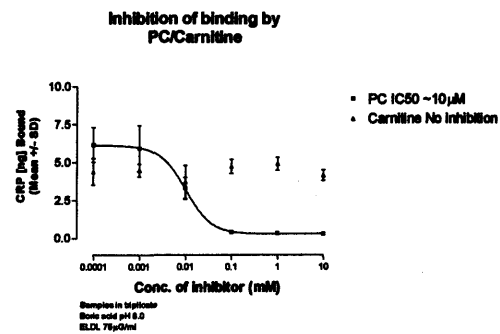
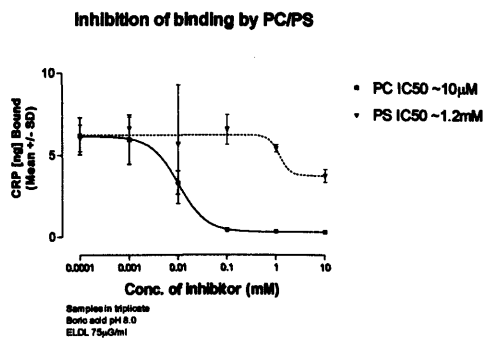


Figure 105 Inhibition of binding of CRP to covalently immobilised ELDL

- the potential inhibitors PS and Carnitine are compared with PC

Oxidised LDL (thermal and copper generated) was also assessed as a potential ligand in a plate assay. However (Table 48) in keeping with the C3 cleavage studies earlier, oxidised LDL was not a very efficient ligand (although calcium dependent binding was evident), and was not pursued further in this context.

Table 48 CRP binding to covalently immobilised copper oxidised LDL

<i>Ligand (~100µg/ml)</i>	<i>Tris-Ca²⁺</i>		<i>Tris-EDTA</i>	
	% offered bound ⁺			
	mean +/- SD [N=3]			
Control LDL (4°C)	1.4 +/- 0.0		0.3 +/- 0.0	
OxLDL (24hr 37°C)	1.2 +/- 0.2		0.4 +/- 0.1	
OxLDL (48hr 37°C)	1.5 +/- 0.0		0.4 +/- 0.0	
CuOxLDL (500nM / 24hr 37°C)	1.5 +/- 0.3		0.3 +/- 0.1	
CuOxLDL (5µM / 24hr 37°C)	2.3 +/- 0.1		0.3 +/- 0.1	
CuOxLDL (50µM / 24hr 37°C)	2.4 +/- 0.1		0.2 +/- 0.1	
CuOxLDL (100µM / 24hr 37°C)	2.6 +/- 0.2		0.3 +/- 0.1	
Buffer PBS pH7.4	⁺ ~100ng CRP offered			

13.6.2.2 C-polysaccharide based approaches

Whilst the above work clearly demonstrates that ELDL can be a robust ligand for a screening assay, technically it is not convenient to use, requiring lengthy isolation of the purified LDL, prior to careful and timely enzymatic modification followed by prompt coupling. Pneumococcal C-polysaccharide, the archetypal ligand, is more stable and robust and using a commercial source (Statens Serum Institut, Denmark) I proceeded to investigate its properties when covalently bound to plates as a ligand to facilitate the screening of inhibitors of CRP binding.

CRP effectively binds this commercial CPS, as evident by precipitation in a simple Ouchterlony, and the complex generates C3 cleavage products (*), both in mouse and human serum (Figures 106, 107 and 108).

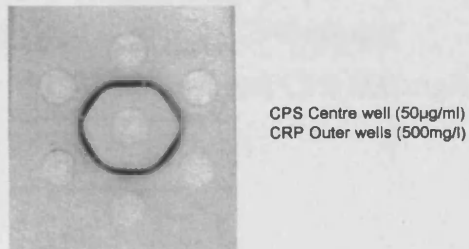
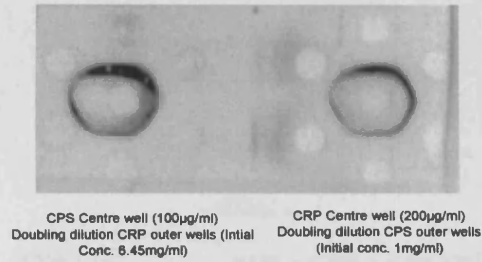


Figure 106 Double diffusion precipitation between human CRP and CPS

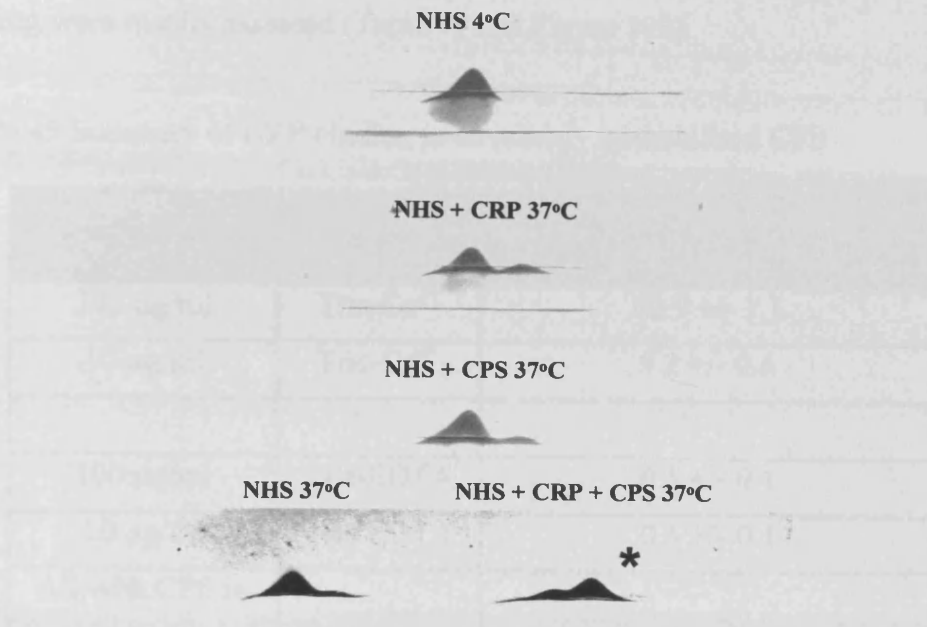


Figure 107 CRP-CPS complexes lead to human C3 cleavage
 - activated profiles indicated by *; CRP 20 mg/l and CPS 200 mg/l

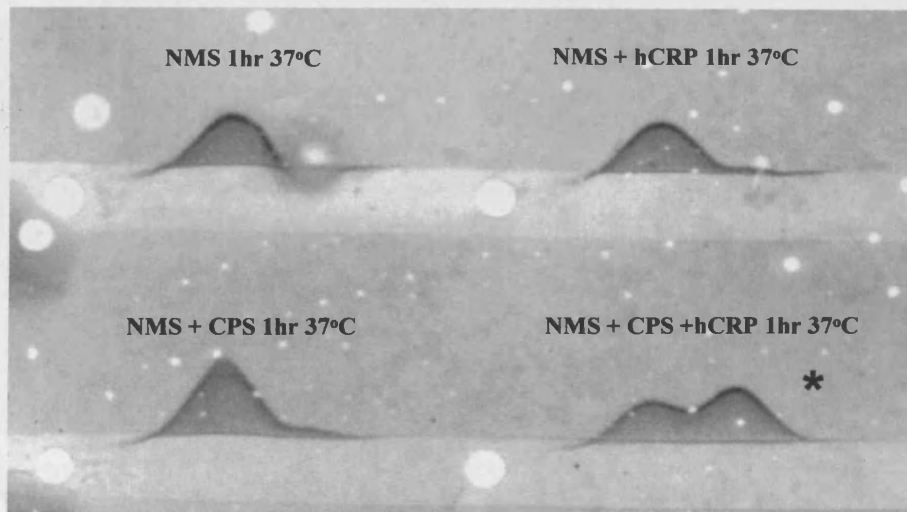
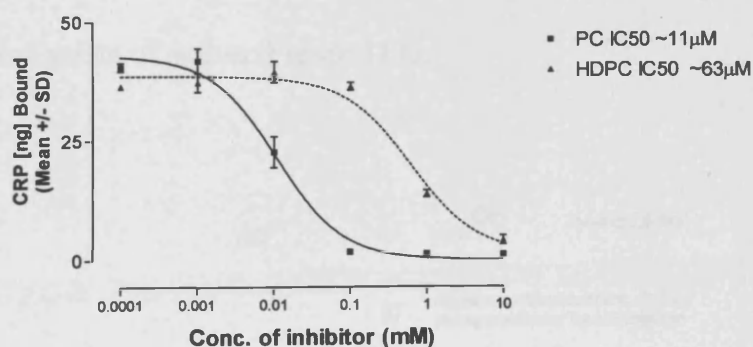


Figure 108 CRP-CPS complexes lead to mouse C3 cleavage
 - activated profiles indicated by *; CRP 20 mg/l and CPS 200 mg/l

It proved a very efficient ligand for our plate based assays and inhibitors of binding were readily assessed (Table 49 and Figure 109).

Table 49 Summary of CRP binding to covalently immobilised CPS

CPS (approx conc. offered)	Buffer (with 1% BSA)	hCRP (ng) bound mean +/- SD [N =4]
100 µg/ml	Tris-Ca ²⁺	42.9 +/- 1.1
10 µg/ml	Tris-Ca ²⁺	9.2 +/- 0.6
100 µg/ml	Tris-EDTA	0.5 +/- 0.1
10 µg/ml	Tris-EDTA	0.6 +/- 0.1
All with CPS in Borate pH 8.0		



Samples in triplicate
 Boric acid pH 8.0
 CPS 100µG/ml
 EDTA binding 0.5ng
 No inhibitor 42.9ng

Figure 109 Inhibition of CRP binding to covalently immobilised CPS

- ligands PC and HDPC studied

Binding was calcium dependent and the IC₅₀ values for PC (~10 µM) and HDPC (~60 µM) were of the same magnitude of those from the ELDL plate assays.

13.6.3 A novel bisphosphocholine C-reactive protein inhibitor

At this stage we were therefore ready to assess our novel CRP inhibitor, having established assays for screening compounds presently and in the future if needed. As described in the introduction the previous observation of decamerisation of human SAP proved to be the platform for rational drug design of a novel bisphosphocholine inhibitor of CRP binding. This molecule was initially synthesised by Professor R. Ganellin (UCL) and then using a novel and more efficient pathway in greater quantities in the laboratory of Professor S. Ley (University of Cambridge). The final product's structure and molecular integrity were confirmed by NMR and HPLC.

The final synthetic pathway used is summarised below (Figure 110) as well our proposed mechanism of action (Figure 111).

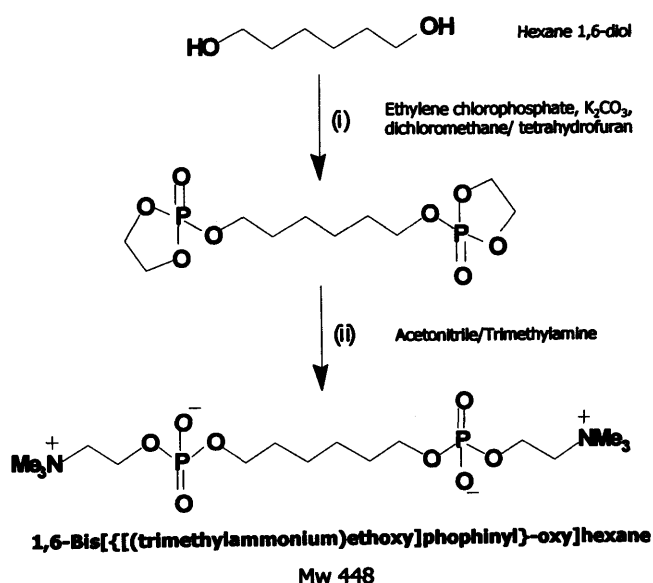


Figure 110 Synthesis of bisphosphocholine, RMM

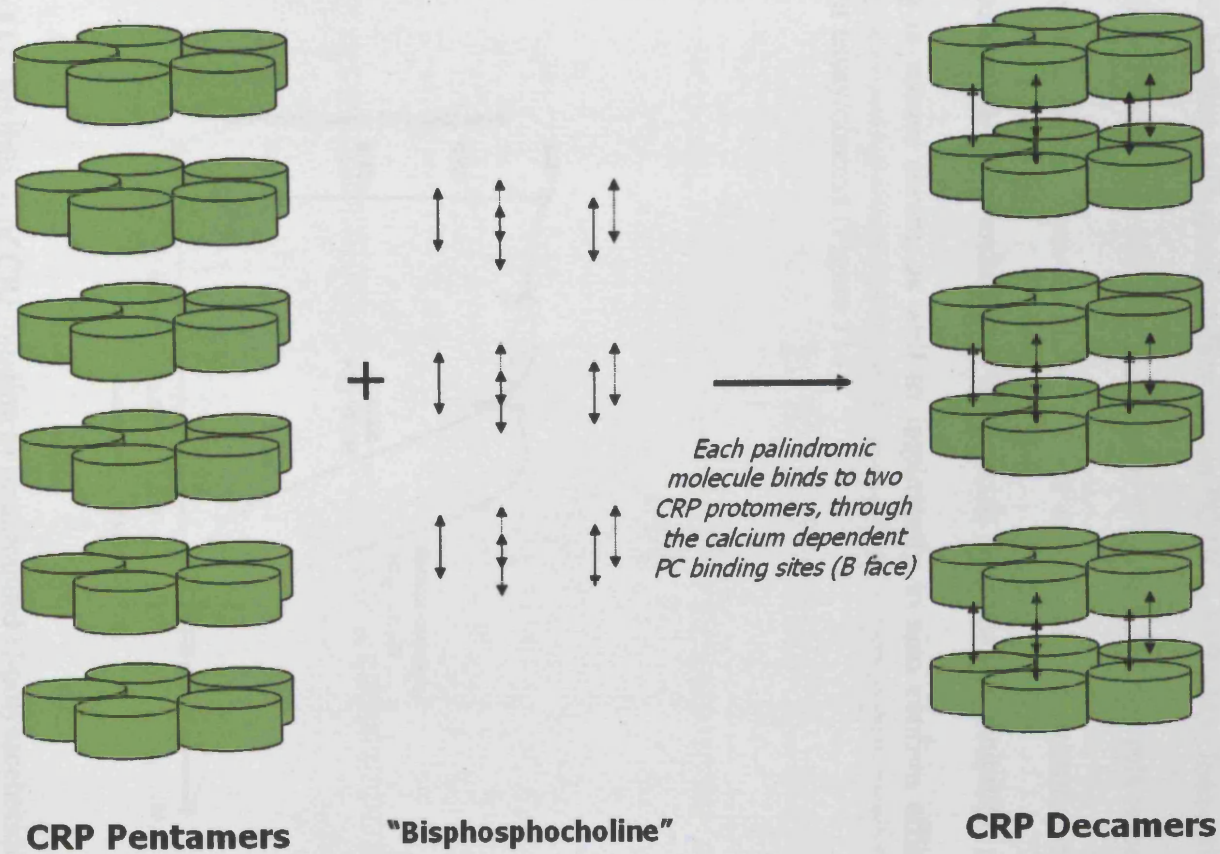


Figure 111 Proposed mechanism of action of RMM

13.6.3.1 Demonstration of efficacy *in vitro*

In TC buffer (Figure 112), RMM ($IC_{50} \sim 2 \mu M$) was a very effective inhibitor of CRP binding to covalently immobilised CPS, being the first ligand tested that inhibited binding with greater efficacy than PC ($IC_{50} \sim 19 \mu M$). Interestingly in a serum based assay this efficacy was muted (Figure 113), and this is probably a result of a different bioavailability. Prior to animal experimentation, a fluid phase assay was also undertaken in order to demonstrate inhibition of ligand binding in mouse serum, as well as importantly to also confirm efficacy in a different assay/context (Figure 114).

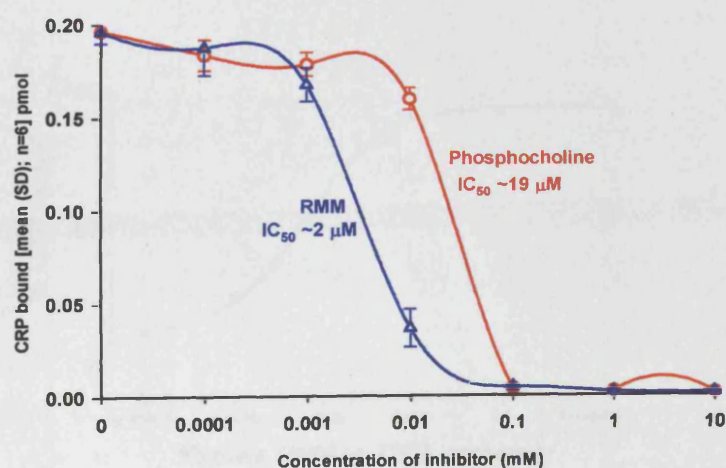
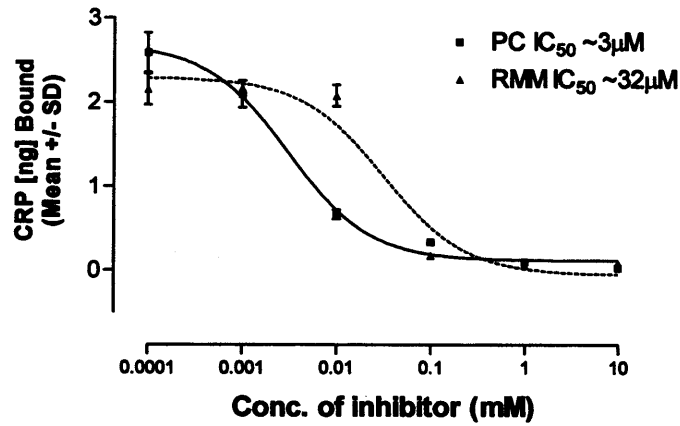


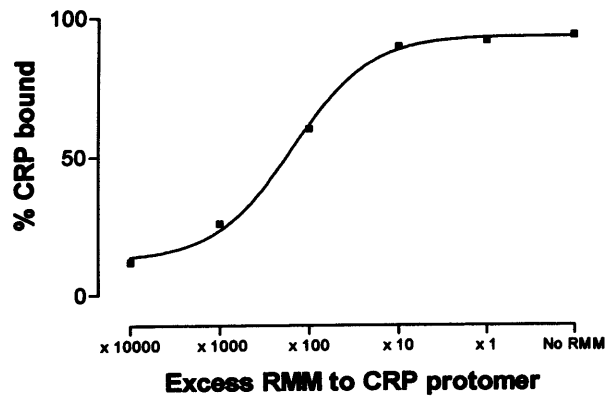
Figure 112 Inhibition of CRP binding to immobilised C-polysaccharide - experimental conditions as in Figure 109



Samples in triplicate
 Pooled normal human serum (CRP <5mg/l)
 CPS 100μG/ml
 EDTA binding 0.05ng
 No inhibitor 2.18ng

Figure 113 CRP binding to covalently immobilised CPS and its inhibition in serum

- the ligands PC and RMM were compared in human serum



15μl pooled Sepharose-PE
 40μl spiked serum
 CRP measured by rocket immunoelectrophoresis

Figure 114 Inhibition of CRP binding to Sepharose-PE in mouse serum

- human CRP *spiked* mouse serum

Figure 115, importantly confirms that complement activation (C3 cleavage), is inhibited when CRP is ligand bound to RMM. Using a classic haemolytic assay of complement activation, Table 40 (section 11.6), we also confirmed this finding.

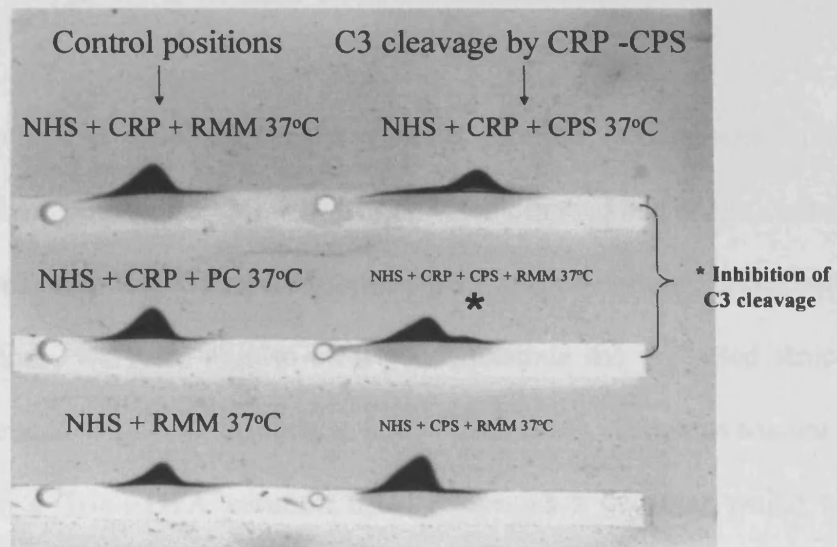


Figure 115 RMM inhibits C3 cleavage by CRP-CPS complexes in human serum
 - CRP 15mg/l; PC 6 mM ; RMM 0.3 mM; CPS 30 mg/l; Incubation at 37°C for 2 hours

13.6.3.2 Demonstration of structural effects

A number of independent techniques were used to investigate the effects of RMM on the molecular assembly of CRP.

Gel filtration chromatography

Gel filtration chromatography separates proteins, peptides, or oligonucleotides on the basis of size. Both molecular weight and three dimensional shape contribute to the degree of retention. Using a calibrated gel filtration column, under varying buffer conditions, we were able to clearly demonstrate the predicted structural changes associated with CRP binding to RMM (Table 50). Previous studies have shown that in a Tris-EDTA solution hSAP elutes as a decamer whilst hCRP elutes as a pentamer; the formation of stable decamers by isolated SAP in the absence of calcium is an *in vitro* artefact, resulting from pentamer pentamer interactions, which are largely electrostatic [357, 514]. When run in a PC containing solution CRP eluted as a pentamer, whilst the change to RMM led to immediate decamerisation. At vast excess of drug CRP eluted once again as a pentamer. In the presence of both ligands, an excess of 10 fold RMM to PC was needed to decamerise CRP. Multimeric CRP, presumed as evident by elution in the void volume, occurred in the presence of HDPC, consistent with the micellar structure this compound assumes in aqueous solution. This series of experiments therefore confirmed decamerisation as a specific, calcium dependent, PC-ligand binding site phenomenon related, to the palindromic structure of RMM (Figure 116).

Table 50 Molecular assembly of pentraxins by gel filtration: effect of ligand binding

<i>Control</i>			V_E (ml)	M_w
<i>Protein</i>				
Blue dextran			7.87 *	2000000
Thyroglobulin			8.67	669000
Ferritin			9.95	440000
Catalase			11.73	232000
Aldolase			12.03	158000
Human albumin			13.28	66500
<i>Pentraxin</i>	<i>Ligand</i>	<i>Condition</i>	V_E (ml)	<i>Molecular Assembly</i>
hSAP	-	Tris-EDTA	11.09	Decamer
hCRP	-	Tris-EDTA	12.58	Pentamer
hCRP	-	Tris-Ca ²⁺	13.06	Pentamer
hCRP	x 10 PC	Tris-Ca ²⁺	12.9	Pentamer
hCRP	x 1 RMM	Tris-Ca ²⁺	11.61	Decamer
hCRP	x 10 RMM	Tris-Ca ²⁺	11.57	Decamer
hCRP	x 1000 RMM	Tris-Ca ²⁺	11.81	Decamer
hCRP	x 10000 RMM	Tris-Ca ²⁺	12.85	Pentamer
hCRP	x 10 RMM	Tris-EDTA	12.6	Pentamer
hCRP	x 200 RMM/x 10 PC	Tris-Ca ²⁺	11.71	Decamer
hCRP	x 100 RMM/x 10 PC	Tris-Ca ²⁺	11.79	Decamer
hCRP	x 50 RMM/x 10 PC	Tris-Ca ²⁺	12.17	Mixed
hCRP	x 10 RMM/x 10 PC	Tris-Ca ²⁺	12.86	Pentamer
hCRP	x 10 HDPC	Tris-Ca ²⁺	6.94	Multimeric (void)
hCRP	x 10 HDPC	Tris-EDTA	12.61	Pentamer
100 mg/l	<i>Molar excess with respect to CRP protomer</i>		* Void volume	
<i>Column: Superdex 200 HR10/30</i>				

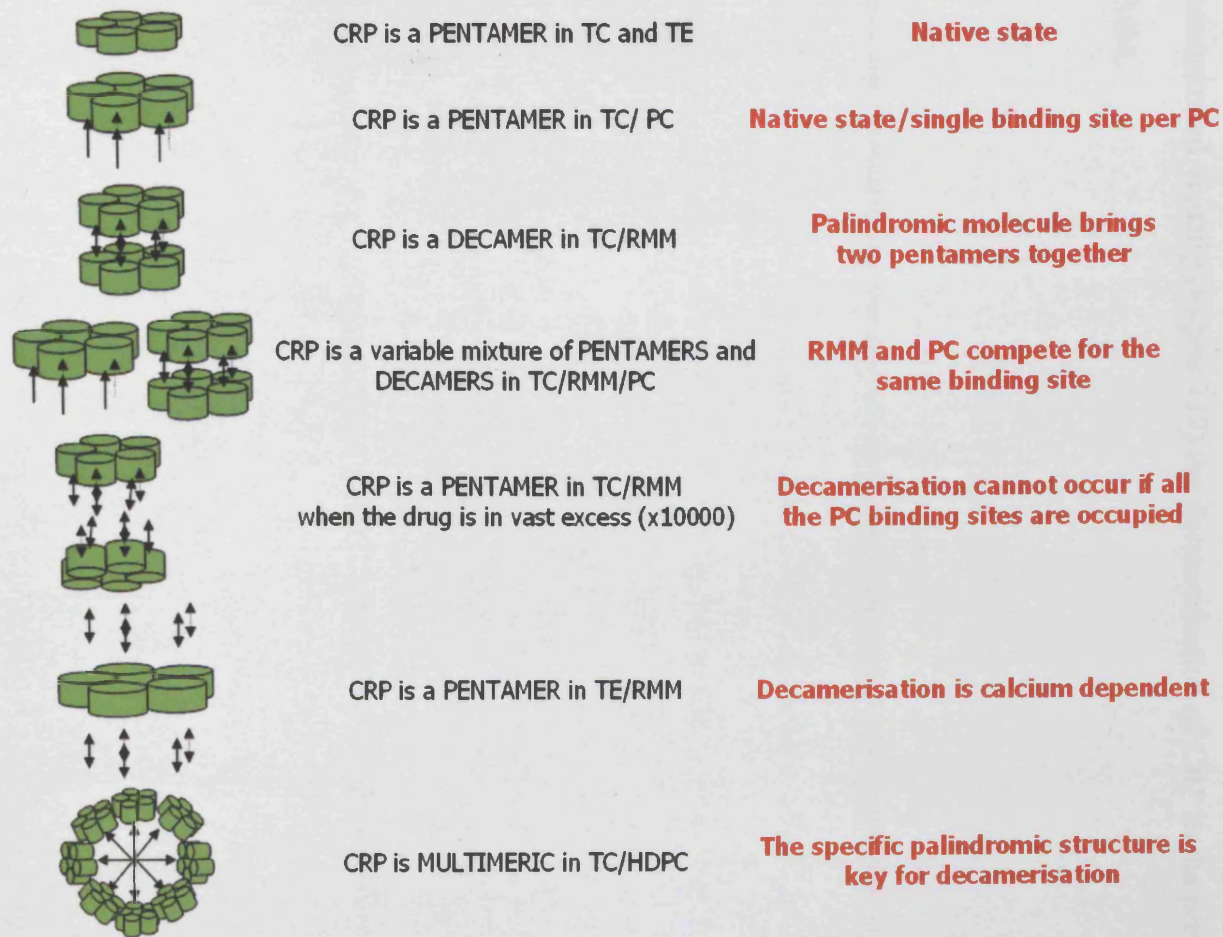
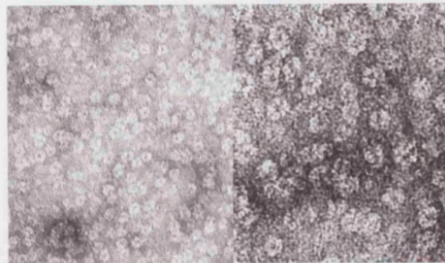


Figure 116 Representation of molecular assembly of CRP with different ligands as revealed by gel filtration

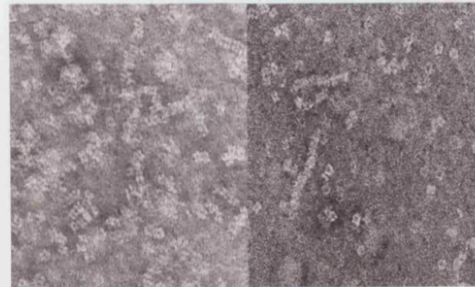
Electron microscopy

Osmand [61] studied CRP by negative-stain electron microscopy and confirmed that the protein was composed of five subunits arranged in cyclic symmetry. Using electron microscopy we studied the effects of RMM on CRP assembly and demonstrated visually (Figure 117) the decamerisation of CRP in the presence of RMM.

Pentameric CRP
(x180K and x360K)



Decameric CRP
(x180K)



Transmission electron microscopy of 2% aqueous uranyl acetate negatively stained pure human CRP in the presence or absence of RMM (1000 fold excess).

Figure 117 Electron microscopy: face on and side views of drug induced CRP decamers

Tandem electrospray mass spectrometry

With appropriate sample desolvation and instrument pressures nanoelectrospray ionization mass spectrometry can be used for the analysis and mass measurement of non-covalent protein complexes, such as the protomeric assembly of the pentraxins, since it is possible to preserve native structure and higher-order non-covalent interactions. In the present study, we used the technique with Dr A. Aquilina and Professor C. Robinson of the University of Cambridge to study the effects of the presence of RMM on the higher-order structure of CRP.

Figure 118 demonstrates a calcium dependent decamerisation of CRP by the addition of RMM. The drug induced decamer differs from the non-ligand decamer by a mass equivalent to approximately 5 drug molecules (Figure 119); the non-ligand decamer is an A:A face artefact and there remains a very minor amount of non-ligand decamer in the +RMM spectrum. The extra mass observed beyond that calculated from multiples of the mass of a protomer of CRP arises from residual solvation of the protomers by water molecules and buffer salts, a feature often observed for non-covalent interaction of macromolecular complexes in the gas phase. The stability of the structure can be assessed by dissociation of monomeric CRP from the pentamer/decamer by increasing the sample cone voltage and notably in the presence of RMM, there is marked stability of the decamer (Figures 120 and 121).

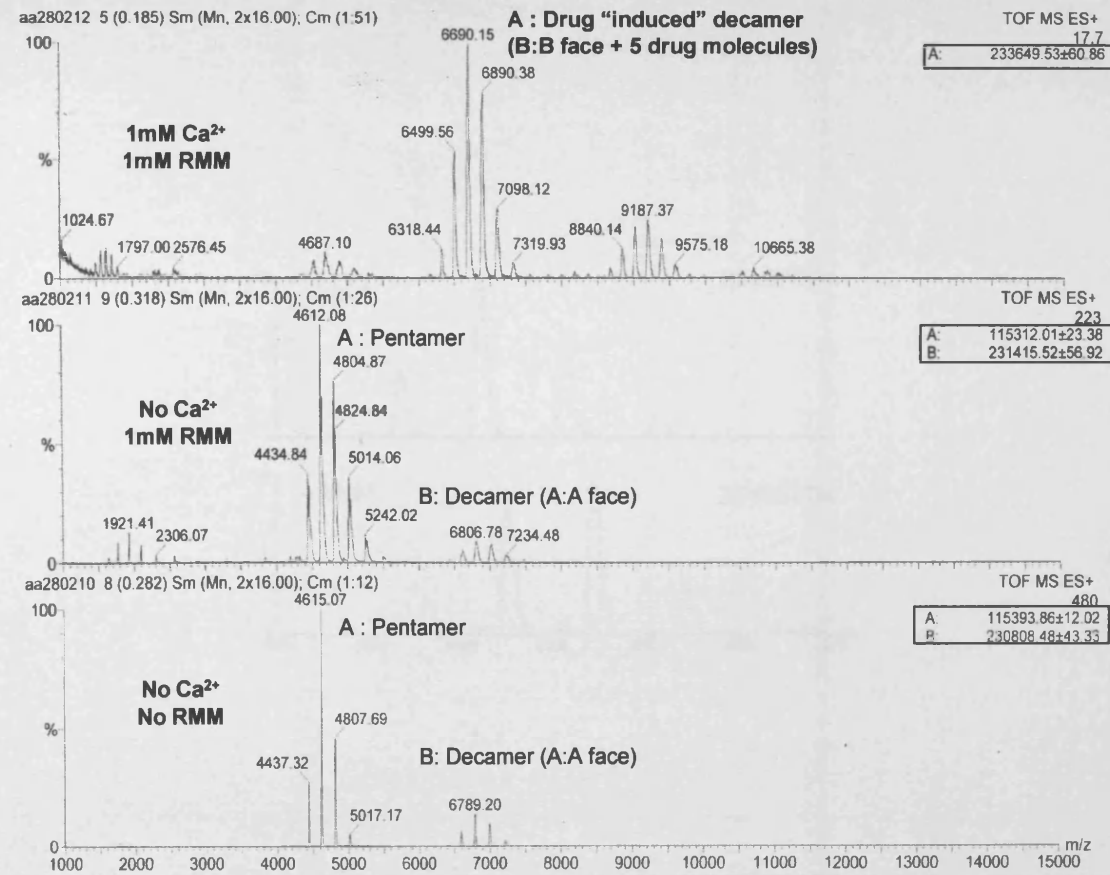


Figure 118 Mass spectrometry and CRP-drug interactions
 - 10 μ M hCRP [pentamer] in 200mM ammonium acetate pH 8

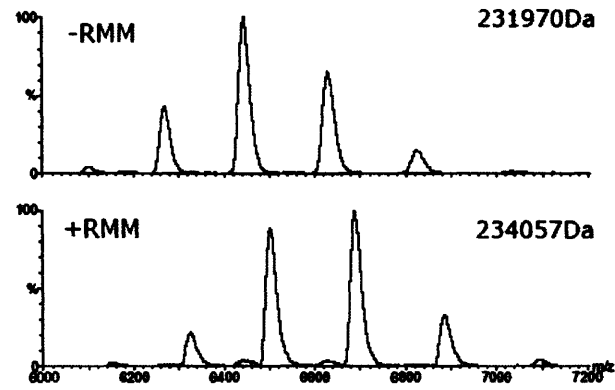


Figure 119 Calculated molecular weights of CRP decamers in presence and absence of RMM

- the difference in weights equals the mass of ~5 RMM molecules

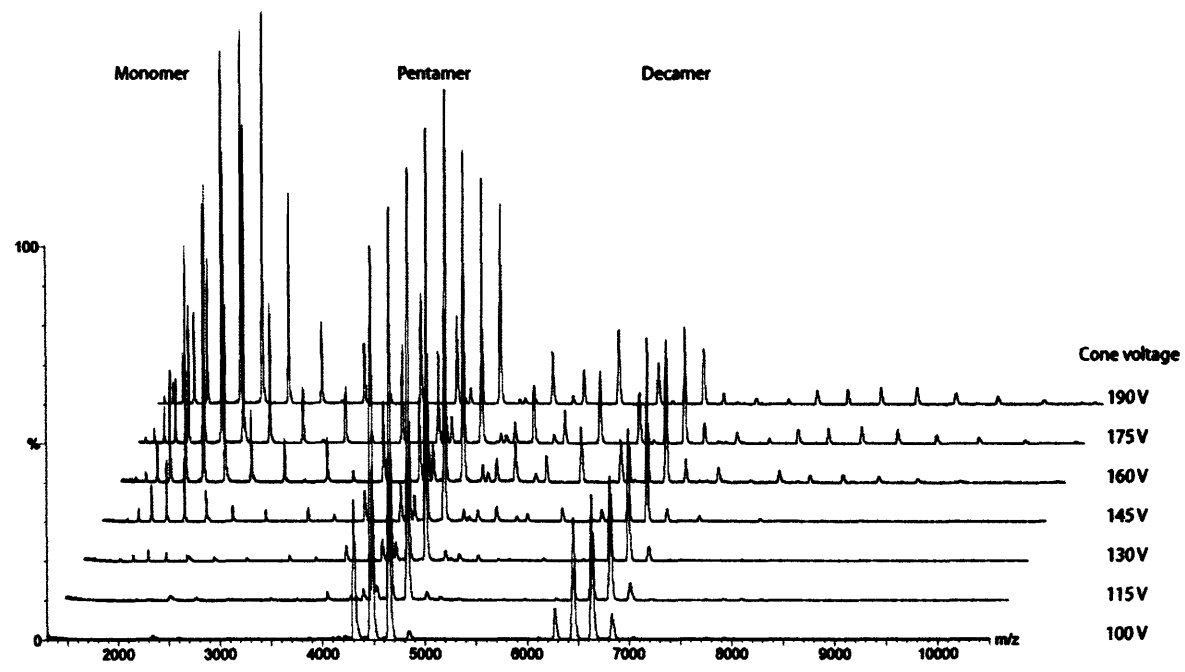


Figure 120 Effect of increasing cone voltage on the structure of native pentameric CRP

- 10 μ M hCRP, 1mM Ca²⁺, 200mM ammonium acetate, pH 8

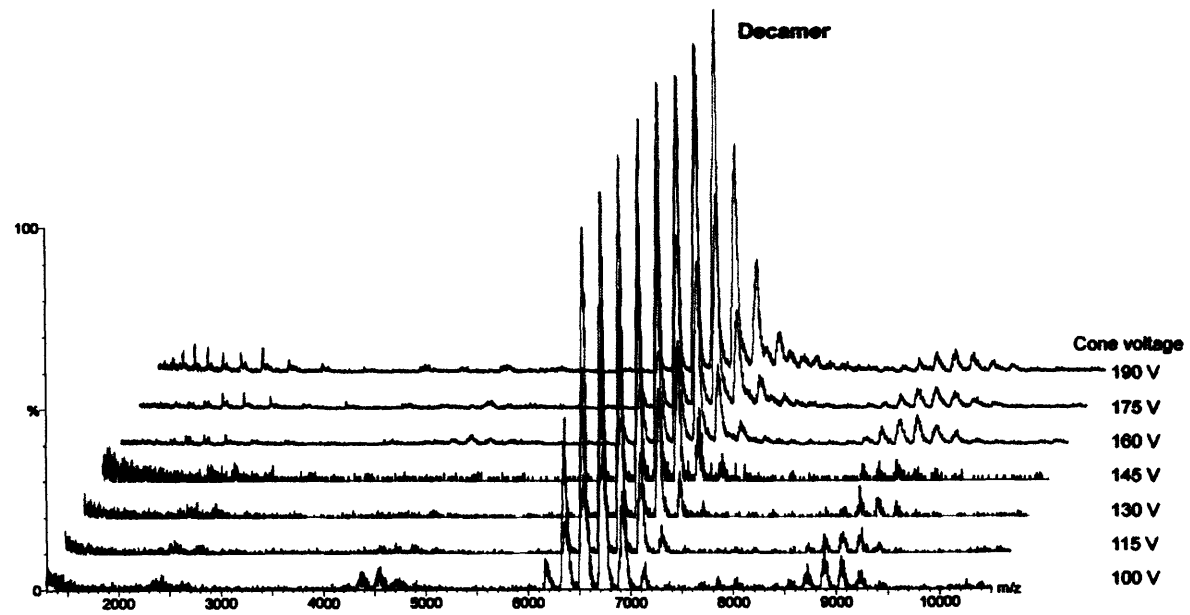


Figure 121 Drug induced decamer formation markedly stabilises the structure of human CRP

- 10 μ M hCRP, 1mM RMM, 1mM Ca²⁺, 200mM ammonium acetate, pH 8

X-ray Crystallography

Finally crystallography (performed and analysed by Professor S. Wood and colleagues, Southampton University) confirms decamerisation (Figure 122). Having previously designated the two planar faces of the human pentraxin molecules as A, bearing the single small α -helix present in each protomer, and B, bearing the calcium dependent ligand binding pocket of each protomer, the X-ray crystallographic structure of the CRP complex with RMM, confirms the arrangement of two native pentameric CRP molecules lying B face to B face and cross linked by 5 palindromic RMM molecules with their phosphocholine head groups located in the calcium dependent ligand binding pockets on each protomer.

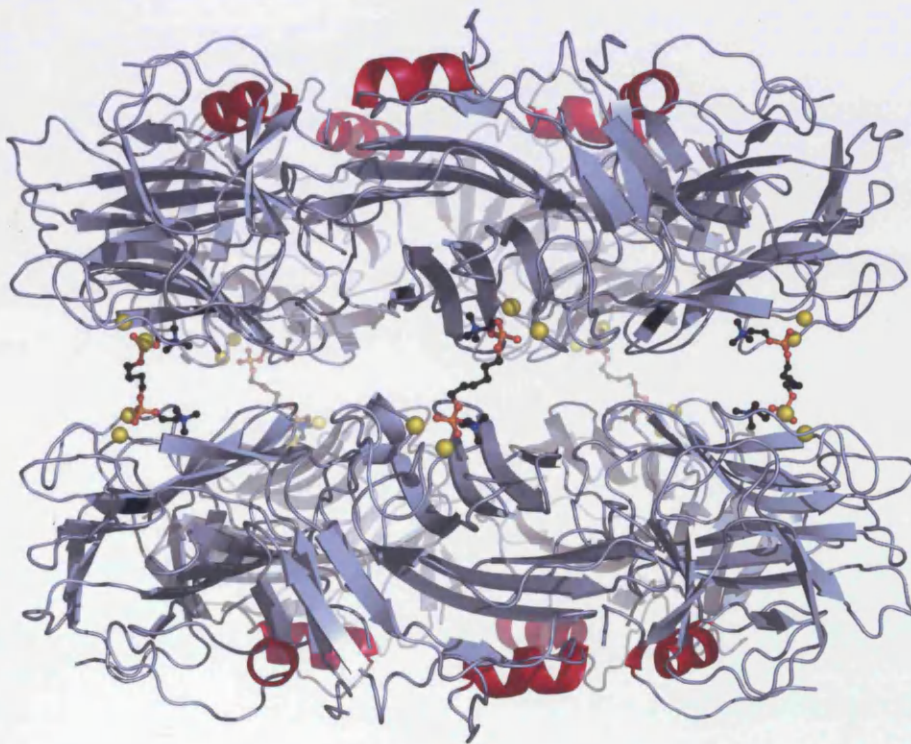


Figure 122 X-ray crystallography of drug induced CRP decamerisation

- View of complex perpendicular to five-fold axis and along the local two-fold axis relating pairs of subunits; calcium ions are yellow; drug molecules are red.

13.6.3.3 Ligand bound C-reactive protein loses immunoreactivity in the

MIRA

As there was no direct assay available to measure RMM concentrations we sought a robust and reliable method of demonstrating CRP binding of RMM, and indirectly presumed decamerisation. It is already known that the calcium dependent binding site of CRP is a major epitope for antibodies raised against CRP [515, 516], and simply its occupation by ligand may affect reactivity greatly. Certain monoclonal antibodies may bind CRP only in the presence of calcium, and the calcium dependent binding can be inhibited by PC, indicating that the antigenic determinant is at or near the PC-binding site of CRP [516].

The Roche MIRA assay is a microparticle enhanced turbidimetric assay for human CRP. The assay uses two microparticle types differing in size and reactivity of the coated antibodies, which provides accuracy and precision across a wide range of CRP concentrations [343, 344]. The in house monoclonal antibodies, with different epitope specificities, are designated 36F12 and 21F12, and the binding of 21F12 to CRP is calcium ion dependent (Dr S. Eda, Personal communication, Roche Diagnostics, Basle; the assay buffers being accordingly adjusted to 20mM CaCl₂). The so called high reactive antibody, 36F12, is coated on large 221nm microparticles and the low reactive antibody, 21F12, is coated on small 127nm microparticles. The high reactivity antibody on large particles dominates the immunoreaction at low analyte concentration and provides the desired high analytical sensitivity; the low reactivity antibody coated to smaller microparticles provides less analytical sensitivity but tolerates very high analyte

concentrations. The optimised reaction mixture is a ratio of 9:1, small to large particles.

With this in mind we demonstrated that ligand bound CRP was not detected in this particular automated CRP assay, although it was clearly detected in other assays: the Dade Behring BNII Nephelometer, a different and independent automated latex enhanced immunoassay, as well as by traditional immunoelectrophoresis, in which a polyclonal anti-human CRP antibody is used in the presence of EDTA. Tables 50 and 51 demonstrate the loss of MIRA reactivity with different ligands, which is not mirrored in the BNII assay whilst Figure 123 demonstrates the effect in buffer or serum. Figure 124 confirms the presence of CRP by electroimmunoassay despite masking in the MIRA.

The MIRA assay thus provided a method of demonstrating ligand binding by CRP.

Table 51 Ligand binding and loss of MIRA reactivity

Fluid phase ligand	Concentration/Molarity	% CRP detected with respect to buffer reading
Tris-Ca ²⁺	-	100
CPS	62.5 mg/l	0.6
CPS	250 mg/l	0.7
CPS	500 mg/l	0.6
21F12	1000mg/l	0
21F12	100 mg/l	65.5
21F12	10 mg/l	90.2
21F12	1 mg/l	92.4
21F12	0.1 mg/l	93.6
21F12	0.01 mg/l	93.8
PC	4.34 mM *	6.9
RMM	4.34 mM *	4.1
	* approx. 1000 fold excess with respect to CRP protomer	100mg/l CRP spiked

Table 52 No loss of immunoreactivity in the BNII assay

- 100mg/l CRP spiked

Spiked hCRP + Buffer	MIRA concentration (mg/l)	BNII concentration (mg/l)
Tris-Ca ²⁺	99.1	104.5
RMM (4.34mM)	4.3	105
PC (4.34mM)	7.3	105.5

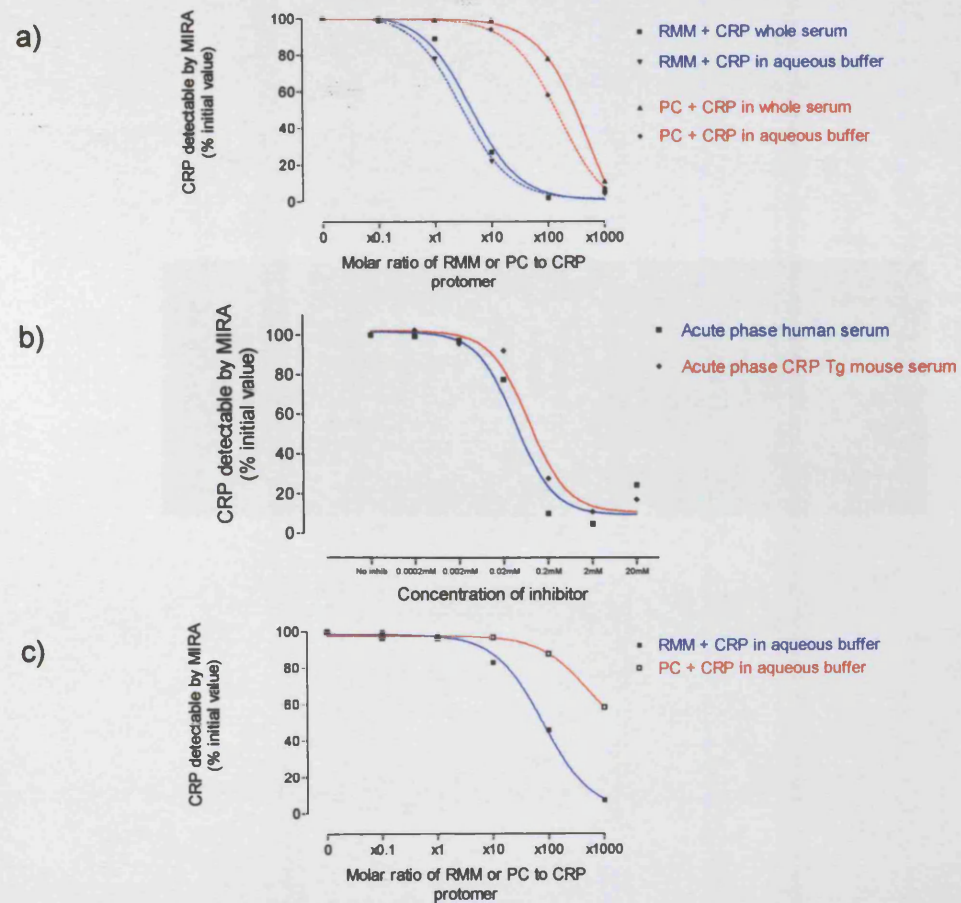


Figure 123 RMM masks MIRA CRP immunoreactivity in serum and aqueous buffer, at high and low CRP concentrations - Normal assay a) in buffer (100mg/l CRP) and b) in serum (100mg/l CRP); and c) high sensitivity assay (5mg/l CRP)

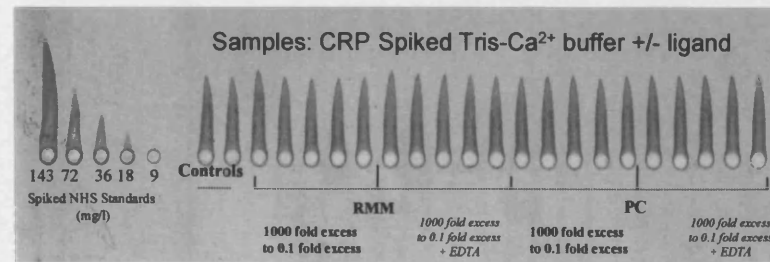


Figure 124 Rocket immunoelectrophoresis plate to visually confirm no loss of CRP antigen

- despite the loss of MIRA reactivity under varying conditions, immunoreactivity in a different assay persists

The formation by RMM of CRP dimers in which the calcium-dependent ligand binding site is occluded, clearly masks a relevant epitope needed by the particular antibody in question (21F12) and this makes the CRP-RMM complex undetectable (Figure 125).

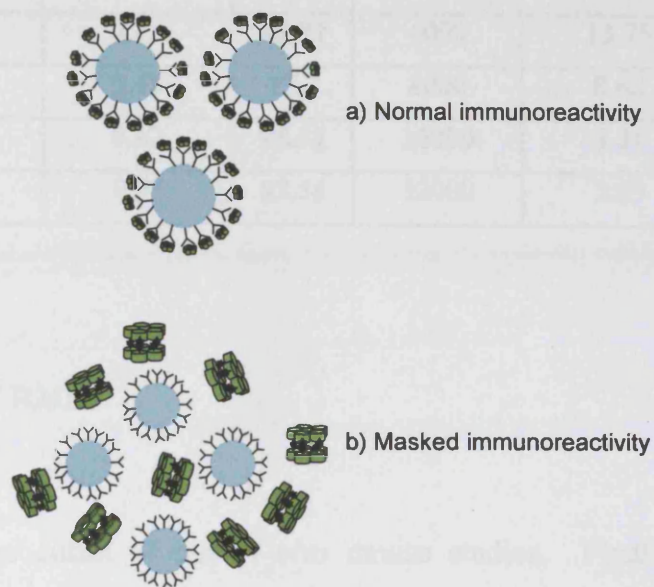


Figure 125 Diagrammatic representation of masking of immunoreactivity

By increasing the relative concentration of RMM to CRP, but accounting for the absolute amount of CRP (Table 53), we demonstrated that the relative loss of MIRA reactivity falls. Greater blockade of MIRA reactivity at RMM: CRP molar ratios that produce decamers is presumably related to the complete occlusion of the B face, in B-B dimers. When all the binding sites are occupied, RMM-CRP pentamers are formed and the antibody can presumably access its epitope again; experimentally this is seen as a lower rate of masking relative to the amount of CRP present.

Table 53 Indirect evidence of saturation binding of RMM to CRP

- relative reversal of loss of MIRA reactivity by presence of vast excess of drug

Buffer, TC, reading (mg/l)	Molar ratio of drug to protomer	MIRA reading	% <i>masked</i> wrt <i>buffer</i>	Molar ratio of drug to protomer	MIRA reading	% <i>masked</i> wrt <i>buffer</i>
163.75	100	1.5	99.08	1000	32.42	80.20
95.38	200	0.71	99.26	2000	21.98	76.96
41.75	400	0.49	98.83	4000	13.75	67.07
17.83	800	0.48	97.3	8000	8.62	51.65
7.26	1600	0.12	98.35	16000	5.31	26.86
2.07	3200	0.05	97.58	32000	2.03	1.93

13.6.3.4 *In vivo* activity of RMM

We had three goals at the outset of our *in vivo* mouse studies. Firstly to demonstrate efficacy in terms of *in vivo* decamerisation and loss of ligand binding properties, secondly to consider the effect of RMM on CRP turnover and finally to demonstrate a tangible reversible of a biological phenomenon mediated by CRP recognising a natural ligand. The first two questions were addressed by combining the loss of MIRA reactivity for CRP, with *ex vivo* assessment of CRP binding to immobilised phosphoethanolamine. By administering the drug not only as an intra-venous bolus with CRP, but also following continuous sub-cutaneous absorption from surgically inserted osmotic pumps (7 days), we were also able to demonstrate true *in vivo* interaction between human CRP and RMM.

Unlike CPHPC, which causes a dramatic enhancement in SAP turnover, there was no enhanced turnover of passively administered human CRP *in vivo* with RMM in the limited experiments performed, although there was clear evidence of circulating decamers, along with the abolition of ligand binding *ex vivo* (Figures 126 and 127; Table 54). Further work interestingly now suggests that if delivered at an appropriate concentration and duration, RMM does enhance CRP turnover (Personal communication, Professor M.B. Pepys).

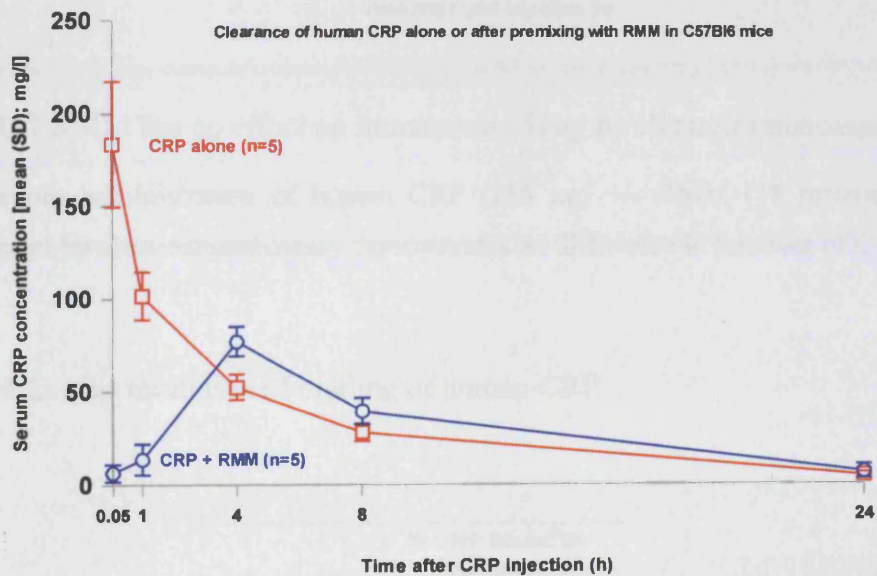


Figure 126 RMM masks MIRA CRP immunoreactivity *in vivo*

- intra-venous administration of human CRP (215 μ g) +/- RMM (18 micromoles)

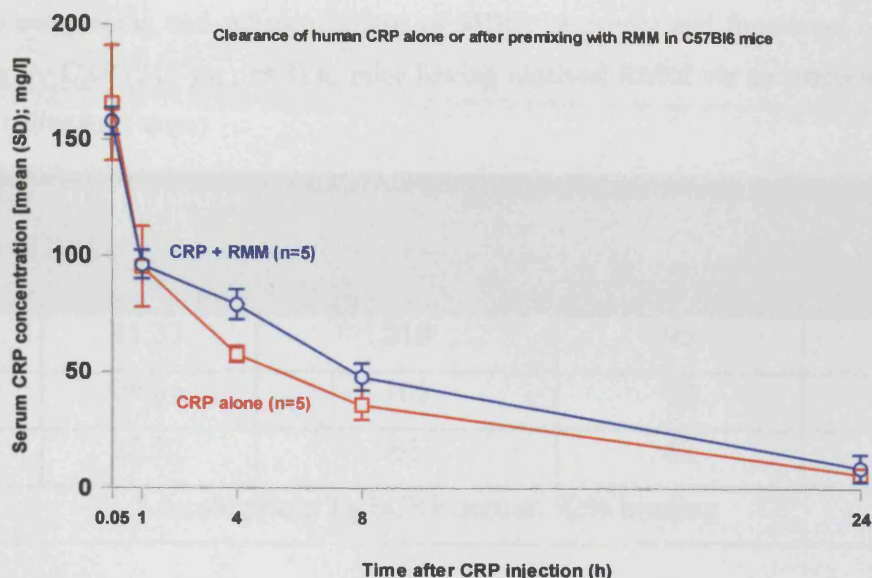


Figure 127 RMM has no effect on immunoreactivity by electroimmunoassay

- intra-venous administration of human CRP (215 μ g) +/- RMM (18 micromoles); measurement by electroimmunoassay demonstrates no difference in turnover of CRP

Table 54 *Ex vivo* inhibition of binding of human CRP

Time post CRP injection	% CRP bound to Sepharose-phosphoethanolamine	
	CRP alone	CRP + RMM
3 min	96	17
1hr	92	46

Continuous delivery of drug by osmotic pumps gave similar results, with a therapeutic level inferred of drug, which was functional (Tables 54 and 55). Whilst as yet we have not completed formal toxicology studies, no adverse effects were noted in the mice.

Table 55 Demonstration of *in vivo* complexing of CRP and RMM

– *in vivo* complexing and subsequent loss of MIRA reactivity and functional binding, following i/v CRP (215 µg ; n=3) to mice having received RMM via an osmotic pump (0.78M, 1µl/hr for 7 days)

Time	MIRA (mg/l)	Rocket immunoelectrophoresis (mg/l)	% masked reactivity	% binding to Seph PE *
3 min	11.37	210	95	57
1hr	29.86	105	72	71.3
4hr	23.72	63	62	92.2
* Acute phase Tg hCRP serum: 92% binding				

Table 56 Osmotic delivery of RMM or vehicle and *in vivo* loss of MIRA reactivity

– *in vivo* complexing and subsequent loss of MIRA reactivity following i/v CRP (215 µg; n=3) to mice having received RMM via an osmotic pump (0.78M, 1µl/hr for 7 days)

Time	MIRA (mg/l)		% masked reactivity
	RMM	TC	
3 min	62.77	212.97	70.5
1 hr	82.72	137.48	39.8
4 hr	75.53	90.65	16.7

In vivo human CRP is known to be protective against lethal *Streptococcus pneumoniae* challenge in mice [100, 103]. Abrogation of this effect would be an important proof of principle for effectiveness of RMM, whilst awaiting other, more technically complex, *in vivo* models (e.g. rat myocardial infarction). This model in particular is important because it is presumed that the protective effect of CRP arises precisely because of the avid ligand binding interaction that occurs between it and the C-polysaccharide in the *S. pneumoniae* cell wall.

Preliminary work was necessary to establish the best conditions in which to test RMM. Tables 57, 58, 59 and 60 demonstrate the binding of CRP to different *S. pneumoniae* strains *in vitro* (in buffer, as well as serum, using direct and indirect measures of CRP binding) and how this binding is inhibitable with calcium chelation, as well as by ligands including RMM.

Table 57 Calcium dependent CRP binding to *S. pneumoniae* strains Wt and D39-6

Strain	EDTA (~10mM)	Amount of CRP bound in pellet fraction (ng)
Wt (R6, unencapsulated strain)	No	3.5
	Yes	1.2
D39-6 (Type 2 encapsulated serotype)	No	7.3
	Yes	1.4
Pelleted $\sim 5 \times 10^7$ bacteria Offered 64ng radiolabelled hCRP		

Table 58 RMM inhibits CRP binding to *S. pneumoniae* strain D39-6

Strain	EDTA ~10mM	RMM ~165µM	Amount of CRP bound; pellet fraction (ng)
D39-6	No	No	2.5
D39-6	Yes	No	0.3
D39-6	No	Yes	1.6
Pelleted $\sim 2.5 \times 10^6$ bacteria Offered 64ng radiolabelled hCRP			

Table 59 CRP binds to both *S. pneumoniae* strains WU2 and A66.1

- as evident by loss of recovery of offered CRP (MIRA determination of supernatant)

Strain	CRP offered (µg)	CRP recovered (µg)	Pellet ~
WU2 Type 3	16	0.06	4.5×10^8
A66.1 Type 3	16	2.9	9×10^7

Table 60 CRP binds specifically to strain WU2 in serum

- *S. pneumoniae* WU2: pellet 2.5×10^8 ; normal human serum spiked to ~ 200 mg/l human CRP; inhibition of CRP binding by calcium chelation, PC and RMM.

Bacteria	Ligand	Conc. of supernatant by RIE (mg/l)
No	Tris-Buffer	218
Yes	Tris-Buffer	122
Yes	EDTA 10mM	202
Yes	PC 8.69mM	220
Yes	RMM 8.69mM	202

When given passively human CRP did not protect against lethality from i/p administration of strain D39-6 into female C57/BL6 mice, despite adjustments in the dose of bacteria and the amount of human CRP injected at baseline (Table 61).

Table 61 *In vivo* lethality with strain D39-6 of *S. pneumoniae*

- no protection by human CRP (as compared to vehicle); n= 10 per group of age and weight matched female C57/BL6 mice; CRP or buffer (TC) i/p at -15 minutes

5 x 10³ bacteria i/p = LD100 at 72hrs

No protection from 80µg hCRP i/p

Partial protection with 5 x10² bacteria and 800µg hCRP i/p
(Log rank P =0.02 at 72hrs)

Using strain WU2 and age/weight matched female C57/BL6 mice (8 week old / 20-25g), intra-peritoneal administration of CRP, 15 minutes prior to administration of bacteria, significantly protected against the lethal effects of intra-peritoneal *S. pneumoniae* (Figures 128 and 129). Moreover RMM given passively significantly abrogated this effect (Figures 130 and 131). In the first experiment (Figure 130) with RMM employing 1000 organisms per mouse, survival was enhanced by CRP as compared to buffer (P=0.02; Breslow) whilst the RMM treatment reversed this; the difference between the RMM + CRP treated group and the CRP alone treated group being highly significant (P=0.02 Log Rank and P= 0.01 Breslow). In the second experiment (Figure 131) with RMM employing 100 organisms per mouse, survival once again for the CRP treated group as compared to buffer was significantly improved (P=0.03 Log Rank and P=0.01 Breslow). Once again RMM treatment reversed this; the

difference between the RMM + CRP treated group and the CRP alone treated group being highly significant ($P= 0.03$ Breslow).

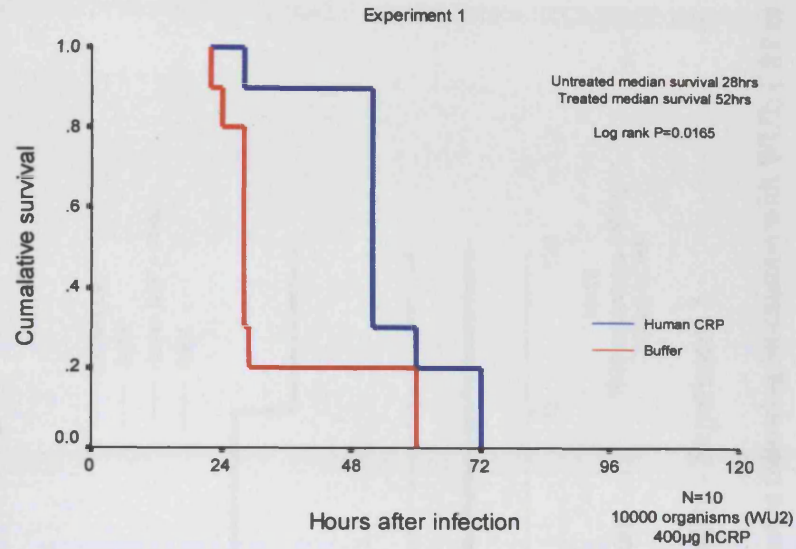


Figure 128 Protective effect of human CRP in *S. pneumoniae* lethality - Experiment 1

- n= 10 per group of age and weight matched female C57/BL6 mice; CRP or buffer (TC) i/p at -15 minutes

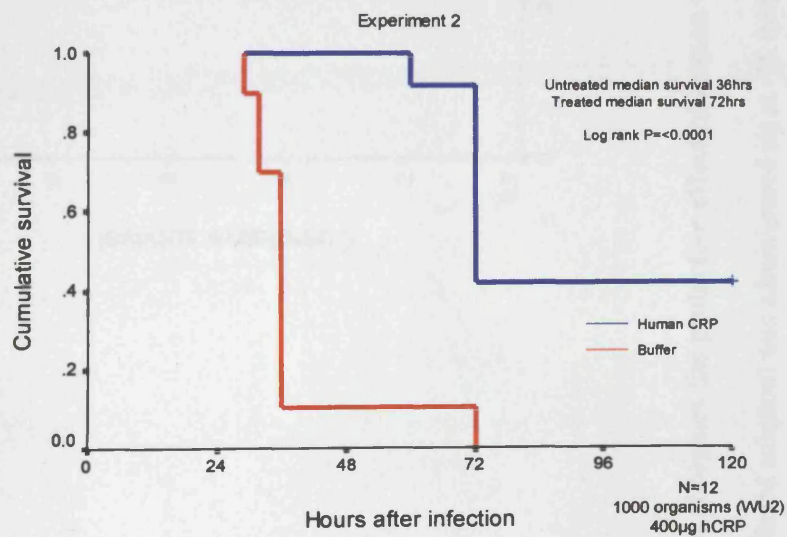


Figure 129 Protective effect of human CRP in *S. pneumoniae* lethality - Experiment 2

- n = 12 per group of age and weight matched female C57/BL6 mice; CRP or buffer (TC) i/p at -15 minutes

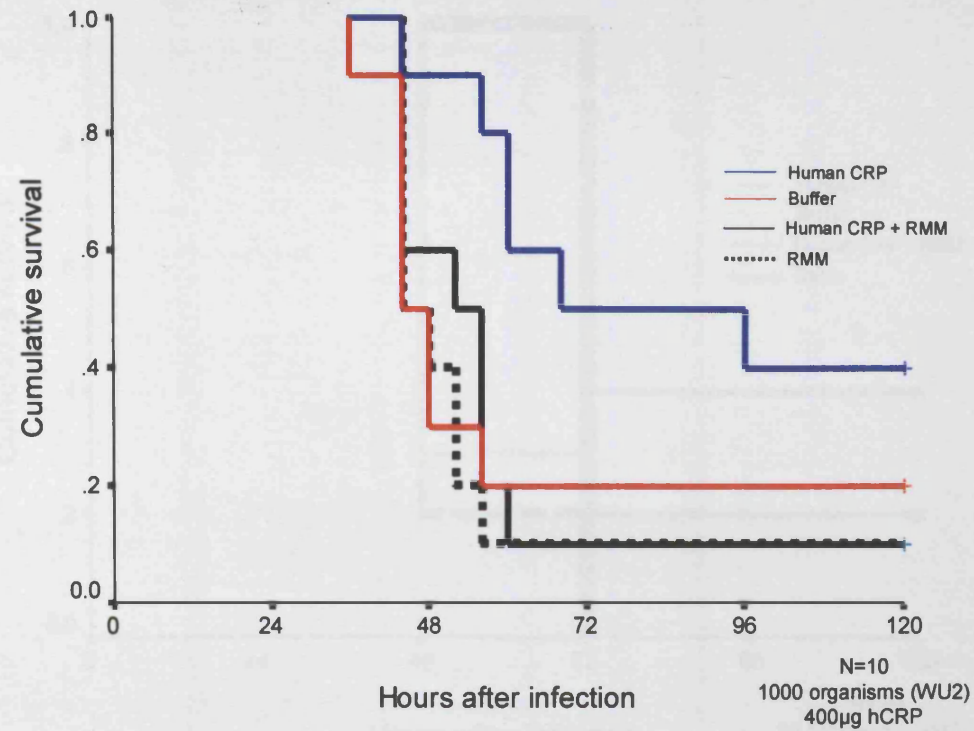


Figure 130 RMM abrogates the protective effect of human CRP in *S. pneumoniae* lethality - Experiment 3

- RMM (100µl, 200mM solution) was administered i/p at -15 minutes, 2 hours, 4 hours and 6 hours following inoculation with WU2; CRP or buffer (TC) was given i/p at -15 minutes; n= 10 per group of age and weight matched female C57/BL6 mice

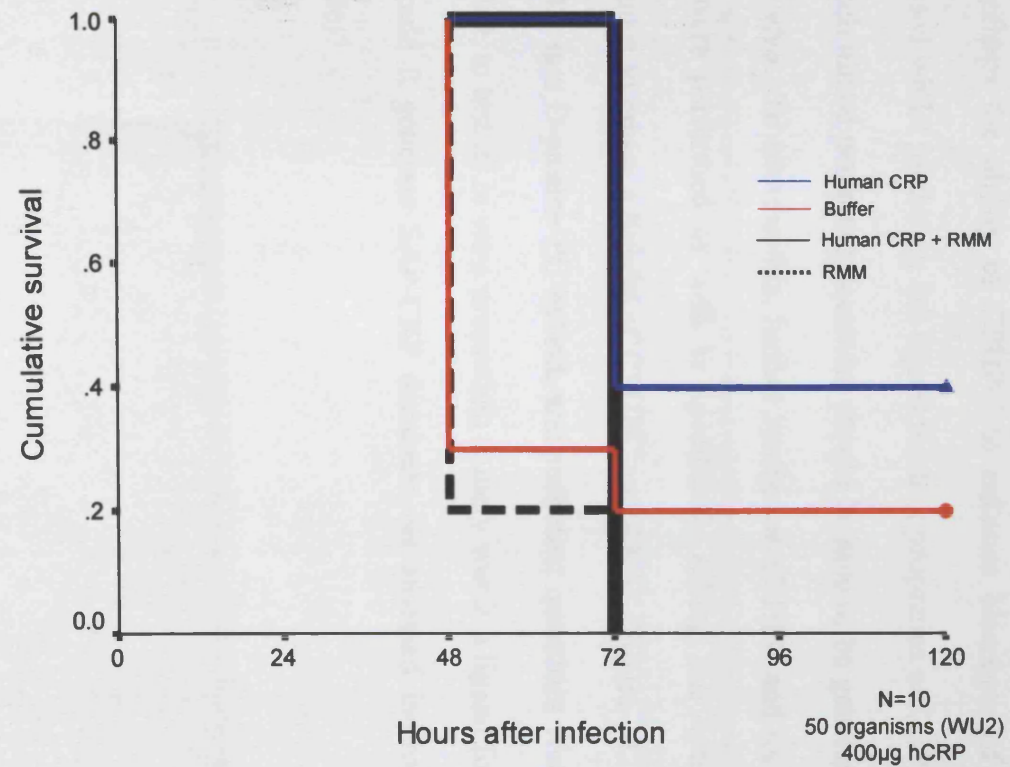


Figure 131 RMM abrogates the protective effect of human CRP in *S. pneumoniae* lethality -Experiment 4

- RMM (100µl, 200mM solution) was administered i/p at -15 minutes, 2 hours, 4 hours and 6 hours following inoculation with WU2; CRP or buffer (TC) was given i/p at -15 minutes; n= 10 per group of age and weight matched female C57/BL6 mice

13.6.4 Mixed pentraxin assembly

Having successfully demonstrated clear proof of principle with RMM we extended our studies to further novel pentraxin interactions. We hypothesised that perhaps the ability of CPHPC to enhance clearance of SAP could be harnessed whilst inhibiting the ligand binding properties of CRP. We speculated that such mixed pentraxin decamers should be able to be generated both *in vitro* and *in vivo*. To address this, further studies on CPHPC and its interaction with CRP were performed as well as specifically asking our synthetic chemistry colleagues to create a hybrid of CPHPC and RMM (Figure 132). The product, AJC042, is a D-proline-PC hybrid, and sufficient quantities were produced and validated, to test its *in vitro* properties; namely was it a ligand for CRP and SAP and could it generate SAP-CRP decamers (as assessed indirectly in the first instance)?

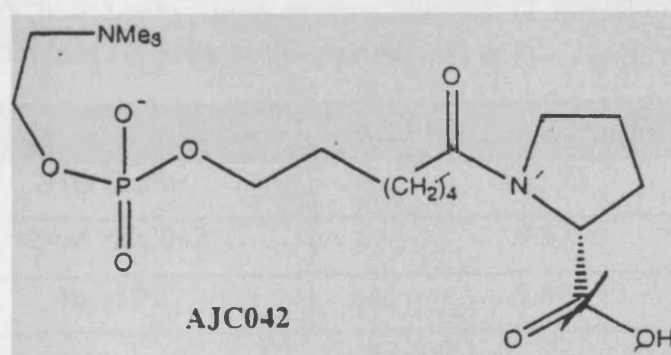


Figure 132 Structure of AJC042

In the absence of a ligand, isolated human SAP auto-aggregates and precipitates in the presence of calcium [357]. Inhibition of aggregation of SAP in the

presence of calcium can therefore be used to screen for SAP ligands. Table 62 shows how AJC042 prevented calcium induced aggregation of SAP.

Table 62 AJC042 prevents calcium induced aggregation of pure human SAP

- as assessed by spectrophotometry; using an extinction coefficient of 1.71 for 1mg/ml; 100mg/l starting concentration of human SAP

Ligand *	Calcium (3mM)	Abs 320	hSAP (mg/l) in supernatant
Tris Buffer	No	0.0052	92.6
Tris Buffer	Yes	0.1780	11.3
AJC042	Yes	0.0027	104.4

* ~4mM AJC042 vs ~4μM SAP protomer

As already demonstrated loss of MIRA reactivity can be used to screen for CRP ligands and AJC042 in TC buffer, spiked with human CRP, behaved similarly *in vitro* (Table 63).

Table 63 AJC042 is a CRP ligand as demonstrated by loss of MIRA reactivity

- CRP (100mg/l) spiked TC buffer with either AJC042 or PC

Ligand	CRP MIRA reactivity (mg/l)
Tris Buffer	105.73
4mM AJC042	9.37
4mM PC	9.56

I hypothesised that co-incubation of SAP and CRP with AJC042 should therefore lead to further loss of MIRA reactivity, if as predicted, mixed SAP-CRP decamers are formed, and behave like CRP decamers 'immunologically'. Tables

64 and 65 demonstrate this precise effect, with enhanced loss of MIRA reactivity when both SAP and CRP are present (with a graded effect as the concentration of SAP is increased), supporting our prediction, for both buffer and serum. Note that alone SAP does not interfere with the MIRA assay.

Table 64 AJC042 and loss of MIRA reactivity with SAP and CRP in buffer

- CRP (100 mg/l) spiked buffer in presence of calcium, measured on the MIRA, with drug (or vehicle) and increasing concentrations of human SAP. The increased loss of MIRA reactivity in the presence of CRP, SAP and drug is taken to imply co-decamerisation.

Ligand (4mM)	Spiked SAP (mg/l)	CRP MIRA reactivity (mg/l)
Tris-Buffer	0	103.72
Tris-Buffer	100	102.34
AJC042	100	15.23
AJC042	50	27.85
AJC042	20	40.28
AJC042	10	39.52
AJC042	0	39.38

Table 65 AJC042 and loss of MIRA reactivity with SAP and CRP in serum

-CRP (100 mg/l) spiked into previously SAP/CRP depleted serum, measured on the MIRA, with drug (or vehicle) and increasing concentrations of SAP. The increased loss of MIRA reactivity in the presence of CRP, SAP and drug is taken to imply co-decamerisation.

Ligand	Spiked SAP (mg/l)	CRP MIRA reactivity (mg/l)
Tris-Buffer	200	97.83
Tris-Buffer	0	92.84
AJC042 : 8mM	200	35.85
AJC042 : 8mM	0	60.33
AJC042: 4mM	200	51.96
AJC042 : 4mM	100	65.76
AJC042: 4mM	0	75.50
AJC042 : 400 μ M	200	71.56
AJC042 : 400 μ M	0	101.59

In our plate assay CPHPC was shown to inhibit CRP binding to immobilised ligand with an IC_{50} in the millimolar region (see Figure 104). Our collaborators at Southampton University (Professor S. Wood), using isothermal calorimetry, estimate a K_d of 15 μ M for SAP/D-proline and 4 μ M for CRP/PC (*cf.* 400nM for CRP/RMM and 10nM for SAP/CPHPC). Whilst testing AJC042, we also studied CPHPC and intriguingly, under certain conditions it avidly cross links CRP and SAP, as demonstrated by the robust technique of size exclusion/gel filtration chromatography. The presence of PC reverses the effect and using

immunoassays on the relevant elution fractions, co-elution of mixed decamers was confirmed immunochemically (Tables 66 and 67).

Thus although CPHPC in itself is not an avid CRP ligand, presumably when bound to SAP, the ligand binding properties change such that mixed decamer formation is favoured; most likely the binding of multiple CPHPC molecules by each SAP pentamer creates an array of ligands for CRP which although independently weak, provide sufficient avidity to favour formation of a stable decamer.

Table 66 Mixed pentraxin assembly with CPHPC as assessed by gel filtration

hSAP (200 mg/l)	hCRP (200 mg/l)	CPHPC (4 mM)	PC (4 mM)	TC/TE	Elution volume (ml)	
					"Decamer"	"Pentamer"
Yes	Yes	No	No	TE	11.15	12.78
No	Yes	Yes	No	TC		13.01
Yes	No	Yes	No	TC	10.70	
Yes	Yes	Yes	No	TC	11.02 *	
Yes	Yes	Yes	Yes	TC	10.65	13.05

Column: Superdex 200 HR10/30

Table 67 Co-elution is confirmed by immunoassay of the appropriate (*) fractions

Relevant fraction	Concentration (mg/l)		
	hCRP (MIRA; mg/l)	hCRP (BNII; mg/l)	hSAP (mg/l)
	<i>Masked immunoreactivity</i>	<i>No masking</i>	<i>Rocket immuno-electrophoresis</i>
<i>B11</i>	0.92	9.16	12
<i>B12</i>	1.01	17.90	24
<i>B13</i>	0.97	10.70	15
<i>B14</i>	0.63	5.11	8
<i>B15</i>	0.23	1.16	3

Once again utilising the loss of MIRA reactivity, also allows for the demonstration of this novel effect. Furthermore competition for ligand binding sites can be inferred by co-incubation of both AJC042 and CPHPC (Table 68).

Table 68 Effect of CPHPC and AJC042 on MIRA reactivity in spiked serum

- CRP (100 mg/l) spiked CRP/SAP depleted serum

Ligand	Spiked hSAP mg/l	CRP MIRA reactivity (mg/l)
Tris-buffer	0	112.98
Tris-buffer	200	113.26
AJC042 : 4mM	0	85.47
AJC042 : 4mM	200	58.38
CPHPC : 800 μ M	0	107.82
CPHPC : 800 μ M	200	10.99
CPHPC : 800 μ M	400	1.54
AJC042 (4mM) and CPHPC (800 μ M)	200	82.78

These are important *in vitro* observations of the ability for mixed decamers to form with two compounds, one a D-proline palindrome, the other a D-proline-PC compound. However CPHPC has been given *in vivo* to patients with human amyloidoses [94]. Was there evidence of mixed decamer formation *ex-vivo*? The BNII assay for human CRP is not influenced by ligand bound CRP in the same way as the MIRA assay, and we used this to study the very limited serum samples available from patients who had been given high dose CPHPC. Table 69 suggests the effect demonstrated above occurs *in vivo*, with preliminary

evidence furthermore of the loss of binding properties for CRP that is complexed with SAP. Data with respect to SAP concentration and CPHPC concentration was available within the laboratory and is included for reference [94].

Table 69 MIRA reactivity and binding to immobilised ligand in patients receiving CPHPC

- evidence of *in vivo* mixed decamer formation (loss of MIRA reactivity) and loss of ligand reactivity *ex-vivo* (reduced binding to Sepharose-PE) in patients on a continuous CPHPC infusion.

Patient/Time post infusion	CPHPC (μ M)	SAP mg/l (Rocket immuno-electrophoresis)	CRP mg/l (MIRA)	CRP mg/l (BNII)	% CRP bound to Seph-PE
JB T 0	0	37	6.2	5.5	57
JB T 6hr	165	7	2.9	5.4	0
PMT 0	0	67	3.4	2.8	53
PMT 6hr	52.1	3	1.4	2.8	26

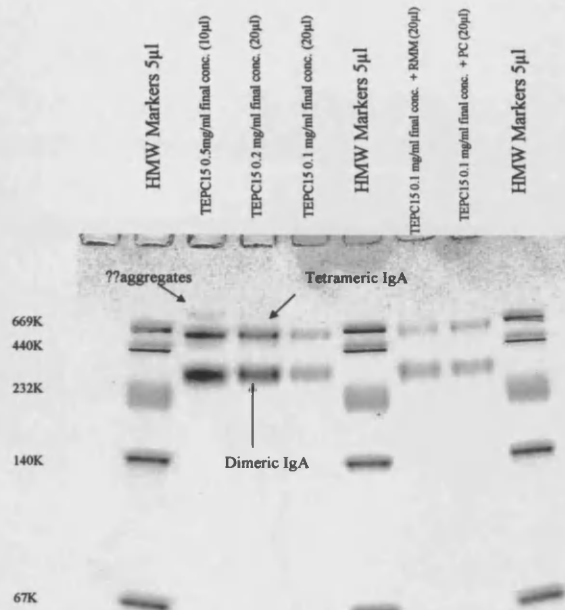
13.6.5 RMM and ligand binding to a monoclonal IgA (kappa)

recognising phosphocholine

Phosphocholine is a target of a number of the autoantibodies present in the conditions of systemic lupus erythematosus and primary antiphospholipid syndrome. Whilst the role for anti-phospholipid antibodies in the disease manifestations are debated (which intriguingly include a very high cardiovascular mortality), finally we speculate that a bisphosphocholine molecule may have applications in removing or preventing the potentially

deleterious effects of such antibodies. Albeit with now limited supplies of RMM, and TEPC-15 a mouse monoclonal IgA (κ) that recognises PC, preliminary work presented below does indeed suggest that RMM has the potential to cross link this antibody. TEPC-15 was predominantly either tetrameric or dimeric at least by Native-PAGE, whilst SDS-PAGE clearly revealed the heavy and light chains (Figures 133 and 134). I looked for changes in structure by size exclusion chromatography after incubation with either RMM or PC (with no discernible effects being present by Native-PAGE). In buffer alone TEPC-15 interacts with the column (perhaps because of trapped lipid or specific/non-specific binding interactions with the column). The presence of PC seems to overcome this, suggesting some specificity in the interaction with the column (Figure 135). However quite clearly with RMM, as compared to PC, the gel filtration profiles are indicative of an effect whereby (Figure 136) increasing concentrations of RMM lead to multimeric complexing of TEPC-15.

These are preliminary and limited observations but merit further work.



Thyroglobulin (669K), Ferritin (440K), Catalase (232K), Lactate Dehydrogenase (140K) and Albumin (67K).

Figure 133 Native PAGE for TEPC-15

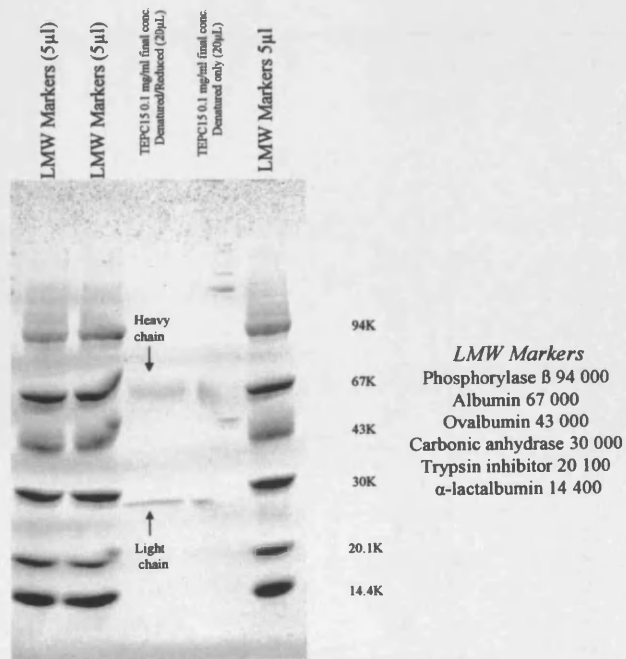


Figure 134 Denatured/Reduced SDS gel for TEPC-15

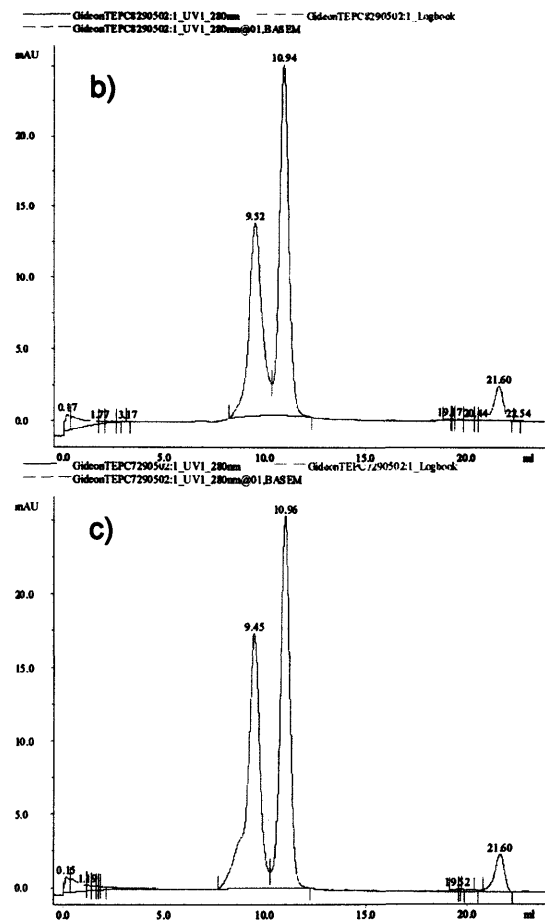
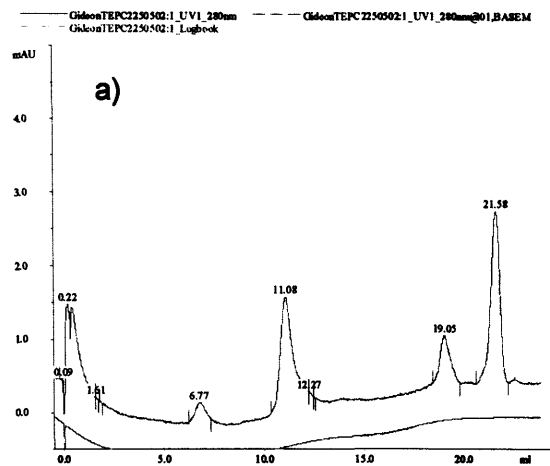


Figure 135 Gel filtration profiles for TEPC-15 in the presence and absence of PC

- a) Tris-NaCl, b) PC 133 μM and c) PC 13.3 mM; TEPC-15 at 200 mg/l

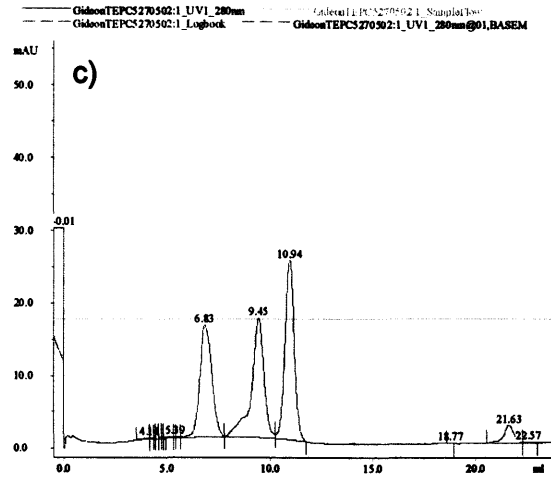
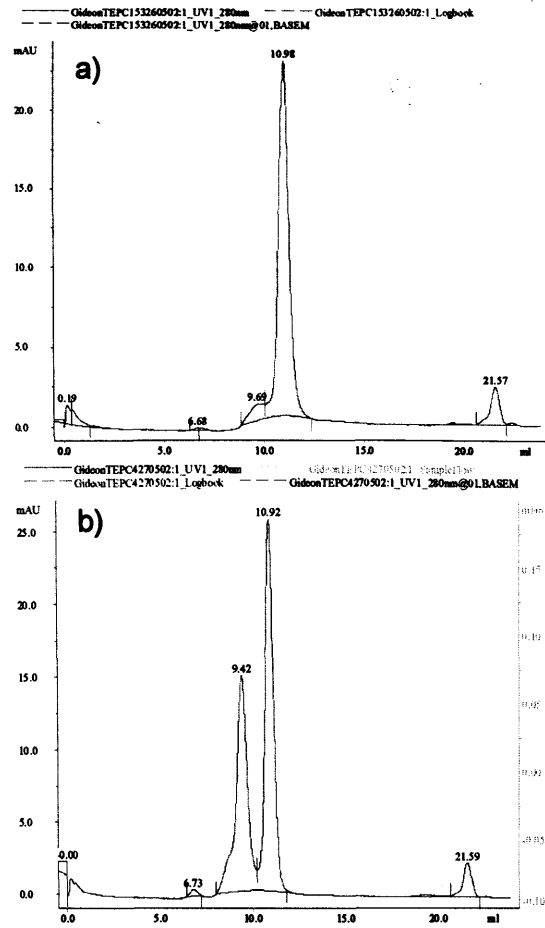


Figure 136 Clear graded effect of increasing concentrations of RMM on the elution volumes of TEPC-15

- a) 133 μ M, b) 1.3 mM and c) 13.3 mM RMM; TEPC-15 at 200 mg/l

13.7 Discussion

Understanding the biological properties of the ligand binding protein CRP remains a challenge for many investigators, and has been made more pressing by the evidence, both epidemiological and experimental, that inhibition of CRP-binding and its effects, may actually be an important therapeutic target.

Harnessing and extending an established platform, based on previous research with human SAP, has led to our synthesis of an entirely novel and unique inhibitor of CRP ligand binding. This chapter outlines how this was accomplished by:

- Generating robust platforms for testing potential inhibitors
- Clearly demonstrating inhibition of CRP ligand binding, and its downstream effects, *in vitro*, to immobilised and fluid phase physiological ligands
- Studying and confirming, by a number of independent methods, the predicted mode of action of our lead compound
- Documenting *in vivo* activity in a biologically meaningful model
- Extending our observations to other novel drug molecules and protein targets

All work has limitations and drug development is a lengthy process. We remain at an early stage and throughout have been limited physically by available drug.

Sufficient quantities are required for formal pharmaco-kinetic, -dynamic, and – toxicological studies. We are unable to measure the concentration of drug at present and poorly appreciate its turnover *in vivo*. Although structural work, including now X-ray crystallography and latterly isothermal calorimetry is more advanced, greater detail of the bioenergetics and biophysics of the interaction between RMM and CRP would be desirable, not just for completeness sake but also to guide improvements in future drug molecules. Equilibrium dialysis studies, density ultracentrifugation and computer modelling could all throw light on the interactions taking place, as well as suggesting possible modifications. Much of the data above is by its very nature indirect, particularly using the so-called “MIRA effect”, which cannot be further clarified without the ability to directly alter the assay properties (not something we as end users can readily achieve). Nevertheless it represents a very effective and efficient automated tool that might aid screening other similar ligand binding inhibitors of CRP.

With respect to the present inhibitors described, it has been known for some time that after binding of the divalent calcium cation, CRP binds phosphate monoesters with a stoichiometry of one mole per mole of CRP subunit [45]. Replacement of the phosphate monoester group by other acidic groups or by conversion to a phosphodiester markedly diminishes or abolishes the ability to bind. Our findings from our plate assays are fully in keeping with this. PC is bound by CRP with much higher affinity than other phosphate monoesters, as assessed indirectly in inhibition assays.

We estimated that RMM had an IC_{50} approximately 10 times less than that of PC, and again calorimetry studies, that measure the related but distinct binding affinity, confirm this qualitative ballpark figure (K_d for CRP/RMM 400nM). The enhanced affinity of a bisphosphocholine ligand is fully expected for a molecule with the structure of CRP, as it is already known that the binding affinity of a ligand increases tremendously when multiple PC residues are present on a macromolecular structure. The dimerisation of CRP by RMM, and the increased stability of this entity, is in keeping with this. Changing the chain length or structure may, or may not, also of course generate a more avid inhibitor of CRP and this is something currently being addressed. Finally a CRP mutant incapable of binding to PC has been described in the literature [120], and this would certainly be an interesting protein with which to study in the context of our inhibitor, as a final proof of specificity.

All *in vivo* models are beset with confounders (from technical aspects to animal heterogeneity) and lethality experiments are no exception. Further refining of the model to include continuous drug delivery or use of a human CRP transgenic strain would of course be ideal. Finally extending the *in vivo* data to a rat myocardial infarction model is a priority as Streptococcal lethality in mice was chosen as the simplest biological model for the initial proof of principle.

The ability of the pentraxins to co-decamerise is interesting. The fact that CPHPC has a low affinity for CRP alone, but a clear affinity when ligand bound to SAP is quite intriguing, but probably just shows that despite distinct differences between pentraxins, sufficient similarity exists in the structure and

binding properties, for interactions to occur in, albeit experimental, settings. No immediate physiological role for this effect is apparent.

Whether or not the concept of mixed dimers can be extended to decamers of SAP with other unrelated proteins (or even CRP) remains to be seen. Unlike SAP, CRP turnover was not increased by decamerisation in these initial experiments, and this fits with a previous study investigating turnover *in vivo* with CPS [422]. Later experiments (Personal communication, MBP) do seem to demonstrate increased turnover, where the delivery rate and concentration of drug was higher (and in rats not mice). Although both proteins are cleared via the hepatocyte [26], different subtleties may exist. Further work in mice and rats with RMM will clarify this as will *in vivo* testing of AJC042 where the effect on clearance of a mixed decamer can be studied.

13.8 Summary

In summary, this chapter describes from laboratory bench to animal house, the development of a novel palindromic CRP inhibitor that through binding to the phosphocholine binding sites, decamerises an important human protein and in so doing inhibits its biological functions.

14 Discussion

C-reactive protein, the archetypal acute phase reactant continues to attract an enormous amount of interest (Figure 137), not only within scientific circles but within the media, especially in the America.

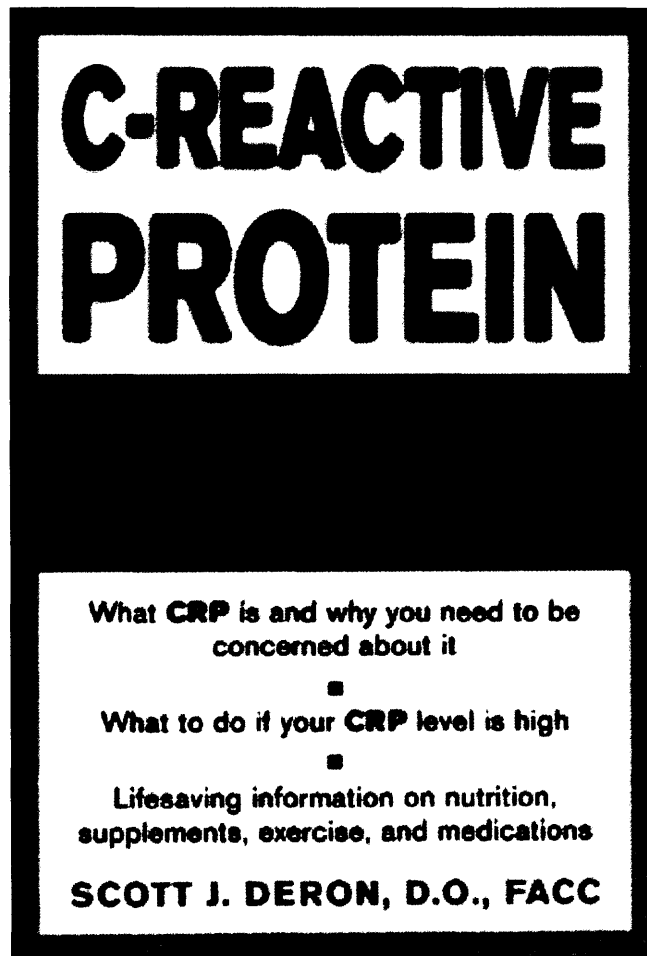


Figure 137 Cover page of a general health book on CRP

- <http://www.amazon.com>

As attempts are made to further understand its function as a molecule, and mediator within the complexities of the acute phase response, we must be wary of premature and unsubstantiated claims becoming *de facto* truths, particularly under the influence of the substantial publicity surrounding this ancient molecule. The title and purpose of this thesis was to address the question of whether CRP may enhance tissue damage, and therefore be a potential therapeutic target. In addressing the question this thesis has started with an epidemiological approach to the problem, before focussing on animal models of disease and finally developing a rational and unique pharmacological approach. The findings presented in this thesis demonstrate clearly:

- 1) A strong association between increased values of CRP and future coronary heart disease, but not one that necessarily exceeds that of traditional risk factors. Independently CRP has a modest association with future coronary heart disease events;
- 2) A failure to confirm, in a transgenic setting, a protective role for CRP following local or systemic endotoxin challenge;
- 3) A lack of causality for human CRP in a transgenic mouse model of atherosclerosis, despite clear up-regulation of the protein, and evidence of its deposition in advanced plaques;
- 4) An exciting development of a low molecular weight inhibitor of CRP binding that specifically dimerises the protein efficiently preventing ligand binding, opening the way for pharmacological manipulation of CRP.

There is often a loss of clarity in the discussions regarding CRP. Certain potential areas of interest within cardiovascular medicine have driven research priorities recently, and each area does not necessarily overlap clinically or pathophysiologically. For this reason conclusions drawn from one study addressing one topic do not necessarily impact on another focus of interest. Broadly then within the spectrum of cardiovascular disease CRP has attracted attention for its:

- 1) Predictive power for future cardiac events in otherwise healthy individuals;
- 2) Predictive power for future cardiac events in individuals post myocardial infarction;
- 3) Predictive power for future cardiac events in individuals with acute coronary syndromes, including those undergoing interventions;
- 4) Utility in targeting individuals (both within primary and secondary cardiovascular disease prevention programmes) who may benefit from HMG CoA-reductase inhibitors in particular, despite “normal” lipid parameters;
- 5) Potential direct pathogenic role in atherosclerosis;
- 6) Potential direct pathogenic role in myocardial infarction (and other similar inflammatory conditions);
- 7) Potential to therefore be a therapeutic target, such that a CRP inhibitor may be a novel anti-inflammatory drug.

With colleagues I started by addressing in the largest and most rigorous evaluation so far, the predictive value of CRP in coronary disease in the general population. The large size of our study provides excellent power and precision in its estimates and our conclusion was that the prognostic value of CRP has been overstated by overestimation of its predictive power, and underestimation of established risk factors.

In reply Dr. Paul Ridker of Brigham and Women's Hospital was widely reported as saying our study was "creating controversy where none existed". Moreover he went on to say that "I would never want to be a patient in Great Britain...I suppose in Great Britain they don't want people to be healthy...This is about public health and letting patients and their primary physicians know there is overwhelming evidence that the test works."

(http://www.missouri.edu/~chemrg/current_news/Article_Inflammation.html)

Such criticism appears based on firm opinion rather than evidence. We were attempting to answer a specific question, namely what does CRP determination add to our understanding of heart disease risk? Everyone agrees that coronary heart disease risk prediction is important because intervention prior to an event has the potential to save many lives. However whilst inflammation is clearly involved in the processes we study as end points, this does not mean to say that a measure of inflammation adds to any of the known risk factors. The recently published INTERHEART study (Yusuf and colleagues study of risk factors for myocardial infarction in 52 populations worldwide [374]) shows that more than 80% of the global burden of coronary heart disease, irrespective of the ethnic

origin, can be attributed to five main conventional cardiac risk factors—abnormal lipids, diabetes mellitus, cigarette smoking, hypertension, and lack of exercise. Including other important factors such as abdominal obesity, alcohol intake, psychosocial factors and lack of consumption of fruits or vegetables, then accounted for most, if not all, of the population attributable risk of myocardial infarction. The effect of the risk factors were particularly striking in young men (population attributable risk about 93%) and women (population attributable risk about 96%), indicating that most premature myocardial infarction is preventable, with already measured risk factors. American studies have also confirmed this, casting significant doubt on the so called "only 50%" hypothesis [371-373]: a frequently cited concept that individual major risk factors for coronary heart disease are absent in perhaps >50% of those with CHD. Thus long term follow up from three prospective cohort studies (Chicago Heart Association Detection Project in Industry, Multiple Risk Factor Intervention Trial, and the Framingham Heart Study) found that for fatal coronary heart disease, exposure to at least one clinically elevated major risk factor ranged from 87% to 100% i.e. suboptimal levels of cholesterol, raised blood pressure, cigarette smoking, and diabetes [371].

It seems likely then that the explanatory power of the major, established cardiovascular risk factors has been systematically underestimated. So those that criticised our work for over adjustment by factors that tend to reduce the predictive ability of CRP, are correct , but miss the essence of the study; new

markers of risk should not simply be added to global risk scores because they are currently fashionable [517-520].

Of course our conservative analysis and estimation of increment in risk, an odds ratio for CRP of 1.5 (a 50% increase in risk), may still be a significant finding from a clinical and a public health perspective and one that may be clinically important. The problem remains though that there is a likelihood that further larger long term studies, with even better adjustment for confounding factors, will reduce this odds ratio further. As an example there was much interest in the effect of a polymorphism in the angiotensin converting enzyme gene and risk of coronary restenosis after percutaneous coronary intervention, as well as in myocardial infarction. However weaker associations between the angiotensin converting enzyme DD genotype and restenosis were found in larger and more rigorous studies than in other earlier, smaller studies [521]. Other putative associations with cardiovascular disease have also largely fallen by the wayside, as studies increase in size and are pooled e.g. *Chlamydia pneumoniae* [522] and *Helicobacter pylori* infection [523]. Publication bias or detection biases can produce artefactual associations and adequately powered investigations are needed to properly address important questions relating to association/causation. It is a well recognised phenomenon in epidemiology, that early publication bias favours positive papers, and as larger studies appear there is the so called “regression to the mean or truth” and often the association disappears.

To ultimately answer the question all are in agreement that no single study alone is sufficient. We acknowledge that our study could be criticised for its geographical location, its failure to measure lipid parameters such as LDL-cholesterol and HDL, and its time frame which meant that most patients were not using aspirin or statins. Additionally we could not for practical reasons measure other inflammatory markers such as SAA, total white cell count or pro-inflammatory cytokines (although ESR, a less specific but summative marker of inflammation was assessed and its predictive power was similar to that of CRP, suggesting that inflammation *per se* and not CRP itself is relevant). It was also telling that the odds ratio in the Iceland study was very similar to that of the up to date meta-analysis, again suggesting that the data was robust. We accept that we couldn't and didn't fully address the role of CRP and the metabolic syndrome nor was the study powered to look at CRP and diabetes directly or specific gender differences. We also only addressed the role of CRP in prediction of future coronary heart disease risk and not in the management of patients with acute coronary syndromes or those whom might benefit from statin treatment. Further collaborative research continues therefore to be needed, with long term follow up, significant groups of individuals as cases and appropriate controls, and critical appraisal of the results [524, 525].

Finally it may be the case that CRP does have a practical role to play but traditional risk factors must not be underutilised, and any use of CRP must be justified for the correct reasons. Hence perhaps it will prove to be the case that CRP has a very significant role in day to day practice, if it can be shown that in "live use" clinicians prefer CRP based algorithms to those already followed.

CRP could for example be used as a good global marker of risk, which patients and physicians may more easily understand and utilise as a target for interventions.

There are many features of CRP the molecule, not the analyte, which suggest it may contribute to some processes in atherosclerosis. However whilst it remains the most studied of inflammatory markers it is somewhat self fulfilling to conclude that because CRP has stood out as a risk marker it must have a pathological role in the disease as well. CRP was measured because it is robust and relatively easy to measure. However we know that diverse markers of inflammation such as ESR [168], total white cell count [17] and SAA [179] also predict cardiovascular disease. In testing for causation rather than just association, it is however not as easy to manipulate experimental paradigms for these inflammatory markers; whilst one can easily design an experiment to see the effect of CRP *in vitro* or *in vivo* this is not a meaningful strategy for ESR or total white cell count.

The remainder of this thesis attempted to experimentally address the role of CRP *in vitro* and *in vivo*. Its strength lies within our grasp of the problems one faces when you turn to such experimental work. So we are very much aware of the areas of controversy that exist for CRP and the experimental hazards inherent in studying this molecule. There are many pertinent questions (not all with adequate answers) and issues worthy of thought including:

1) Reliability and plausibility of *in vitro* studies

a. Contamination of CRP preparations with immunoglobulins and endotoxin

i. Is recombinant CRP made in *E. coli* the same as recombinant CRP made in insect cells and the same as isolated human CRP from ascites/serum? Are *in vitro* reports therefore that CRP is pro-inflammatory merely a result of contamination? Hence the conclusion of one group who carefully investigated a wide range of effects on endothelial cells ascribed to CRP and found that azide (see below) and LPS, but never CRP itself, were responsible for the cell activation events [526]. Injection into animals of different CRP preparations, with subsequent assessment of inflammatory responses has shown acute phase responses and pro-inflammatory effects induced by commercially available recombinant human CRP produced in *E. coli*, with no such effects using genuine human CRP isolated from human subjects [527].

ii. Can low concentrations of immunoglobulins in the preparations explain the reported binding to cell surface receptors? Hence work showing that the apparent binding of human CRP by Fc γ receptors on human cells is artefactual and reflects either use of whole IgG anti-CRP antibodies, or contamination of commercial CRP preparations with traces of IgG. When recombinant

human CRP or highly purified human CRP was used, or CRP binding was sought using F(ab)₂ anti-CRP antibodies, no binding of CRP by Fc receptors on human cells was detected [296]. This has been confirmed by others [297, 298].

iii. Are adequate steps taken to demonstrate specificity of effects described? Accordingly in our own *in vitro* studies of the effects of CRP on vascular reactivity [304], we demonstrated specificity in a number of ways (notably pre-absorption of CRP by pure ligand and inhibition of binding with RMM), but still concluded that despite demonstrating specific, direct effects on vascular function *in vitro* further clarification of the effect, if any, of CRP on vascular reactivity in humans *in vivo* would require clinical studies using specific inhibitors of CRP.

b. Presence of azide in CRP buffers

i. Have adequate steps been taken to remove the cellular toxin and nitric oxide donor, sodium azide from CRP used experimentally? Independent groups are now reporting that many findings claimed for CRP are in fact artefactual. 0.1% NaN₃ is the preservative used for commercially available CRP and a potential source of NO; the acute vasoactive properties of commercially available CRP preparations have now been clearly attributed to NaN₃ and subsequent production of NO by catalase [526, 528-531].

- c. Has the quality and specificity of other reagents used, not only the purified CRP, but antibodies for example been adequately confirmed? Have the assays employed to measure CRP been calibrated appropriately?
- d. Design of meaningful experiments
 - i. Can you explain the functions of an acute phase serum protein with a dynamic range of 10000 fold by studying it out of the context of an acute phase response? Is the absence of serum proteins and/or acute phase cytokines in the experimental milieu acceptable? How do you model the time course of the acute phase response, and hence CRP production, in an *in vitro* setting?
 - ii. Are experiments with modified CRP, which has to be generated by exposure of the protein to 6M urea, biologically meaningful?
 - iii. Are cell culture studies generally inappropriate in the context of this particular serum protein, and prone to misleading conclusions? It is recognised that CRP binds to necrotic and apoptotic cells (particularly through PC recognition), but might out of context binding generate artefactual phenomenon?

2) Reliability and plausibility of *in vivo* studies

a. Specificity of transgenic production of CRP, or passive administration of protein, across species boundaries

- i. Is the regulation of synthesis of transgenic human CRP in mice, including sites outside of the liver, sufficiently close to man to generate genuine transferable results?
- ii. Is the turnover of transgenic human CRP either in normal or hyperlipidaemic murine serum representative of man?
- iii. Is there a *biological cost* to the mouse of transgenic production of a human protein?
- iv. Are the consequences of ligand binding comparable for human CRP in human serum with human CRP in mouse serum? Is mouse or rat complement sufficiently similar to man to contemplate studying the effects of CRP *in vivo*? Are the intricacies of complement regulation mirrored adequately enough to allow conclusions about CRP to be drawn? It is known for example that human CRP requires human C1q to activate guinea pig complement [48] and similarly rat CRP appears not to activate its own autologous complement when ligand bound, but human CRP does activate rat complement [75, 201] (although some dispute the idea that rat complement doesn't activate its own complement [532]).
- v. Are other models any better? Is there intrinsic value in studying rabbit CRP expressed under a nutritional

promoter in mouse serum? Should a mouse CRP knockout be generated even though murine CRP is not the predominant pentraxin acute phase protein in mice? Might targeted transgenic expression of human CRP be informative?

vi. Why are there such profound differences in CRP across species in terms of regulation of production and effects of ligand binding? Do such differences have an effect on experimental models of CRP function? Are such differences insurmountable experimentally? Is a pharmacological approach, in man, the only logical approach?

b. Design of meaningful experiments

i. The peak acute phase response in terms of CRP production is approximately 48 hours after an insult or injury. Could passive use of CRP in experiments mean that peak values are achieved at time points undergoing different pathophysiological effects than normal? Could CRP be exposed therefore to non-physiological ligands at non-physiological times? Could such binding effects of CRP be displacing/competing with other ligands and so generating an artificial pro-inflammatory effect?

ii. Do the present animal models reflect the important pathophysiological mechanisms that CRP appears to predict epidemiologically i.e. events rather than disease

itself? Is the apoE knockout mouse simply a model of atherosclerosis with little bearing on atherothrombosis? Is a rat coronary artery occlusion study really synonymous with human myocardial infarction with staggered coronary artery occlusion? Would ischaemia-reperfusion models be better?

iii. Are sufficient experiments performed to overcome chance in the results? Are the effects seen reproducible in independent laboratories?

iv. Are the correct animals being studied; is there a compelling argument for CRP researchers to use large animals, where CRP biology is more closely related to man [12, 533]?

3) Commercial pressure to utilise CRP

a. Is there undue emphasis from outside the clinical arena (Figure 138) on the adoption of CRP into clinical practice?

The image shows a screenshot of the CRPhealth.com website. At the top, there is a navigation bar with links for 'Home', 'Online Presentations', 'Video Library', 'Slide Library', 'Publications', and 'Resources'. Below this is a search bar and a 'Get' button. The main content area features a 'JUPITER TRIAL' banner and a 'Forum Message' section. A list of sponsors is displayed at the bottom, including AstraZeneca, Novartis, GlaxoSmithKline, and Dade Behring. A quote is overlaid on the image: "In brief, CRPhealth.com provides in a single website a full range of information regarding inflammation and vascular risk that is appropriate for both physicians and lay audiences."

Figure 138 Reproduction of the www.CRPhealth.com website

By studying animals that produce transgenic human CRP under an inflammatory stimulus we have come as close as we can in small animal models to the best available tests of causality. All of the caveats and concerns outlined above apply equally to our work, but we have tried to tackle potential confounders and pitfalls where possible throughout our studies: the purity and integrity of the CRP can be vouched for and demonstrated, azide is removed where appropriate, the endotoxin concentration is insignificant, the antibodies used in detecting CRP are specific and the animals models chosen are established; our epidemiological study is the largest ever performed and our assays for measuring CRP precise, specific and reliable; finally we have in our sights a rationally designed inhibitor of CRP binding that inhibits CRP *in vivo*.

We found no protection from endotoxaemia in transgenic mice, despite the findings of others and we notably didn't show enhanced atherogenesis in apoE knockout mice producing transgenic CRP. With respect to endotoxaemia one recent publication that claims protective effects of CRP against *Vibrio vulnificus* LPS, used commercially sourced CRP from the horseshoe crab, *Limulus polyphemus* [534]. This material was completely uncharacterised with regard to its purity and functional integrity and as already discussed *Limulus* CRP is heterogeneous in amino acid sequence, is only about 20% homologous with human CRP, is dodecameric rather than pentameric, is heavily and heterogeneously glycosylated, whilst human CRP is not a glycoprotein, and *Limulus* is an arachnid separated from primates by about 700 million years of evolution. In other studies no control proteins were used [431], whilst in the work with rabbit CRP transgenic mice, the control animals were not even of the

same strain [429]. Our present failure to find protection by exogenous human CRP suggests that this rather artificial phenomenon is unlikely to be of general significance. Our apoE study [491] didn't tally with the first small study [436] but does fit with two other subsequent more robust analyses [492, 493]. One comment on our work dismissed it as "not terribly important or helpful...mouse models of atherosclerosis are interesting, but by no means definitive. I don't think you learn much from these studies where you create a disease in mice rapidly...and when you look at studies involving 10 000 patients where you find that CRP is a risk factor, a handful of mice is not important."

(<http://www.medicinenet.com/script/main/art.asp?articlekey=47289>)

This appears not to appreciate the literature [326, 366, 535]. Firstly whilst CRP is a risk marker in its own right, when confounders and traditional risk factors are accounted for, it adds very little further information. Secondly animal models are very instructive in modelling disease, so long as their limitations are acknowledged. We accept that the xenogeneic combination of human CRP, regulated by murine factors and functioning in a murine cellular and extracellular environment is artefactual but if CRP were playing a fundamental role in atherogenesis one would expect to see an effect. A pharmacological approach is therefore the most logical way forward in addressing the problem since the only definitive way to confirm or exclude a possible pathogenic role of human CRP in atherosclerosis and/or atherothrombotic events will be use of drugs that specifically target CRP *in vivo* and inhibit its presumed functions, specifically those that result from phosphocholine binding. The bisphosphocholine compound that we have rationally designed and studied is an attractive candidate

for development as a cardioprotective and neuroprotective treatment in acute myocardial infarction and stroke respectively, and may well have wider applications in other acute and chronic disorders associated with increased CRP production. It also provides a starting point for development of specific CRP inhibitor drugs that could be used to establish whether or not CRP plays a causal role in atherosclerosis.

The studies presented and further studies now performed in rats undergoing myocardial infarction do show that RMM can specifically inhibit biological functions of human CRP by its bringing together of two pentamers. Further toxicological and pharmacokinetic/pharmacodynamic studies will be needed as will specific studies of what effects this drug has in its own right, aside from any specific binding to CRP. Is it binding to any other ligands in the serum, such as phosphocholine specific antibodies, including antibodies against OxLDL?

Importantly human studies will be needed early as the limitations expressed above about animal studies are such that only clinical studies in man can yield as definitive as possible answers. There could well be, however, concerns about such studies. The conservation of the pentraxin proteins over many millions of years evolution cannot be ignored, precisely because we don't understand the protein fully. A strong protective role must be postulated to exist, and it must surely be the conservation of PC recognition and the abundance of such ligands in inflammatory and infectious situations, along with the similarities to antibody once CRP is ligand bound with respect to complement, that has made the protein so robust. The judicious use of complement once activated, and many contend

that complement activation by CRP does not deplete the late complement components, may be important to how organisms handle foreign and self antigens.

An interesting study on targeted and restricted complement activation on acrosome-reacted spermatozoa was recently published in JCI, and perhaps is pertinent [536]. Upon exposure of human spermatozoa to autologous serum or follicular fluid, it was found unexpectedly that acrosome-reacted spermatozoa activated the complement cascade efficiently through C3 but not beyond. The study suggested that complement activation was initiated by CRP and was C1q, C2, and factor B dependent. This pattern is consistent with engagement of the classical pathway followed by amplification through the alternative pathway. C3b deposition was targeted to the inner acrosomal membrane, where it was cleaved to C3bi. Factor H, and not membrane cofactor protein (MCP; CD46), was the cofactor responsible for C3b cleavage. The authors of this study proposed that this localised deposition of complement fragments aided the fusion process between the spermatozoa and egg, in a role similar to that of complement in immune adherence. They hypothesised that C3bi, a ligand for integrin-related complement receptors, facilitates sperm-egg interactions.

More generally, and of significance to this discussion, they speculated that such a targeted and restricted form of complement activation on host cells was a common strategy to handle modified self. In conditions like apoptosis, ischaemia/reperfusion injury, and probably other types of alterations in host cell structure targeted and restricted activation of the complement system perhaps is a

preferable means to activate the complement system. CRP may play an important role in this. In contrast to the desirable explosion of the cascade on microbes, complement activation on an apoptotic cell or during ischaemia-reperfusion is less robust, yet sufficient to facilitate the safe and relatively non-inflammatory removal of altered self, rapidly and safely in order to enhance wound healing and promote tissue sterility.

Extreme caution will be needed in designing appropriate human experiments. However any binding effects of the drug will be limited to an immediate effect on CRP in the circulation. CPHPC, the specific SAP ligand binding drug molecule which has been used in trials of treatment of amyloidosis, appears so far to be safe. This of course does not mean RMM would be but it is certainly encouraging that a plasma pentraxin protein can be removed from the circulation without immediate concerns.

In conjunction with a CRP inhibitor there are many further experiments that would be of interest. By already extending the studies in apoE mice to include 18 month old mice, we hope to look at transgenic production of human CRP in a model including plaque rupture. Other experimental adjustments such as high fat diets, manipulation with statins or inducing specific vascular injuries may also prove interesting to study. Pigs may also be a possible species for studies looking at myocardial infarction or atherosclerosis. One study has already reported that serum CRP values correlated with macrophage accumulation and coronary artery disease in hypercholesterolaemic pigs [537], and further work could be insightful, if appropriately designed and executed; hence in the study

cited an antibody against human CRP was used to detect porcine CRP, which is sub-optimal. Our limited studies in a murine model of arthritis are also worth extending, particularly as discussed by backcrossing human CRP transgenic mice onto a susceptible strain. And although controversial, extra-hepatic expression of CRP has been described in the mouse model [435] and by some in man [538]. Local expression of complement has been shown to be important in renal transplant rejection [539]; is there a similar role for CRP produced in the kidney or other tissues? Finally very recently a genome screen identified that a polymorphism in the Factor H gene was associated with development of age related macular degeneration (AMD) [540-543]. Indeed it was estimated that in individuals homozygous for the risk allele, the likelihood of AMD was increased by a factor of just over 7 fold. The polymorphism is in linkage disequilibrium with the risk allele and represents a tyrosine-histidine change at amino acid 402, in a region of the complement factor H protein that binds heparin and CRP. There is already some evidence that minor elevations in CRP are associated with age related macular degeneration [544, 545] and complement mediated damage has been speculated previously to be important in the pathophysiology of this very important eye disease [546, 547]. There is thus a clear link with CRP that experimentally needs addressing.

Clinically further studies of CRP in different settings are a priority. Areas of great interest include the metabolic syndrome, associated fatty disease of the liver, diabetes, statin use and risk stratification in acute coronary syndrome management. However it will prove more important to ensure that the studies are of sufficient size and duration with appropriately employed sensitive CRP

assays to adequately answer questions. The creation of a CRP collaborative centre has been suggested by John Danesh, to allow deposition of the raw data on studies, and so facilitate pooling of results [168]. Genetic association studies will also continue to be important and are already underway.

14.2 Summary

In conclusion the studies presented in this thesis largely cast doubt for a very strong role clinically or pathologically for CRP in atherosclerosis but generate optimism that a pharmacological approach can be utilised to understand the *in vivo* properties of this protein in health and disease. This has the potential to generate a drug molecule that could be clinically very significant in myocardial infarction and stroke, where the evidence remains for a pathogenic role for CRP.

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