The impact of cytokine modulation in acquired and inherited inflammatory disease and AA amyloidosis

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Declaration

I, Thirusha Lane, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, this has been declared within the thesis.

Signature:

(Electronically signed)

Date: 11th May 2016

Abstract

Background

Inflammatory disorders (IDs) cause significant morbidity. SAA and CRP are acute phase proteins used to diagnose and monitor IDs. SAA is also the precursor to AA amyloidosis (AAA), a serious complication of chronic inflammation, causing renal failure and poor quality of life (QoL). The aim of treatment is thus to prevent AAA or to halt its progression to renal failure, protect renal allografts, and restore QoL. Novel biological therapies have transformed the landscape, but their longer term effects are yet to be elucidated.

Aims

We set up a nurse-led clinic to explore safety and efficacy of modulation of cytokines IL-1 and IL-6 in patients with IDs and AAA, and studied the changing epidemiology of AAA. We also sought to investigate whether CRP was itself pro-inflammatory.

Results

Suppressing SAA using anti-IL-1 and anti-IL-6 agents resulted in stabilisation of amyloid deposits and in some, amyloid regression and improved renal function. Treatments were safe and effective in dialysis and renal transplantation, even where the underlying cause of AAA was uncertain. Improvement was seen in almost all anti-IL-1-treated CAPS patients. Common adverse events were infections. QoL improved when treatment was effective. Referral rates of AAA have remained steady whilst other types have increased. The commonest causes of AAA are changing; unknown aetiology has increased. Injection of purified CRP into volunteers did not provoke inflammation.

Conclusions

Suppressing SAA by effectively treating the underlying ID can lead to improved renal function and regression of amyloid, even when the cause is uncertain. Anti-cytokine agents offer the possibility of targeted therapies to suppress systemic inflammation. Work reported here shows safety, even in dialysis and transplantation. AAA is becoming less common as some IDs have become easier to control. Single cytokine blockade has proved useful in AAA of unknown aetiology and treatment generally improves symptomatology and inflammation. CRP is itself not pro-inflammatory.

Ethical Approval

Independent Research Ethics Committee (REC) approval was sought and received for all clinical studies, including retrospective work. The applicable REC reference number is quoted in each chapter.

Acknowledgements

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Chapter One:

Introduction

1.1 The Innate Immune System

The mammalian immune system is composed of two arms, the acquired or adaptive immune system and the innate immune system. Innate immunity is a genetically encoded, hardwired system that provides a rapid response to danger signals (1). The systemic autoinflammatory syndromes are recognised as disorders of innate immunity, and are entirely distinct from the classical autoimmune diseases such as systemic lupus erythematosus or rheumatoid arthritis, diseases rooted primarily in adaptive immunity (2). There is no doubt, however, that the two immune systems are intertwined, and that the range of self-reactive immunological disease represents a continuum between the two (3).

Two major classes of receptors involved in innate immunity are the Toll-like receptors (TLRs) which are membrane-bound, and the nucleotide-binding oligomerisation domain (NOD)-like receptors (NLRs) which are cytosolic. Both receptor classes recognise pathogen-associated molecular patterns, PAMPs, and the NLRs in particular appear to sense endogenous danger signals, the so called damage-associated molecular patterns (DAMPs). More than 20 NLR-encoding genes have been identified in man. The majority of proteins appear to have a leucine-rich repeat domain at the C-terminal; this is thought to be the ligand binding motif, a nucleotide-binding oligomerisation domain that is central to the formation of multimeric complexes, and an N-terminal effector domain. These regulate activation of caspases (a group of intracellular cysteine proteases) which, in turn, activate a variety of cellular responses including apoptosis pathways, transcription of nuclear factor κB (NFκB) and cytokine activation (4). NLRP3, also known as cryopyrin, is the most studied and best understood member of the NLR family, and contains an N-terminal pyrin domain, a nucleotide binding domain and a leucine-rich repeat domain at the C- terminal. NLRP3 is the key component of the NLRP3 inflammasome.

1.2 Autoinflammatory pathogenic mechanisms

1.2.1 The NLRP3 inflammasome

The NLRP3 inflammasome is a multimeric cytosolic complex. Upon activation by PAMPs or DAMPs, NLRP3 associates with an adaptor protein, ASC (apoptosis-associated speck-like protein with a caspase activation and recruitment domain) and a pyrin domain. The NLRP3-ASC complex oligomerises, and this oligomerisation results in cleavage of procaspase-1 to caspase-1, which in turn cleaves inactive pro-IL-1β to the mature, active IL-1β (5, 6). This is summarised in Figure 1.1.

IL-1β is a key proinflammatory cytokine with pleiotropic effects (7). Binding of IL-1β to its receptor IL-1R1 induces intracellular signaling and transcription of other pro-inflammatory genes; hence dysregulation of IL-1β production has serious pathological consequences. Its transcription, activation and release are tightly regulated, and the activity of IL-1β after release is further regulated by IL-1Ra, an endogenous antagonist of IL-1α and IL-1β. IL-1β leaves the cell and then binds to the IL-1 receptor (8). The binding of IL-1β to its receptor type I, IL1-RI, and to the IL-1 accessory receptor (IL-1RAcP) stimulates intracellular signalling and transcription of other pro-inflammatory genes. The primary role of the NLRP3 inflammasome is therefore to tightly regulate activation of IL-1β. Once released into the extracellular environment the activity of both IL-1β and IL-1α is further regulated by IL-1Ra, which antagonises their binding to IL-1RI (9, 10).

Pivotal to the recent developments in our understanding of the role of IL-1β in autoinflammatory diseases has been the success of agents which block IL-1 in these disorders. Anakinra (a recombinant IL-1 receptor antagonist), rilonacept (an IL-1 trap) and canakinumab (a fully human anti- IL-1β monoclonal antibody) are the three anti-IL-

1 agents that have been widely studied and used in the clinical arena, and there are phase II clinical trials with another anti- IL-1β antibody, XOMA 052.

Anti-IL-1 therapies have been successfully used in many inflammatory conditions, even those not directly associated with mutations in *NLRP3*, providing evidence of the pleiotropic effects of IL-1 and the NLRP3 inflammasome; some of these diseases are discussed in more detail in Section 1.3 below. Whilst direct inhibition of assembly of the NLRP3 inflammasome by means of blocking IL-1 has been successful in many conditions, other inflammatory molecules are known to either activate the NLRP3 inflammasome by mechanisms other than that described above, or by activating completely separate pathogenic inflammatory pathways.

1.2.2 Other players and mechanisms driving inflammation

1.2.2.1 ASC

It has been shown that the recruitment of ASC by NLRP3 is a fundamental step in inflammasome activation, and that if ASC recruitment fails the NLRP3 inflammasome complex does not assemble. Formation of the NLRP3-ASC complex is established between the pyrin domains (PYDs) in each molecule. ASC is composed of two death domains, the PYD and a caspase activation and recruitment domain (CARD); it therefore acts as an adaptor molecule not only in inflammation (as discussed above), but also in apoptosis (11). Furthermore, it has been shown that ASC can, via CARD-CARD interactions, recruit members of the caspase family (12); it can also oligomerise into functional complexes like the pyroptosome, a potent caspase-1 activator responsible for pyroptosis (13); and it can act as a scaffold for other molecular platforms involved in caspase activation (14).

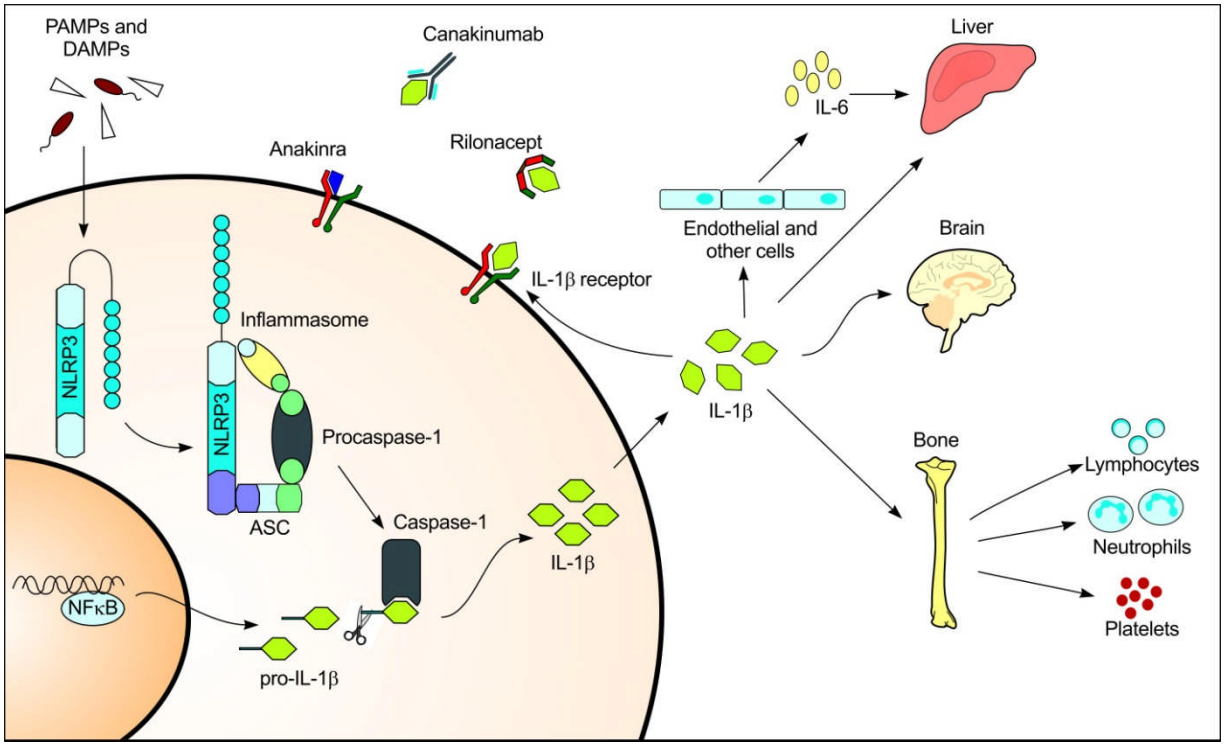


Figure 1.1. Activation of the NLRP3 inflammasome leads to the release of IL-1β which stimulates widespread inflammatory responses. Pathogen-associated and danger-associated molecular patterns (PAMPs and DAMPs) enter monocytes and macrophages and are recognised by pattern recognition receptors such as NLRP3. This results in activation of NLRP3, leading to its interaction with the other components of the NLRP3 inflammasome (ASC and procaspase-1) and activation of the NLRP3 inflammasome. The active NLRP3 inflammasome cleaves procaspase-1 to produce the active form, caspase-1. Caspase-1 in turn cleaves pro-IL-1β to yield the active cytokine, IL-1β. IL-1β is released from the cell and stimulates inflammatory responses: The production and release of the acute-phase proteins is stimulated by direct action on the liver or indirectly by stimulation of the production of IL-6 from endothelial and other cells; IL-1β acts on the hypothalamus to induce fever; IL-1β stimulates bone to induce bone resorption and breakdown of cartilage, and production and enhanced activation of lymphocytes, neutrophils, and platelets. The anti-IL-1 therapies anakinra, rilonacept and canakinumab act by blocking binding to the IL-1 receptor, trapping the IL-1β molecule or by binding to it, respectively.

1.2.2.2 Pyrin

Pyrin is part of the TRIM family of proteins. It contains a PYD at its N-terminus, bZIP basic, B-box and coiled coil domains, as well as a B30.2/SPRY domain at its C- terminus. Direct interaction of pyrin with ASC via the cognate pyrin domain suggests possible molecular mechanisms for the inflammatory features of familial Mediterranean fever (FMF). There are data to support both an anti-inflammatory and a pro-inflammatory role for wild-type (WT) pyrin. Chae and colleagues demonstrated that mice deficient for *MEFV* showed increased cytokine production and subsequent endotoxin-induced lethality (15). Other groups have shown that the B30.2 domain of WT pyrin interacts with caspase-1, thereby inhibiting the maturation and processing of IL-1β (16, 17). These data support the anti-inflammatory role of WT pyrin; mutations in *MEFV* would therefore confer a loss of function.

Other groups have shown a pro-inflammatory role of WT pyrin whereby overexpressing WT pyrin resulted in activation of the inflammasome (18) or silencing WT pyrin resulted in reduced IL-1β production (19). Experimental conditions in these reports are varied and these conflicting results notwithstanding, the role of pyrin in the regulation of IL-1β is undeniable, and this has been proven by the successful treatment with anti-IL-1 therapies of some FMF patients with incomplete colchicine response (20, 21) or complete colchicine resistance (22-24). It remains to be seen whether the conflicting reports actually point to subtle variations in sensing mechanisms associated with different genotypes, inheritance patterns and epigenetic conditions.

The physiological role of pyrin has long been sought. Recently, the group of Xu (25) showed that pyrin acts as a sensor of the modification and inactivation of Rho GTPases by bacterial toxins. Toxin B of *Clostridium difficile* (TcdB) plays a key role in the pathogenesis of *Clostridium difficile* by glucosylating Rho GTPases in order to modulate the

host actin cytoskeleton for the purposes of virulence (26). Xu et al demonstrated that the glucosyltransferase activity of TcdB could induce caspase-1 activation in bone marrow derived murine and human macrophages and that this activation is absent in Asc null cells, suggesting a requirement of a PYD-containing sensor. Pyrin knockdown completely inhibited TcdB-induced inflammasome activation, thereby uncovering the crucial role of pyrin in immunity.

1.2.2.3 ***IL-18 and IL-1α***

A member of the IL-1 cytokine superfamily, IL-18 is an important regulator of acquired and innate immunity, and is expressed in a variety of cells including macrophages. IL-1α is a pleiotropic cytokine involved in various inflammatory processes; it is produced by monocytes and macrophages as a proprotein, which is proteolytically processed and released in response to cell injury, making it a key player in the regulation of immune responses. A recent study demonstrated that secretion of both IL-18 and IL-1α is upregulated in endotoxin-stimulated monocytes from CAPS patients (27). IL-18 release is also stimulated by inflammasome-activated caspase-1 so this is not an unexpected finding. By comparison, IL-1α is bioactive even as a non-activated precursor and exerts its pro-inflammatory activity when released by dying cells, thereby behaving as a DAMP (28). Another study has shown that IL-1α is also actively secreted in response to cell stress (29), and others have found that, the inflammasome itself can mediate IL-1α secretion, despite IL-1α having no cleavage site for caspase-1 (30, 31).

1.2.2.4 ***Tumor necrosis factor (TNF)-α and the TNF receptor***

TNFα is a pro-inflammatory cytokine secreted mainly by activated macrophages, but also by many other cell types. The biological effects of TNFα are mediated primarily through the TNF receptor 1 (TNFR1), a 55 kDa protein encoded by the *TNFRSF1A* gene. *TNFRSF1A* is part of the death domain superfamily, and TNFR1 is composed of an

extracellular domain containing four cysteine-rich motifs, a transmembrane domain and an extracellular death domain. Binding of TNF to the extracellular domain of TNFR1 leads to trimerisation of the receptor, resulting in inflammation triggered by activation of nuclear factor kappa-B (NF-κB), or apoptosis as a result of caspase cleavage. Under physiological conditions binding of TNF to the extracellular TNFR1 domain results in termination of TNF signalling and shedding of the extracellular domain of the soluble TNFR1 into the plasma. The circulating soluble TNFR1 competitively inhibits binding of free plasma TNFα to membrane-bound TNF receptors, thereby regulating TNF signalling. Mutations in *TNFRSF1A* result in dysregulation of TNF processing via a variety of proposed pathways although the exact mechanism are not completely understood (32, 33). The effects of mutations in *TNFRSF1A* are discussed in further detail in Chapter 1.3.3.

1.2.2.5 Mevalonate kinase

Mevalonate kinase is an enzyme in the isoprenoid pathway, an essential metabolic pathway in the biosynthesis of isoprenoids including cholesterol. The enzyme itself is not involved in inflammation, however, deficiency of mevalonate kinase as a result of mutations in the *MVK* gene is the cause of the autoinflammatory disease known as mevalonate kinase deficiency or hyper-IgD and periodic fever syndrome. The mechanism by which the deficiency of mevalonate kinase triggers the autoinflammatory pathway remains incompletely understood. Bisphosphonates have been used in some experimental models for blockade of the isoprenoid pathway downstream of mevalonate kinase, leading to inflammation and IL-1β secretion, suggesting one possible mechanism (34-36). The clinical syndrome of MKD is described in more detail in Chapter 1.3.4.

1.2.2.6 IL-6

IL-6 is a pleiotropic cytokine with a broad range of biological effects which include pro-inflammatory and immunoregulatory effects. IL-6 production has been detected in many different cell types but the main sources of IL-6 are, during an acute inflammatory episode, macrophages and monocytes at the site of inflammation, and T cells in chronic inflammation (37). IL-6 acts via two pathways, the classical signalling pathway and the alternate signalling pathway. In the classical signalling pathway IL-6 binds to the IL-6Rα present on the surface of hepatocytes and some immune cells such as neutrophils, monocytes, and B and T cells. The main purpose of this pathway is the promotion of growth and differentiation and prevention of apoptosis (38). The alternate (or trans-) signalling pathway is believed to be responsible for the negative effects of IL-6 in inflammation; this is based on the finding that blocking the alternate pathway abolishes maintenance and progression of disease (39). In this pathway, free IL-6 binds to soluble IL-6Rα (sIL-6Rα), and this complex is capable of signalling any cell that has gp130 on its surface, i.e. most cells. Binding of free IL-6 to sIL-6Rα activates and recruits gp130 and JAK1 leading to STAT3 activation. The systemic response to infection or inflammation is fever, leucocytosis and increased synthesis of hepatic acute phase proteins such as serum amyloid A protein and C-reactive protein; IL-6, together with IL1β and TNFα, is responsible for generating this systemic response, and IL-6 is believed to be almost completely responsible for the fever and the hepatic acute phase response (37). In chronic inflammatory conditions serum IL-6 levels have been found to be persistently elevated.

1.2.2.7 Cellular stress

The electron transport chain, the final step in aerobic respiration, takes place in the mitochondrial inner membrane. The end result of the process is the generation of energy in the form of adenosine triphosphate (ATP), as oxygen acts as an electron acceptor.

When the electron transport chain breaks down during episodes of cellular stress, toxic reactive oxygen species (ROS) can accumulate within cells. Studies have demonstrated that ATP-induced ROS production stimulates inflammasome activation in macrophages (40).

Furthermore, extracellular ATP can act as a DAMP. Present at a high concentration inside cells, intracellular ATP is released extracellularly in the event of cell injury. This extracellular ATP induces inflammation by binding P2X7R on inflammatory cells resulting in the stimulation of a series of intracellular processes that leads to inflammasome activation and IL1β secretion (27, 41, 42).

1.3 Systemic Autoinflammatory Diseases

The discovery of pathogenic genetic mutations which result in dysregulation of the innate immune response signalled a major breakthrough in medicine, as the systemic autoinflammatory diseases (SAIDs) were recognised as a spectrum of rare genetic disorders characterised by periodic or chronic systemic inflammation, accompanied by unexplained fever, in the absence of immune cell (T or B cell) activation (43). Also referred to as the inherited periodic fever syndromes (IPFS), or hereditary periodic fever syndromes (HPFS), it is now known that in some SAIDs, the pathogenic mutations may occur de novo. There are now over 30 different syndromes recognised but the four commonest are familial Mediterranean fever (FMF), cryopyrin-associated periodic syndrome (CAPS), tumour necrosis factor (TNF) receptor-associated periodic syndrome (TRAPS) and mevalonate kinase deficiency (MKD).

Although there exists some overlap in clinical features between these disorders – fever and systemic inflammation involving the skin, joints, eyes and serosa - there are major differences in the aetiology, pattern of inheritance and length and frequency of attacks,

between the various syndromes (Table 1.1). Attacks do resolve spontaneously without immunosuppressive, anti-cytokine or anti-inflammatory drugs, although patients experience severe discomfort, pain and poor quality of life during the attacks. Between attacks patients may feel well and return to normal daily activities, although some do suffer continuous low grade symptoms which may be debilitating.

Syndrome	Gene and Location	Protein	Inheritance	Ethnicity	Age at Onset	Distinctive Clinical Features	Attack duration	Attack frequency	Treatment
FMF	*MEFV* Chromosome 16	Pyrin	Autosomal recessive	Mainly Eastern Mediterranean	Childhood <10 years or early adulthood	Erysipelas erythema, monoarthritis (usually of lower limbs), aseptic meningitis	1 to 3 days	Variable	Colchicine
CAPS	*NLRP3* Chromosome 1	Cryopyrin	Autosomal dominant; CINCA may be sporadic	Mainly Northern European	Infancy usually, but atypically in adulthood	Spectrum including fever, arthralgia, urticarial rash, conjunctivitis and towards the more severe end, sensorineural deafness, aseptic meningitis, mental retardation	<24 hours	Varies between dependency on environmental stimuli, to daily, to continuous along the severity spectrum	Anti-IL-1 drugs
TRAPS	*TRNRSF1A* Chromosome 12	TNF Receptor	Autosomal dominant	Mainly Northern European	Childhood or early adulthood	Migratory rash, arthralgia, red eyes, periorbital oedema, abdominal pain, serositis	Days to weeks	Variable, may be continuous	Anti-TNF and anti-IL-1 drugs
MKD	*MVK* Chromosome 12	Meval-onate kinase	Autosomal recessive	North Western European	Infancy, usually <1 year	Macular papular rash, red eyes, arthralgia	3 to 7 days	Once to twice monthly	Anti-TNF and anti-IL-1 drugs

Table 1.1. Summary of features of the systemic autoinflammatory diseases.

between the various syndromes (Table 1.1). Attacks do resolve spontaneously without immunosuppressive, anti-cytokine or anti-inflammatory drugs, although patients experience severe discomfort, pain and poor quality of life during the attacks. Between attacks patients may feel well and return to normal daily activities, although some do suffer continuous low grade symptoms which may be debilitating.

1.3.1 Familial Mediterranean Fever

Familial Mediterranean fever (FMF) (MIM 249100) is the commonest inherited monogenic autoinflammatory disorder, characterised by recurrent self-limiting episodes of fever and systemic inflammation usually manifest as serositis, peritonitis, arthritis, myalgia and sometimes pleurisy and pericarditis (44). Attacks may last between 12 and 72 hours, and common precipitants are physical or emotional stress and menstrual periods. Peritonitis is often intense and misdiagnosed as acute abdomen with about 40% of patients undergoing a negative laparoscopy before FMF has been diagnosed. First presentation usually occurs in childhood or early adulthood. FMF is most prevalent in the eastern Mediterranean coastal populations – Armenians, Sephardic Jews, Arabs and Turks – but is known to affect other groups found along the Mediterranean basin, including Italians, Greeks and Spaniards (45-47).

FMF is the result of mutations in *MEFV*, the gene encoding the 781-amino-acid protein pyrin (previously known as marenostrin), located on chromosome 16p13.3, and the mutation is inherited in an autosomal recessive fashion. *MEFV* is 10 exons long and mutations most commonly occur on exon 10, the longest exon on the gene. Over 300 sequence variants have been identified to date (48); although some are more frequently seen than others and pathogenicity is variable. For example, M694V, most commonly seen among Sephardic Jews, is associated with a more severe phenotype, whilst E148Q

(common amongst Europeans and Turks) and V726A are associated with reduced penetrance and milder phenotype; however, some who are compound homozygous for the V726A/E148Q alleles are also known to have a more severe form of the disease (49). Consequently, genetic testing holds only a 70% to 80% positive predictive value, and the diagnosis of FMF remains clinical. The Tel Hashomer revised criteria for diagnosis are listed in Table 1.2.

Pyrin is a member of the death domain superfamily, the members of which are known to play important roles in the assembly and activation of apoptotic and inflammatory complexes. Pyrin is expressed in granulocytes and cytokine-activated mononuclear cells and expression is up-regulated in the presence of activators of inflammation such as TNFα and interferon (IFN) γ (50, 51). The range of genotypic and phenotypic variation in FMF suggests multiple pathogenic mechanisms, and the field appears to be divided broadly between the theories of a pro-inflammatory role and an anti-inflammatory role for pyrin as discussed above. Colchicine is the treatment of choice for FMF and its long term safety and efficacy is well known and well-documented (52). Those who are unresponsive (22-24), or incompletely responsive (20),(21) to colchicine may be successfully treated with anti-IL-1 therapies.

Major Criteria	Minor Criteria
Recurrent febrile episodes with serositis	Recurrent febrile episodes without signs of serositis
AA amyloidosis	Erysipelas-like erythema
Good response to colchicine	FMF in a first-degree relative

Table 1.2. Revised Tel Hashomer Criteria for the diagnosis of FMF. The diagnosis is definitive in the presence of 1 major and 2 minor criteria, or 2 major criteria. The diagnosis is probable if only 1 major criterion and 1 minor criterion are present.

1.3.2 Cryopyrin-Associated Periodic Syndrome

Previously described as three separate syndromes, cryopyrin-associated periodic syndrome (CAPS) is now known to comprise a spectrum of disorders. At the milder end of the spectrum is familial cold autoinflammatory syndrome (FCAS) (MIM 120100) which shows some phenotypic overlap with Muckle-Wells syndrome (MWS) (MIM 191900) towards the middle of the disease spectrum. At the more severe end of the spectrum is Chronic Infantile Neurological Cutaneous Articular (CINCA) (MIM 60711) Syndrome also known as neonatal onset multisystem inflammatory disease (NOMID). CAPS is characterised by recurrent episodes of fever accompanied by urticarial rash, arthritis, myalgia, iritis and fatigue. These attacks may last up to 12 hours and may be triggered by physical or emotional stress, infection or cold temperatures. The more severely affected may suffer profound fatigue, sensorineural deafness, joint deformation or central nervous system (CNS) symptoms manifest as headaches and raised intracranial pressure on lumbar puncture. The worst affected are those with CINCA/NOMID who have severe joint deformities often preventing mobility, and severe CNS manifestations including chronic aseptic meningitis, cerebral atrophy, visual loss due to optic nerve atrophy, seizures and mental retardation.

CAPS results from mutations in the gene encoding cryopyrin, *NLRP3*, resulting in dysregulation of the activity of the pro-inflammatory cytokine, interleukin-1β (IL-1β). *NLRP3* is located on chromosome 1q44, and there are now more than 140 known pathogenic sequence variants, most of which are single nucleotide substitutions (48). FCAS and MWS display an autosomal dominant inheritance pattern in about 75% of cases; CINCA syndrome on the other hand is often the result of de novo mutation, and in approximately 40% of affected individuals, conventional genetic sequencing methods reveal no identifiable mutation (53). CAPS most commonly affects Northern Europeans

and North Americans (54), but has been identified in other ethnic groups such as South Asians and North Africans.

Cryopyrin is expressed in peripheral blood leucocytes and chondrocytes and is the most studied and best understood member of the NLR family. It is the key component of the NLRP3 inflammasome, as previously discussed. Treatment of CAPS involves inhibiting the activity of IL-1, by blocking binding to its receptor or by means of anti-IL-1 antibodies (55).

1.3.3 Tumor Necrosis Factor Receptor-Associated Periodic Syndrome

Tumor necrosis factor (TNF) receptor-associated periodic syndrome (TRAPS) (MIM 142680) is characterised by self-terminating episodes of fever accompanied by localised and systemic inflammation presenting as abdominal pain, pleurisy, myalgia and a geographical rash. TRAPS may first present in childhood or early adulthood, and flares may persist for several weeks. As with FMF the abdominal pain may be intense, mimicking acute abdomen, and often results in unnecessary laparotomy before the diagnosis of TRAPS has been made. Although first described in, and predominantly affecting Northern Europeans, TRAPS does also affect other ethnic populations such as Japanese, Southern Europeans and African Americans (56-58).

TRAPS is associated with mutations in the TNF receptor superfamily 1A (*TNFRSF1A*) gene located on chromosome 12p13, and the pattern of inheritance is autosomal dominant. More than 100 pathogenic variants have been identified to date, and all bar six are single nucleotide substitution mutations (48). Phenotype-genotype studies reveal that some variants, particularly those involving the highly conserved cysteine residues, are associated with a severe phenotype, whilst others, such as the R92Q polymorphism are

associated with a milder phenotype (or a 'carrier' phenotype) and incomplete penetrance (56).

There are several proposed pathways by which mutations cause systemic inflammation, although the precise mechanisms remain unclear. One proposal is that TNFR1 mutations may lead to impaired cleavage of the extracellular domain (receptor shedding), which is the commonest method of TNFR1 inactivation; another is by impaired trafficking to the cell surface and of retention of mutant receptors within the endoplasmic reticulum. Both proposed mechanisms may give rise to enhanced or prolonged signalling (59). Other studies have shown that impaired intracellular trafficking and retention of mutant TNFR1 within the endoplasmic, and/or cytoplasmic aggregation of the mutant protein triggers the unfolded protein response (UPR) and release of pro-inflammatory cytokines including IL-1β (32, 33). For patients with a relapsing-remitting disease course, corticosteroid treatment during flares can be effective. For patients with a more chronic phenotype, long term treatment is either by blockade of the TNF receptor, although this has not been successful in many patients or, more successfully, by blocking IL-1 (60-63).

1.3.4 Mevalonate Kinase Deficiency

Previously known as the hyperimmunoglobulin D and periodic fever syndrome (HIDS), MKD (MIM 260920) presents as recurrent episodes of fever, associated with lymphadenopathy, malaise, rash, arthralgia and gastrointestinal symptoms of abdominal pain, diarrhoea and vomiting. MKD is a rare autosomal recessively inherited autoinflammatory disease resulting from mutations in the gene encoding mevalonate kinase, *MVK*. Located on chromosome 12q24, more than 200 mutations have been identified in MVK, more than half of which are thought to be pathogenic (48). The precise pathogenic mechanisms by which mevalonate kinase deficiency results in the autoinflammatory phenotype remain unclear. Mevalonate kinase is known to be involved

in the cholesterol, farnasyl and isoprenoid biosynthesis pathway; it has been demonstrated that a downstream consequence of isoprenoid deficiency is activation of pro-inflammatory cytokines (64). Magnitude of deficiency of MKD can vary between patients and complete deficiency is associated with the rather severe phenotype of mevalonate aciduria, which is associated with mental retardation, myopathy, cerebellar ataxia, failure to thrive, and often fatality in early life (65). Current treatment of MKD is by IL-1 blockade, although there are reports of the successful use of anti-IL-6 therapy (66).

The systemic autoinflammatory diseases are generally compatible with normal life expectancy, however a proportion of these patients develop AA amyloidosis as a serious and often life-threatening complication.

1.4 Amyloidosis

Amyloidosis is a disorder of protein folding whereby endogenous proteins become misfolded and auto-aggregate in an abnormal but highly stable fibrillar conformation in the extracellular space, progressively disrupting organ structure and function (67). Amyloid deposition may be localised and incidental, having little or no sequelae, or, at the opposite end of the spectrum, may be more widespread causing multi-organ damage and failure, and death. Over 20 endogenous proteins are known to be amyloidogenic, and the type of amyloid is classified according to the precursor protein. Accurate diagnosis and typing is of the utmost importance, as management and treatments vary greatly between different amyloid types.

1.4.1 Precursor proteins and fibrillogenesis

Under the correct in vitro conditions almost any polypeptide can be driven towards misfolding and aggregation (68), but not many are amyloidogenic in vivo (Table 1.3). There are three circumstances under which amyloidogenesis will take place in vivo: Firstly, when there is a prolonged period of excess or overproduction of a normal endogenous protein, e.g. sustained periods of elevated levels of serum amyloid A protein (SAA) as a result of a prolonged acute phase response, is the cause of AA amyloidosis; Secondly, when there are normal levels of an inherently amyloidogenic endogenous protein, present over a prolonged period of time, such as in wild-type transthyretin (ATTRwt) amyloidosis; and lastly, when there are highly amyloidogenic mutant proteins, such as variant transthyretin in hereditary transthyretin (ATTRv) amyloidosis, or variant fibrinogen in fibrinogen (AFib) amyloidosis.

Amyloid fibrils in vivo are usually composed of fragmented precursor proteins which are partially or completely dissociated into their monomers, or which have undergone partial cleavage (69, 70). However, there are some circumstances in which amyloid fibrils may be composed of fully intact precursor proteins as in lysozyme amyloid (71) and certain forms of TTR amyloid (72). Despite being structurally and functionally diverse in their native states, during amyloidogenesis the precursor proteins all assume the remarkably similar but extremely abnormal configuration of a common core structure made up of anti-parallel β-strands forming sheets lying with their long axes perpendicular to the long axis of the fibril (73). And whilst the precise mechanisms of fibrillogenesis remain unclear, the mechanism proposed by David Booth and colleagues (74) for in vitro lysozyme amyloid fibril formation is widely believed to be similar for all amyloid types.

Amyloid Type	Precursor Protein	Clinical Syndrome
AL	Monoclonal immunoglobulin free light chains	Systemic amyloidosis associated with monoclonal plasma cell dyscrasias
AH	Monoclonal immunoglobulin heavy chains	Systemic amyloidosis associated with monoclonal plasma cell dyscrasias
AA	Serum amyloid A protein	Systemic amyloidosis, primarily affecting the kidneys, associated with chronic inflammation
ATTRwt	Wild type plasma transthyretin	Wild type transthyretin amyloidosis with cardiac-dominant disease
ATTRv	Genetically variant transthyretin	Familial amyloid polyneuropathy and/or cardiomyopathy (autosomal dominant)
AFib	Genetically variant fibrinogen A alpha chain	Non-neuropathic with renal-dominant disease (autosomal dominant)
AGel	Genetically variant gelsolin	Predominant cranial nerve involvement with lattice corneal dystrophy (autosomal dominant)
ALys	Genetically variant lysozyme	Non-neuropathic with prominent visceral involvement (autosomal dominant)
AApoAI	Genetically variant apolipoprotein AI	Predominantly non-neuropathic with prominent visceral involvement (autosomal dominant)
AApoAII	Genetically variant apolipoprotein AII	Non-neuropathic with prominent renal involvement (autosomal dominant)
Aβ2M	β2-microglobulin	Periarticular and/or systemic amyloidosis associated with long-term dialysis
Aβ	β-protein precursor (and rare genetic variants)	Cerebrovascular and intracerebral plaque amyloid in Alzheimer's disease
ALect2	Normal plasma leukocyte chemotactic factor 2	Sporadic systemic amyloidosis; lowly progressive proteinuric renal failure
ACys	Genetically variant cystatin C	Cerebral haemorrhage with cerebral and systemic amyloidosis

Table 1.3. Classification of amyloidosis by precursor protein.

1.4.2 Non-fibrillar components

Amyloid deposits in vivo also contain various non-fibrillar constituents. Glycosaminoglycans and proteoglycans such as heparan sulphate and chondroitin sulphate are found universally in amyloid deposits. Although their function remains uncertain, their presence in all amyloid deposits implies a significant role, perhaps in stabilisation of the deposits (75), or in fibrillogenesis (76). The normal plasma glycoprotein, serum amyloid P component (SAP), acts as the precursor to another universal non-fibrillar component of amyloid deposits, tissue amyloid P (TAP) (77). TAP binds to amyloid fibrils in a reversible calcium-dependent manner (78). Whilst the physiological role of SAP is not fully understood, in vitro studies have demonstrated that SAP binding has a stabilising effect on the amyloid fibril, protecting it from degradation by proteases and phagocytic cells (79). The universal presence of TAP in all amyloid deposits has formed the basis of the specialised in vivo nuclear medicine imaging technique, SAP scintigraphy, in which the localisation of ^{123}I-labelled SAP to amyloid deposits in the organs is used to diagnose visceral amyloid (80). It is also the basis of the anti-SAP approach to therapeutic clearance of visceral amyloid (81).

1.5 Systemic AA amyloidosis

Previously known as secondary or reactive amyloidosis, AA amyloidosis is a rare complication of chronic infections or inflammatory diseases. The AA amyloid fibrils are derived from the plasma acute phase reactant serum amyloid A protein (SAA), which is synthesised by hepatocytes under the transcriptional regulation of pro-inflammatory cytokines, (82). The median plasma concentration of SAA in health is < 3mg/L, with > 90% of the population having levels < 10 mg/L, however, during a severe acute phase response, levels can increase more than 1000-fold (83). Prolonged elevation of SAA is a prerequisite to development of AA amyloidosis.

The human SAA gene family is made up of four discrete loci on chromosome 11, containing *SAA1* and *SAA2*, two highly homologous genes, and *SAA3* and *SAA4*, which are more distantly related. SAA1 and SAA2 are the predominant proteins in the acute phase response (82), however, AA amyloid fibrils are almost always derived from SAA1, due perhaps to differences in the processing or catabolism of the two proteins, or possibly due to specific amino acid sequences in SAA1 which promote amyloidogenicity. Polymorphisms in the gene encoding the SAA1 isotype may confer increased susceptibility to the development of AA amyloidosis (84). In humans there are three main *SAA1* alleles, *SAA1.1, SAA1.3 and SAA1.5.* Associations have been found between particular alleles and the risk of developing AA amyloidosis. One study linked homozygosity for the *SAA1.3* allele with increased risk of developing AA amyloidosis, a shorter latency to development of AA amyloidosis and a more severe phenotype (85, 86). Other studies have linked *SAA1.1* homozygosity with a predisposition to AA amyloidosis in patients with FMF (87, 88) and juvenile idiopathic arthritis (JIA) (89).

The lifetime incidence of AA amyloidosis in those with chronic inflammatory disorders is 1 to 5% (90), and in the developed world the commonest predisposing conditions are the inflammatory arthritides, followed by chronic sepsis, inflammatory bowel disease and the SAIDs. Developments in anti-cytokine therapies have significantly impacted on the prevalence of AA amyloidosis secondary to SAIDs. Renal dysfunction and its sequelae dominate the disease course in AA amyloidosis with 80% of sufferers presenting with nephrotic range proteinuria and > 50% of patients in renal failure at presentation (UK National Amyloidosis Centre unpublished data).

Treatment of AA amyloidosis is aimed at controlling the underlying inflammatory condition, thereby suppressing the acute phase response and reducing the supply of the amyloidogenic precursor protein. Outcomes in AA amyloidosis are favourable when SAA

concentrations are maintained below 10 mg/L (91) and complete suppression of inflammation with SAA concentration maintained at < 5 mg/L is associated with preservation of, or improvement in, renal function, and regression of amyloid (92, 93).

1.6 Hepatic Acute Phase Proteins

C-reactive protein (CRP) and SAA are acute phase proteins synthesised by hepatocytes under the transcriptional regulation of pro-inflammatory cytokines, in particular IL-1, IL-6 and tumor necrosis factor (TNF) α (82). An explosion of interest in measurement of CRP and SAA in relation to cardiovascular disease occurred following the original observations in patients with known cardiovascular disease (94, 95)and subsequent findings in the general population, that baseline values of CRP and SAA are statistically significantly associated with risk of future coronary events. Although their physiological roles remain uncertain, they are of enormous value due to their clinical relevance in the acute phase. The rapid increase in serum concentrations, in proportion to the degree of inflammation, infection or tissue damage, and dynamic range in the acute phase response make CRP and SAA ideal biomarkers for objective monitoring of disease activity and response to treatment.

1.6.1 C-Reactive Protein

CRP is an acute phase protein of the pentraxin family, the synthesis and circulating concentration of which increases dramatically in response to most forms of tissue injury, infection and inflammation (96). Normal CRP concentration in healthy individuals is very low at a median 0.8 mg/L, with 90% of individuals having less than 3 mg/L and 99% less than 10 mg/L (97). During the acute phase response the CRP concentration can exceed 500 mg/L, and values around 20-30 mg/L are common during minor illnesses such as upper respiratory tract infection or minor trauma. Ambulant patients in the community with chronic inflammatory diseases, such as rheumatoid arthritis (RA) or Crohn's disease may have circulating CRP values up to 100 mg/L or more for months or even years, and there is no evidence that such increased circulating CRP concentrations have any acute adverse effects. CRP is catabolised by hepatocytes, and has a half-life of 19 hours in the plasma. Human CRP is not glycosylated and is encoded by a single gene with no coding polymorphism. The physiological role of CRP remains unknown. This is because there are no known cases of CRP deficiency or structural polymorphism (96); nor are there any known in vivo depleters or inhibitors of CRP, and so the consequences of absence, dysfunction or inhibition of human CRP cannot be ascertained (98). The non-specific nature of the CRP acute phase response means that CRP values can only be interpreted at the bedside in light of clinical information including medical history, physical examination and results of all available investigations. With this in mind, CRP is used worldwide as the main measure of both inflammation and success of anti-inflammatory therapy.

1.6.2 Serum Amyloid A Protein

SAA, an apolipoprotein constituent of high density lipoprotein (HDL), is a more sensitive marker of inflammation, having a temporally more rapid elevation in the serum during

the acute phase response, and a greater dynamic range than CRP. SAA concentrations may be elevated up to 1000-fold in response to various inflammatory stimuli, including infection, injury, inflammation and neoplasia (82). Whilst the liver is the main site of SAA production SAA expression has also been described in the normal (non-diseased) tissue epithelium (99), in diseased tissues such as atherosclerotic lesions (100) and various carcinomas, and in adipose tissue (101). Whilst the physiological role of SAA remains incompletely understood it is proposed to be involved in cholesterol metabolism, and is known to have an established pathogenic role as the precursor protein in AA amyloidosis (described in Section 1.5 above). As a high-density lipoprotein–associated apolipoprotein, SAA is considered as a modulator of inflammatory processes, playing a part in leukocyte and mast cell tissue infiltration, migration and adhesion (102) and in stimulating production of inflammatory cytokines including IL-1β and IL-6 (103, 104).

1.7 Diagnosing AA amyloidosis

Frequently, the diagnosis of amyloidosis is made at an unfortunately late stage, long after the onset of symptoms, and after prolonged investigation, as often the non-specific and varied presentations give rise to many differential diagnoses. Not infrequently, patients have no inflammatory symptoms at all, and already have renal amyloidosis at presentation.

1.7.1 Histology and immunohistochemistry

To date histological confirmation of amyloid remains the diagnostic gold standard; this is achieved by Congo red staining of amyloidotic tissue with the presence of the pathognomonic red-green birefringence when visualised under cross-polarised light (105). Common biopsy sites include the gastrointestinal tract, kidneys and bone marrow. Abdominal fat pad biopsy is an increasingly popular means of obtaining tissue as this may be performed in the outpatients' clinic relatively quickly, and safely. A disadvantage,

however, is that the yield is frequently insufficient for staining and typing. A positive Congo red stain should trigger further investigations to determine the amyloid fibril type and extent of organ involvement.

Immunohistochemistry is used to ascertain fibril type and should be performed on any tissue in which amyloid has been detected. However, despite recent advances in immunohistochemical techniques, the result is still frequently inconclusive (106), and a diagnosis of exclusion must follow. For example, hereditary forms of amyloidosis may be excluded by genetic screening for mutations in genes encoding known amyloidogenic fibril precursor proteins (107). Treatment of the different types of amyloidosis varies greatly, and so precise identification of the amyloid fibril type is absolutely critical.

1.7.2 SAP scintigraphy

This nuclear medicine imaging technique utilises radiolabelled iodine as a tracer which localises to visceral amyloid deposits in vivo (108). SAP scintigraphy can be used to monitor regression or progression of visceral amyloid and response to therapy (109). SAP scintigraphy also provides information on the distribution of visceral amyloid deposits which may be a useful typing adjunct (110).

1.7.3 Other investigations

Measuring the circulating concentration of amyloidogenic precursor proteins, such as SAA in AA amyloidosis, is important diagnostically, but longer term monitoring is also crucial in order to determine the success of treatment. Echocardiography and electrocardiographs (ECGs), and more recently, cardiac magnetic resonance imaging (CMR) are used to diagnose cardiac amyloidosis and monitor change in cardiac amyloid deposits.

1.8 Diagnosing systemic autoinflammatory syndromes

SAIDs are diagnosed by a combination of clinical assessment, biochemical measurement of SAA and CRP, and genetic sequencing. Clinical assessment involves taking a detailed symptom history and family history, and physical examination to identify the clinical signs of the syndrome as described in Tables 1.1 and 1.2. Biochemical and genetic analysis are discussed in Chapter 2: Methods.

1.9 Measuring quality of life (QoL)

Until fairly recently the so called 'hard' clinical endpoints such a hospitalisation or death were the focus of most clinical trials or disease management programs. When patient-reported outcomes (PRO) were used, they tended to be in relation to disease specific indicators, such as joint pain in rheumatoid arthritis. Whilst such outcome measures are extremely important in disease management, they do not give a sense of the overall health-related QoL. As people are living longer, healthcare must focus as much on quality as length of life. Medicine regulatory bodies such as the United States (US) Food and Drug Administration (FDA) and the United Kingdom (UK) Medicines and Healthcare Products Regulatory Agency (MHRA) have become increasingly interested in patient-reported health-related quality of life, and the pharmaceutical industry, as well as academic and clinical researchers, are now expected to include validated QoL measures as endpoints in clinical studies (111).

The QualityMetric SF-36v2® and other SF QoL questionnaires are widely recognised as leading PRO measures, their validity and reliability having been validated in patients with many different acute and chronic conditions. Although the main intended use of the survey was initially in population studies, the SF-36v2 has become invaluable to healthcare professional as a means of evaluating and monitoring disease and success of

therapy (112, 113). Unlike the standard clinical assessments of health status such as vital signs, laboratory tests and physician examination, the SF-36v2 provides a broad overview of a patient's health status and the effect of the disease/health status on his or her physical, social emotional and mental functioning – i.e. disease burden (114). In addition to the utility of the survey in measuring disease burden, the SF-36v2 may be administered to a single patient at multiple time-points, offering a baseline comparator for long term monitoring in chronic conditions. Thus, a pre-treatment administration, or administration during an episode of disease flare may serve as a baseline measure of health-related QoL, which can then be compared to results of surveys completed during treatment, thereby providing an objective means of documenting the success of therapy. It is easy to administer, taking on average less than 10 minutes to complete, and so may easily be incorporation into clinical practice (115).

The SF-36v2® is composed of 36 questions covering eight health domains or scales:

Physical Functioning (PF): The PF domain is composed of 10 items covering distinct aspects of physical functioning over a range of severe and minor physical limitations, such as lifting and carrying groceries, climbing stairs, bending, kneeling, and walking moderate distances. There is also one item dedicated to describing limitations in self-care activities. The PF items are designed to capture the presence as well as the extent of physical limitations with low scores indicating significant limitations in performing physical activities, whilst high scores reflect little or no limitations in performing physical activities.

Role-Physical (RP): This domain is composed of four items covering a range of physical role limitations, including limitations in carrying out work or other usual activities and reduction in the amount of time spent on work or other usual activities. Low scores indicate interference of physical problems with work or other usual activities.

Bodily Pain (BP): This domain comprises two items: one designed to measure the intensity of bodily pain and one measuring the extent of interference with normal work activities as a result of bodily pain. Low scores indicate high levels of pain which significantly affect normal activities, whilst high scores indicate no pain or impact on normal activities.

General Health (GH): The GH scale comprises five items, including a rating of health from poor to excellent, as well as four items addressing the patient's opinion and expectations of his/ her health. Low scores indicate a perception of poor general health, which is expected to deteriorate. High scores indicate a perception of good general health.

Vitality (VT): This scale is composed of a four-item measure of vitality (i.e. energy level and fatigue). Low scores indicate feeling tired and worn out a lot of the time, whilst high scores indicate feeling energetic all or most of the time.

Social Functioning (SF). This two-item domain is designed to assess health-related physical or emotional problems on the quantity and quality of usual social activities.

Low scores indicate extreme or frequent interference of health-related physical or emotional problems with normal social activities.

Role-Emotional (RE). This three-item scale assesses the impact of mental health-related problems on role limitations in terms of time spent on work or other usual activities, the amount of work or usual activities completed, and the care with which work or other usual activities were performed. Low scores here are indicative of problems with work or other usual activities as a result of emotional problems and high scores reflect no such limitations due to emotional problems.

Mental Health (MH). The five-item MH scale includes items from each of four major mental health dimensions - anxiety, depression, loss of behavioural and/or emotional control, and psychological wellbeing. Low scores point to frequent feelings of

nervousness and depression all or most of the time, whilst high scores indicate feelings of peace, happiness, and calm (115).

Each domain or scale is scored individually, and the result expressed in comparison to American norms. Higher scores represent a better QoL and a change of 10 points or more in a domain between administrations is considered clinically significant.

1.10 The UK National Amyloidosis Centre

The UK National Amyloidosis Centre (NAC) is the UK's only referral centre specialising in diagnosis and treatment advice for the national caseload of patients with amyloidosis. Patients with amyloidosis (diagnosed or suspected) are referred by their local medical teams for diagnosis (or confirmation of suspected diagnosis) and management advice. Patients return every six to 12 months for monitoring of the disease burden, and undergo an extensive battery of tests at every visit. Treatments outside of clinical trials, including chemotherapy, renal replacement therapy and solid organ or stem cell transplantation take place at the patients' local hospitals.

Contained within the UK NAC is the Fever Clinic which specialises in the diagnosis and treatment of rare and ultra-rare autoinflammatory diseases. Following the discovery of the causative role of excessive IL-1 production in causing the symptoms of CAPS, the first CAPS patients were treated with anakinra here, in 2002. Since then several dozens of patients with CAPS and other SAIDs have been prescribed anakinra. Following successful clinical trials with canakinumab, the National CAPS Treatment Service was established at the NAC. This service comprises a medical clinic where patients are reviewed annually, alongside a specialist nurse clinic which patients attend approximately every eight weeks to receive their canakinumab injections and to undergo safety and

efficacy monitoring. Patients undergo annual ophthalmology, audiometry and neurology assessments and brain MRI, where indicated, and quality of life assessments.

Aims

This thesis is composed of clinical studies of inflammatory disorders and AA amyloidosis, involving patients and healthy volunteers.

AA amyloidosis is one of the most serious complications of chronic inflammation. It causes renal dysfunction which if left untreated, inexorably progresses to renal failure. Renal replacement therapy comprising chronic dialysis and kidney transplantation is costly to the National Health Service (NHS), is fraught with complications and unwanted side effects, and has a negative effect on quality and length of life. An obvious target of therapy is thus prevention of the development of AA amyloidosis, or once it has developed, halting its progression to end stage renal failure, or protecting renal allografts.

The diseases studied within this thesis are characterised by sustained inflammation associated with a substantial acute phase response. The main focus of this body of work is the safe and effective treatment of inflammatory disorders – SAIDs such as CAPS, acquired inflammatory disorders such as rheumatoid arthritis, and inflammatory disorders of unknown aetiology. SAIDs are rare diseases characterised by seemingly unprovoked episodes of inflammation which manifest as a catalogue of symptoms including fever, rigors, rash, arthralgia, myalgia, serositis and pleurisy, amongst others. Although between attacks some patients may be relatively well, periods of flare are debilitating and may impact severely on normal physical and social development, schooling, relationships and employment. Other inflammatory diseases such as rheumatoid arthritis, juvenile

idiopathic arthritis and Castleman's disease can have the same sequelae, as can prolonged inflammation of uncertain aetiology. Early diagnosis and treatment of these diseases is essential for these reasons, but also in order to prevent or halt amyloid deposition.

In recent years huge leaps have been made in the understanding of disease pathways, pathogenic mechanisms and molecular origins in the fields of autoinflammation and autoimmunity. These developments have resulted in the identification of therapeutic targets, and for the very first time, successful pharmacological 'switching off' of disease activity for hundreds of sufferers.

Disease activity and success of therapy are monitored by serial measurements of the hepatic acute phase proteins CRP and SAA. Production of both CRP and SAA are part of the nonspecific acute-phase response to injury, infection and inflammation.

Serial measurements of serum CRP concentration interpreted in light of other clinical and pathological information, contribute powerfully to diagnosis and clinical management of a huge range of infectious and inflammatory disorders. Some published studies however, suggest that CRP may itself be pro-inflammatory. The conflation of epidemiological association with causality, has led to claims that a higher baseline CRP concentration is not only a risk marker for cardiovascular disease but also a risk factor contributing to the pathogenesis of atherosclerosis and atherothrombosis. This might therefore be of particular concern for patients with inflammatory conditions in whom the CRP concentration is very substantially elevated for many years or even decades. We sought to investigate this phenomenon.

SAA is an even more sensitive and dynamic marker of the acute phase response but it is also the precursor of AA amyloidosis, which means that monitoring of its serum

concentration is absolutely crucial to the management of patients with AA amyloidosis, and those who are deemed to be at risk of developing it.

The main aim of this thesis is to study the safety and efficacy of modulation of cytokines IL-1 and IL-6 in inflammatory disorders and AA amyloidosis. In doing so I have also sought to give a more complete picture of this very specialist disease area by characterising the UK population of patients in whom AA amyloidosis complicated systemic autoinflammatory disease, as well as an exploration of the role of CRP, and the evolving epidemiology of AA amyloidosis.

Chapter Two:

Methods

2.1 Methods performed by the author

2.1.1 Patient selection and ethical approval

All the patients included in this thesis were under the care of the NAC. In the 5 year period between 2009 and 2014, more than 5000 patients have been referred to the Centre; approximately 60% of those have been found to have amyloidosis and 15% of referrals are to the Fever Clinic. A clinical database has been maintained with demographic and clinical details (including results of all investigations) of all patients since approximately 1990, and this database, together with the routine medical and research records have formed the source data for this research. Other nurses, doctors and technicians within the Centre have also contributed to collection of the data within this database together with the author, however, every chapter contained herein has been written by the author. Ethical approval for all studies herein were obtained by the author. All patients included in this work have given written informed consent. The healthy volunteers in the CRP Study (Chapter Eight) were not patients of the Centre but were recruited via advertisements. They too gave written informed consent.

2.1.2 CAPS/Inflammation nurse-led clinic protocol

In 2010 I set up the specialist nurse CAPS/inflammation clinical and research service at the NAC. All patients receiving anti-IL-1 and anti-IL-6 therapies at the NAC are managed jointly between this clinic and the medical consultant clinic. Within this specialist nurse clinic I have administered anti-IL-1 and anti-IL-6 therapies, and have conducted regular long term follow up - consultation, assessment and dosing approximately every eight weeks for CAPS patients treated with canakinumab, monthly for patients on intravenous tocilizumab, three- to six-monthly for patients on anakinra - and monitoring of these patients in terms of safety and efficacy of treatments, and general holistic care including QoL surveillance. Further detail on the clinic protocol may be found in Chapter Three.

Within this clinic I also administered purified human CRP to healthy volunteers, as described in detail in Chapter Eight.

2.1.3 Definition of response to therapy in SAIDs

We defined a complete response or complete remission (CR) as normalisation of serum acute phase reactants (SAA $\leq$ 10 mg/L and CRP $\leq$ 5 mg/L) and resolution of chronic disease symptoms and flares/exacerbations. Mild recurrent symptoms such as oral ulcers, or occasional mild headaches or rash or joint aches, which result in symptom scores of 1 or 2, and which were not bothersome to patients, were not considered to be in conflict with the classification of complete response. A partial response or remission (PR) was defined as reduction in, but not normalisation of, SAA and CRP measurements and/or an improvement in disease symptoms, but not complete resolution as defined above. Non-responders (NR) or inadequate responders were patients who had no significant improvement in either or both domains.

2.1.4 Assessment of CAPS disease activity

Assessment of CAPS disease activity at each clinic visit was performed by means of a scoring tool adapted from one used in clinical trials of canakinumab. The tool scores patient-reported (and clinician observed) symptoms experienced since the last clinic visit, out of a maximum score of 20. Mild or moderate symptoms are each allocated one point and severe symptoms are each allocated two points. Examples of pre- and on-treatment clinical assessment worksheets may be found in the Supplemental Information section.

2.1.5 Venepuncture and injection of radio-iodinated SAP

Venepuncture and blood sampling for routine clinical tests including serum SAA and CRP assays was performed by the author and other clinic nurses. Intravenous injection of ^{123}I-SAP in preparation for SAP scintigraphy was performed by the author and other

clinic nurses. SAP scintigraphy is discussed in detail below. Approximately 70 μg SAP, labelled with approximately 180 megabecquerels ^{123}I is injected intravenously into each patient. This equates to an effective radiation dose of approximately 4 millisieverts, which is comparable to exposure to the average UK natural background radiation for 1 year, or to that from an intravenous pyelogram. Thyroid uptake is inhibited by oral administration of six doses of potassium iodide solution at a concentration of 10 mg/mL – the first dose is given just before radio-isotope injection, the second on the same night, and then twice daily for the next two days

2.1.6 Quality of life assessments

The QualityMetric SF36v2® Health Survey is designed to measure functional health and well-being from the patient's perspective. There are eight health domains (physical functioning, role physical, bodily pain, general health, social function, role emotional, mental health and vitality) which are scored individually out of 100 points, and the result expressed in comparison to American norms. Higher scores (closer to 100) represent a better QoL and a change of 10 points or more in a domain between administrations is considered clinically significant. Patients were asked to complete the surveys at various time-points before during and after treatment, and some patients took the survey at multiple time-points, enabling comparison over the follow-up period. Surveys were administered by the author and the CAPS/Fever clinic specialist nurse. Data analysis was performed solely by the author using QualityMetric software.

2.1.7 Statistical analysis

Statistical tests were performed using Graphpad Prism® version 5.04.

2.2 Methods performed or contributed by others

2.2.1 Criteria for diagnosis of amyloidosis and definition of organ response

Organ involvement and organ response were defined according to International Consensus Criteria (116) in combination with SAP scintigraphy – an abbreviated version may be found in Table 2.1. Renal function was measured by means of creatinine levels, albumin levels and estimated glomerular filtration rate (eGFR) from serum, and proteinuria from 24-hour urine collections.

Organ	Definition of Organ Involvement	Definition of Organ Response
Heart	Echocardiogram: Mean IVSd > 12 mm (in absence of other cardiac cause) OR Late gadolinium enhancement on Cardiac MRI	Mean IVSd decreased by 2 mm, 20% improvement in EF, improvement by 2 NYHA classes without an increase in diuretic use and no increase in wall thickness
Kidneys	24 hour non-Bence Jones Proteinuria > 0.5g, or uptake on SAP scintigraphy	50% reduction in proteinuria (at least 0.5 g/day) creatinine and creatinine clearance has not worsened by $\geq$ 25% over baseline
Liver	SAP scintigraphy	50% decrease in abnormal ALP or reduced organ uptake on SAP scintigraphy
Spleen	SAP scintigraphy	Reduced organ uptake on SAP scintigraphy
Adrenal glands	SAP scintigraphy	Reduced organ uptake on SAP scintigraphy

Table 2.1. Definition of organ involvement and response. IVSd = intraventricular septal thickness during diastole; EF = ejection fraction; MRI = magnetic resonance imaging; NYHA = New York Heart Association; ALP = alkaline phosphatase.

2.2.2 Histology and immunohistochemistry

The presence of amyloid in biopsy tissue sections was confirmed by a modified version of the alkaline-alcoholic Congo red staining method described by Puchtler et al. (105). Formalin-fixed deparaffinised tissue sections, 6-8 μm thick, were stained with Congo red and viewed in brightfield and under cross-polarised light. Positive controls were obtained from a known Congo red positive composite block (confirmed by laser microdissection and mass-spectrometry based proteomic analysis) and were always processed in parallel.

Immunohistochemical staining of formalin fixed de-paraffinised 2 μm sections of amyloidotic tissue followed Congo red staining in order to determine the amyloid fibril type (117). Sections were washed with water. Endogenous peroxidise activity was quenched by 30 minute incubation in aqueous (0.3%) hydrogen peroxide (H_2O_2). Sections were rinsed again in phosphate-buffered saline (PBS) containing 0.05% Tween (Calbiochem). Non-specific tissue binding was abrogated by incubation for a further 30 minutes in normal non-immune serum from the species providing the secondary antibody (Vector part of the ImmPRESS Kit) prior to the application of antisera. Thereafter sections were incubated overnight with primary antisera at 4°C. Following incubation they were rinsed with PBS containing 0.05% Tween (Calbiochem) and labelled with secondary antibodies. Sections were washed in PBS and bound enzyme-antibody bound complexes were viewed using a metal-enhanced DAB (Fisher Scientific solution). Immunohistochemical stains were performed using commercial monoclonal antibodies against SAA protein (Euro-Diagnostica, Huntingdon, UK) and AL kappa and lambda (Dako Ltd, Denmark House, Ely, UK). Positive and negative controls were used in each run.

2.2.3 Serum and plasma assays

2.2.3.1 C-reactive protein (CRP)

Serum CRP was measured using a high sensitivity automated microparticle enhanced latex turbidimetric immunoassay (COBAS MIRA) (Roche). The lower limit of detection was 0.2 mg/L with an inter-assay coefficient of variance (CV) of 4.2% at 4 mg/L and 6.3% at 1 mg/L. Standardisation of the assay was based on the WHO International Reference Standard, 1987.

2.2.3.2 Serum amyloid A protein (SAA)

SAA was measured in serum by latex nephelometry (BNII autoanalyser; Dade Behring, Marburg, Germany). The lower limit of detection was 0.7 mg/L, with an inter-assay CV of 2.6% at 15 mg/L and 3.7% at 80 mg/L. Standardisation of the assay was based on the respective WHO International Reference Standards, 1987.

2.2.3.3 Cytokine assays

Procedures for assay of cytokines IL-1β, IL-6, TNFα and IL-10 have been previously described (118, 119). Assays were performed on plasma taken at baseline, 4, 8 and 24 hours, in Chapter Four. The assays were all calibrated with the respective WHO International Standard preparations for each cytokine and spike-recovery assays with control plasma samples were very close to 100% for all cytokines.

2.2.3.4 Routine haematology, biochemistry, coagulation and anti-nuclear antibody panels

Haematology, biochemistry, coagulation and anti-nuclear antibody (ANA) panels were carried out by the Royal Free Hospital Pathology Department according to standardised protocols.

2.2.4 Genetic analysis

Genomic DNA was isolated from patients' peripheral blood using the rapid technique previously described by Talmud et al. (120). Whole blood was obtained from patients in a 2 ml BD Vacutainer EDTA® draw tube Sequence variants were identified by amplification using polymerase chain reaction (PCR) and direct sequencing of the appropriate exons of the gene in question. The following genes and exons were amplified using appropriate primers and commercially available amplification kits:

- *MEFV* exons 1 to 10
- *NLRP3* exon 3
- *TNFRSF1A* exons 2 to 7 including introns 2, 4 and 6
- *MVK* exons 2 to 11.

Analysis was extended to other exons where required, e.g. where no mutation was detected in the exons indicated above. PCR was validated by gel electrophoresis.

2.2.5 Purification of human CRP

Authentic natural human CRP administered to healthy volunteers in Chapter Four was isolated and purified from 38 kg of B+1 paste derived from pooled plasma of US donors undergoing plasmapheresis. The preparation and characterization of this batch of pharmaceutical current Good Manufacturing Practice (cGMP) grade CRP have been previously reported in comprehensive detail, including its purity, full structural and functional integrity and lack of detectable endotoxin in solutions at 3 mg/mL of CRP, which is less than 0.1 EU/mg of CRP (118). In summary, the CRP has been prepared in the BioProducts Laboratory (BPL) under strict cGMP conditions, and has been isolated from the pooled plasma of donors from the US under precisely the same conditions as for all commercial pharmaceutical plasma protein products provided by the BPL. The CRP was isolated from a by- product of the process used to prepare a batch of a licensed

therapeutic immunoglobulin G (IgG) product. The source plasma from each individual donor was tested and was negative for all relevant viral pathogens by immunoassay; small pools of donations were also tested, and were negative, for the major viral pathogens using sensitive molecular genetics methods. The processed protein preparation was subjected to a validated solvent detergent virus inactivation procedure and then provided to the study team as an active pharmaceutical ingredient. It was then virus filtered using a membrane with a maximum pore size of 50 nm, sterile filtered and dispensed for pharmaceutical use in the Royal Free Hospital manufacturing pharmacy. In addition to sterility checking, the product has been independently assayed for endotoxin by *Limulus* amoebocyte lysate (LAL) testing by the Scottish Blood Transfusion Service, showing an endotoxin content of < 0.10 EU/mg which is well within the European Pharmacopeia standard for administration to humans. The GMP CRP has also been tested in human peripheral blood monocyte activation assays (121, 122) by the National Institute for Biological Standard and Control to ensure absence of LAL negative contaminants, as well as any other unsuspected cellular or pro-inflammatory effects specific exclusively to human cells. The results of all purity tests were entirely negative.

2.2.6 SAP scintigraphy

This is a sensitive and specific nuclear medicine technique used to image and quantify amyloid deposits in vivo (123). Highly purified human SAP is radio-labelled with the gamma emitting isotope ^{123}I, and injected intravenously (described above). In patients without visceral amyloid deposits no localisation or retention of radiolabelled SAP occurs and the tracer is rapidly catabolised and excreted. In patients with visceral amyloid however, radiolabelled SAP localises rapidly and specifically to amyloid deposits in proportion to the amount of amyloid present (108). In patients with AA amyloidosis the technique has 100% diagnostic sensitivity, and in patients with AL amyloidosis,

approximately 90%. SAP scintigraphy is the only method available for serial monitoring of the progression or regression of visceral amyloid deposits (124).

Anterior and posterior whole body images are obtained from each patient approximately six or 24 hours after injection of ^{123}I-SAP by means of either a GE Infinia Hawkeye (4 slice) SPECT-CT or a GE Discovery 670 (16 slice) SPECT-CT gamma camera (GE Healthcare, UK).

The whole body amyloid load was classified as follows: 'None' when there was no abnormal localisation of tracer; 'Small' when visceral uptake was visible but the intensity of the blood-pool background signal remained normal; 'Moderate' when abnormal uptake was sufficiently intense such that the blood-pool background signal was partially lost when the grey-scale was normalised to encompass the signal in the target-organ; and as 'Large' when the blood-pool background was lost with adjustment of the grey scale to encompass the target-organ signal. Serial SAP scintigraphy was performed at six-monthly or annual intervals to monitor change in amyloid load. On serial scans amyloid load was considered stable if tracer localisation or uptake remained unchanged. Regression of amyloid was defined as a reduction in tracer uptake in affected organs or an increase in the blood-pool background signal, or both. Progression (or accumulation) of amyloid was defined as an increase in tracer uptake in affected organs, new abnormal tracer uptake in a previously unaffected organ, or a decrease in the blood-pool background signal.

Chapter Three:

Establishment of Nurse-led Clinical and Research Services

3.1 Background

Nurse-led clinics, run by experienced specialist nurses, have emerged increasingly in the last decade in response to a need to provide additional care to that available within medical or physician clinics. Nurse clinics have developed to provide education, health promotion, psychological care and support, to help patients develop coping and self-care strategies, as well as to perform nursing interventions, administer pharmacological treatments and to monitor the patient's condition, disease activity and efficacy of treatments. A literature search reveals countless publications from around the world, testifying to the positive impact that nurse clinics have had, not only on service delivery and clinical outcomes but also on quality of life of the patients who use these services (for example (125-129).

Nurse-led research clinics extend this already successful model of clinical care to patients participating in clinical studies, and to those receiving off-license or experimental treatments outside of a clinical trial. These clinics may be run jointly by specialist and research nurses, or by specialist nurses with an interest in research, and provide the infrastructure for nurse-led, multi-disciplinary, academic and clinical research.

In 2010 Novartis was granted the marketing authorisation for the use of canakinumab as the treatment for CAPS in the UK and Europe. In the UK, treatment with this newly licensed, ultra-high cost drug would be funded by the NHS on condition that the drug was administered only by the expert clinical team at the NAC. This meant that all patients who wished to be treated with canakinumab would need to travel to the NAC every eight weeks for review, monitoring and dosing. It was expected that the availability of the drug outside of restrictive clinical trials would open the door to many more CAPS patients, beyond the few who had participated in the phase III clinical trial. As the medically-led periodic fever syndrome clinic was already full and often over-booked, a new service

delivery model was required. This need sparked the birth of the nurse-led inflammation clinical and research service, which encompassed the NHS-funded National CAPS Treatment Service within the NAC.

3.2 Aims

The primary aim was to establish a protocolised, nurse-led clinical service, in order to safely reconstitute and administer canakinumab to patients with CAPS, and to provide long term monitoring of these patients, as well as holistic care. The secondary aims were to create the infrastructure to closely study the natural history of CAPS and other inflammatory conditions and AA amyloidosis, to safely administer biological therapies on- and off-license, and to investigate the safety and efficacy of these therapeutic agents.

3.3 Patients and methods

3.3.1 Patients, volunteers and diagnosis

Patients were reviewed and diagnosed in the medical clinic and then referred to the nurse clinic for pre-treatment screening and counselling, initiation of therapy and long term follow up. Funding approval had to be obtained before treatments could be initiated. Healthy volunteers (CRP study) were recruited via adverts within the university, and were seen within the research clinic.

3.3.2 Administration of therapies

Canakinumab was administered approximately every eight weeks, at doses of 150 mg, 300 mg or 450 mg to patients weighing $\geq$ 40 kg. Each 150 mg vial containing canakinumab powder was reconstituted with 1 ml of sterile water for injection. Once completely dissolved, the drug was injected subcutaneously in one of the usual sites for subcutaneous

injection. Tocilizumab was administered at four-weekly intervals, at a dose of 8 mg/kg intravenously for patients weighing ≥ 30 kg. The vials containing tocilizumab solution at a concentration of 20 mg/ml were diluted to a final volume of 100 ml with sterile sodium chloride 0.9% solution for injection and infused over one hour. Patients prescribed anakinra and subcutaneous tocilizumab were taught to self-inject using pre-filled syringes. Purified human CRP was administered according to the protocol described in detail in Chapter Eight.

3.3.3 Other methods

Other methods are described in Chapter Two: Methods.

3.4 Results

3.4.1 CAPS nurse clinic protocol

Once diagnosed, and having discussed treatment options with the medical staff, patients would be introduced and handed over to the specialist nurse. A full verbal handover would be given. Patients would then have the opportunity to further discuss treatment options, side effects, pros and cons and procedure for administration of treatment and monitoring with the specialist nurse.Before treatment was initiated, the following assessments are carried out within the nurse clinic:

- Routine biochemistry, including SAA and CRP and haematology (blood tests)
- Screening for TB, HIV and Hepatitis B and C (blood tests)
- Vital signs (resting blood pressure, heart rate, temperature) and weight
- Baseline QoL assessment by means of SF-36v2 health survey
- Baseline CAPS symptom score (see Supplementary Information Section).

Once canakinumab therapy has commenced patients attend every eight weeks for assessment and further dosing (unless contra-indicated); patients on anakinra attended every one to three months initially, and then every six to twelve months once stable and established on treatment. Vital signs, weight and routine blood tests and CAPS symptom score are performed at every visit. Patients are examined for signs of CAPS activity and questioned regarding CAPS symptoms and any other health issues. A clinical summary letter is sent out to the patient, and his/her local doctors after each clinic visit (see Supplementary Information for examples), and includes any requests for intervention or review at the local hospital or GP surgery, where indicated. In between visits patients would be monitored remotely by telephone or email, and patients were required to post in serum samples for weekly or fortnightly monitoring of serum inflammatory markers in the initial phase of treatment, until stabilised.

The quality of life survey is repeated after commencing treatment and annually thereafter. New patients have brain MRI, audiometry, ophthalmology and neurology assessments at the start of treatment and then repeated annually (apart from brain MRI, which is performed annually only in patients in whom this is indicated). Blood tests for anti-nuclear antibodies are performed annually. Patients are reviewed in the medical clinical annually, unless referred back sooner for any complications. A monthly multi-disciplinary team meeting is held monthly, during which patients are reviewed and any changes to treatment are discussed. Figure 3.1 depicts the patient pathway for canakinumab therapy diagrammatically.

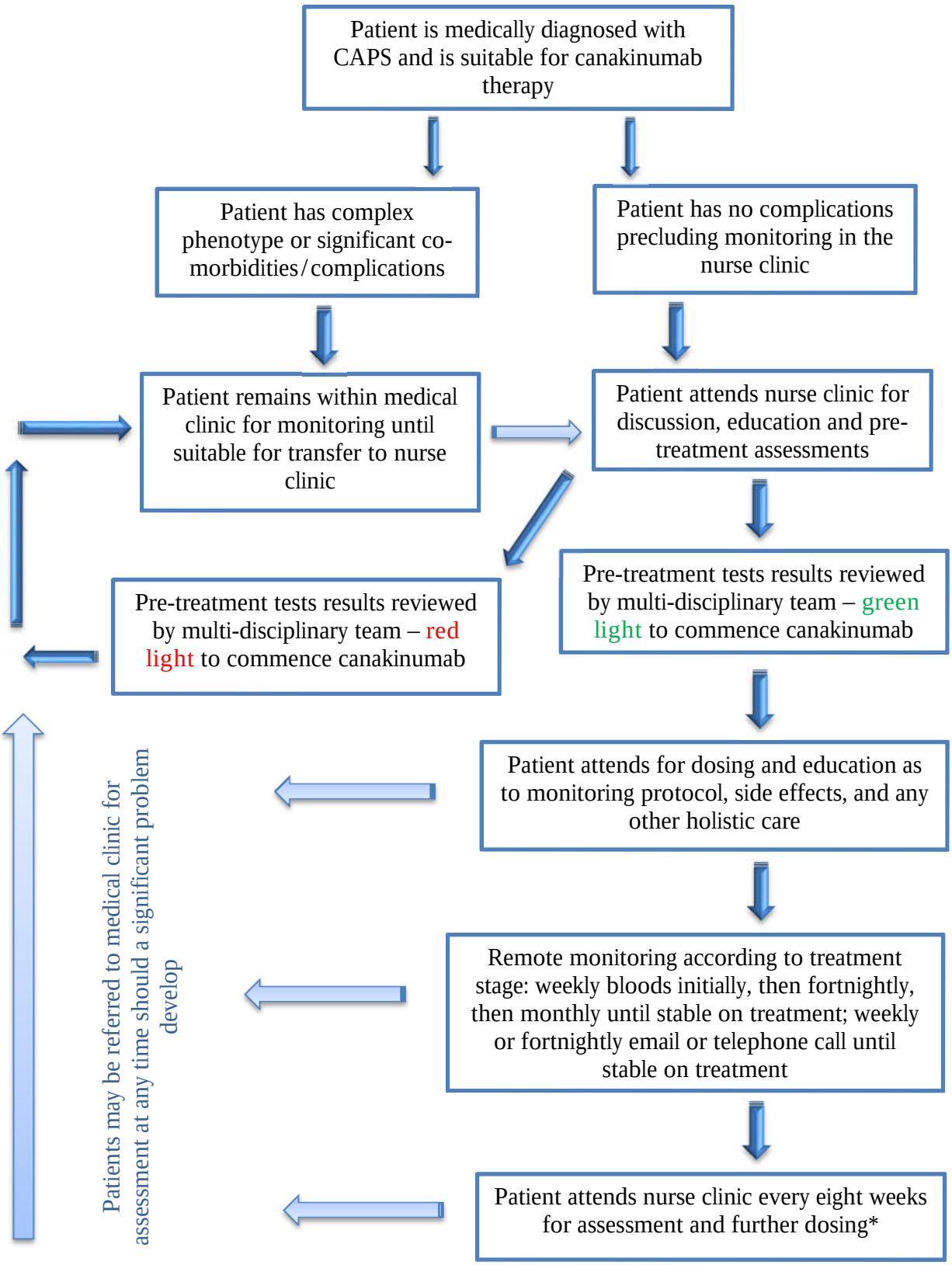


Figure 3.1. The patient pathway for canakinumab therapy. *Patients also reviewed in monthly multi-disciplinary team meetings and annually in medical clinic.

3.4.2 Protocols for other therapies

Protocols for administration of other drugs are described in detail in each relevant chapter.

3.5 Discussion

A great number of publications document the many benefits of nurse-led clinics. In the context of this body of work, the nurse-led clinic has made possible the provision of very specialist, perhaps even niche, care to patients with complex inflammatory conditions requiring treatment with biologic therapies, and careful and close monitoring. Medical clinics are under great time and capacity pressure, and so the creation of a clinical service where education, intervention/medication, assessment, monitoring and holistic care can all be provided by experienced healthcare professionals was an essential step in addressing the unmet need.

Nurse-led clinics also provide the opportunity for nurses to extend their skills and knowledge, whilst providing specialist and holistic care. They provide an effective environment for nurses to feel more valued in their roles and to have ownership over their work. The recognition of nurses as experts within the multidisciplinary team boosts the professional profile of nursing, encouraging other nurses to extend their roles and enhancing recruitment of bright and motivated individuals to the profession.

Finally, in the context of this thesis, the nurse-led clinic has enabled this body of work to be conducted. Combining research with clinical work, especially in such rare diseases where patient numbers are small, is the gold standard. In order to make this happen, the right combination of conditions must exist – adequate funding, infrastructure and human resources - and resourceful humans. The result when these conditions are met is benefit

to the patients, the multidisciplinary team of healthcare professionals and to the NHS as a whole.

Chapter Four:

Characterisation of AA Amyloidosis Complicating Systemic Autoinflammatory Diseases

4.1 Background

The SAIDs are described in detail in Chapter One: Introduction. The syndromes are characterised by a marked acute phase response, with some patients experiencing 100-fold or more increase in circulating concentration of the acute phase protein SAA. When adequately suppressed, these diseases are associated with normal life expectancy, but a proportion of patients develop the life-threatening complication AA amyloidosis, with SAA the amyloidogenic precursor protein. The period of latency between the onset of inflammation and clinical presentation with amyloidosis is variable and may be prolonged, but amyloid progression can be rapid. Renal dysfunction is the predominant presenting feature, and poor outcomes are associated with inadequate suppression of the underlying inflammation (92).

4.2 Aims

We sought to study the presentation, phenotype and outcome of patients who developed AA amyloidosis as a complication of SAIDs in a single national referral centre.

4.3 Patients and Methods

4.3.1 Patients and diagnosis

From our database of 854 patients with a sequence variant in a gene associated with a SAID and 545 patients with confirmed or probable AA amyloidosis, we identified a cohort of 46 patients with AA amyloidosis complicating a SAID. This comprises all patients referred to our centre in the 21 years up to September 2011 in whom a SAID was diagnosed by a combination of clinical presentation, medical history, genetic sequencing and additional investigation, and in whom AA amyloidosis had been confirmed by immunohistochemical analysis, or by whole body ^{123}I-SAP scintigraphy and

additional investigation. Routine investigation and assessment at each visit included clinical evaluation, electrocardiography and echocardiography, and measurements of hepatic and renal function, including 24-hour urinary protein excretion. Patients underwent comprehensive clinical investigation at baseline and were followed up every 3 to 6 months. Ethical approval was obtained for this retrospective study (REC reference number 06/Q0501/42).

4.3.2 Other methods

Other methods are described in Chapter Two: Methods.

4.4 Results

4.4.1 Whole cohort characteristics

The mutations, ethnic origins and age at presentation of all patients in the cohort are shown in Table 4.1. The median age at diagnosis of AA amyloidosis was 38 years with an inter-quartile range (IQR) of 27 to 47 years. In 37 patients there was a clinical history compatible with a SAID; median age at symptom onset was five years (IQR 2-13), and median latency between symptom onset and presentation with AA amyloidosis was 23 years (IQR 17-34). Eight patients gave no history of overt inflammatory symptoms and complete clinical data was unavailable for one patient. In 23 patients (50%) the diagnosis of a SAID was made at our centre following presentation with AA amyloidosis. Renal presentation dominated; however, three patients also had liver amyloid diagnosed scintigraphically, and one had cardiac amyloid, diagnosed post-mortem.

Patient	Ethnicity	Sex	Sequence variants / Mutations	Attack symptoms	Age at SAID onset	Age amyloidosis diagnosed	Latency period
			FMF – *MEFV* mutations				
1	Northern European	F	M694del/E148Q	F, A, P	10	59	49
2	Northern European	F	K695R homo	Asymptomatic	Asymptomatic	61	NA
3	Northern European	M	M694del	F, A, P	10	45	35
4	Northern European	M	M694V het	Asymptomatic	Asymptomatic	78	NA
5	Northern European	M	M694V/E148Q	Asymptomatic	Asymptomatic	63	NA
6	Turkish	F	M694V homo	F, A, J	3	27	24
7	Turkish	M	M694V het	Asymptomatic	Asymptomatic	45	NA
8	Turkish	F	M694V homo	F, A, J	15	24	9
9	Turkish	M	M694V/E148Q	F, A, J, R, Ft	7	27	20
10	Turkish	M	M694V homo	F, A, P	17	26	19
11	Arabic	F	V726A/M694I	F, A	7	36	29
12	Arabic	M	M694V homo	A, J	13	19	6
13	Arabic	F	V726A/ M694V	F, A, J	30	62	32
14	Arabic	M	M694I/ E148Q	F, A	41	58	17
15	Arabic	F	M694V/M680I	F, H	10	35	25
16	Greek	M	M694V het	F, A, P	24	38	14
17	Greek	M	V726A/F479L	F, A, J	3	19	16
18	Greek	M	V726A/E167D/F479L	Asymptomatic	Asymptomatic	67	NA
19	Egyptian	M	M694V/V726A	A, J	21	42	21
20	Egyptian	M	M680I homo	F, A, P, N, V	25	41	16
21	Armenian	F	M680I/V726A	F, A, P	43	58	15
22	Armenian	F	V726A/ M694V	F, A, P, H, N	3	7	4
23	Jewish-Sephardi	F	M694V homo	F, A, P, J, G	6	32	26
24	South Asian	F	S154P het	Asymptomatic	Asymptomatic	37	NA

Table 4.1. Mutations, ethnic origins, sex and clinical characteristics of 46 patients with AA amyloidosis complicating SAIDs - FMF. F=fever, A=abdominal pain, P=pleuritic chest pain, J=joint pain/arthralgia/swelling, R=rash, Ft=fatigue/malaise, H=headache, N=nausea, V=vomiting, G=Gastrointestinal disturbance, M=myalgia, L=lymphadenopathy, E=red/swollen eyes, S=sweats, Ri=rigors, St=sore throat, O=oral ulcers.

Patient	Ethnicity	Sex	Sequence variants / Mutations	Attack symptoms	Age at IPFS onset	Age amyloidosis diagnosed	Latency period
			TRAPS – *TNFRSF1A* mutations				
25	Northern European	F	D42del	F, A, P, R, J	5	20	15
26	Northern European	F	H22Q	F, A, P, R, J, M, L, E	Unknown	30	Unknown
27	Northern European	M	T50M	F, A, R, J, E	<1	31	30
28	Northern European	M	T37I	A, M, F, R, Ft	2	28	26
29	Northern European	F	C33Y	A, M, E, R	2	24	22
30	Northern European	M	D42del	F, A, M, J, R	6	44	38
31	Northern European	F	R92P	F, A, S	18	46	28
32	Northern European	F	C33Y	F, A, M, R, S, Ft, Ri	3	42	39
33	Northern European	M	T50M	A, S, M, Ri	3	39	36
34	Northern European	F	D42del	F, A, P, R, J, G, Ri	4	46	42
35	Northern European	M	C33Y	F, A, P, R, J, E	1	46	45
36	Northern European	F	Y38S	F, R, J, M, E	4	75	71
			CAPS – *NLRP3* mutations				
37	Northern European	M	A439V	Asymptomatic	Asymptomatic	52	NA
38	Northern European	M	T348M	R, F, M, J	1	29	28
39	Northern European	M	T348M	R, E, F,	<1	15	14
40	Northern European	M	R260W	R, M	<1	23	22
41	South Asian	M	R260W	R, E, F, J	10	29	19
42	South Asian	F	R260W	R, E, F, J	7	45	38
			MKD – *MVK* mutations				
43	Northern European	M	L234P / V337I	F, L, V, G, A, H	<1	23	22
44	Northern European	M	I268T / V377I	F, L, V, G, N, O, J, Ft	1	15	14
45	Northern European	F	I268T / V377I	F, L, G, R, J, St	<1	26	25
46	South Asian	M	S52N / D386N	Asymptomatic	Asymptomatic	47	NA

Table 4.1 continued. Mutations, ethnic origins, sex and clinical characteristics of 46 patients with AA amyloidosis complicating SAIDs – TRAPS, CAPS and MKD. F=fever, A=abdominal pain, P=pleuritic chest pain, J=joint pain / arthralgia / swelling, R=rash, Ft=fatigue / malaise, H=headache, N=nausea, V=vomiting, G=Gastrointestinal disturbance, M=myalgia, L=lymphadenopathy, E=red / swollen eyes, S=sweats, Ri=rigors, St=sore throat, O=oral ulcers.

4.4.2 FMF patients

79% of the 24 FMF patients were born outside Europe, the majority in the eastern Mediterranean region. The median age at diagnosis with AA amyloidosis was 41 years (IQR 28-58). Six patients had a family history of FMF and only one had a family history of AA amyloidosis. Two patients were untreated: one was asymptomatic with normal inflammatory markers, and one died of metastatic cholangiocarcinoma almost immediately after the diagnosis of amyloidosis and genetic testing. The remaining 22 were given a trial of colchicine: 19 patients achieved complete remission (CR) and one achieved partial remission (PR). Two were intolerant - one continued to take low daily doses (< 1mg) despite severe gastrointestinal side-effects until she died of progressive cardiac amyloidosis five years later; the other remains untreated, on long term dialysis. Follow-up data was available for 18 patients for a median follow-up period of 11 years. Two patients were on dialysis at presentation and remained so at last follow-up. Of the rest, seven patients experienced improvement of proteinuria; however, only three had stabilisation of CKD. Eight patients showed amyloid regression on SAP scan, and the remainder showed a stable load over time. Two patients underwent renal transplantation.

4.4.3 TRAPS patients

Ten of the 12 patients had a family history of TRAPS, and nine (from four known kindreds) had relatives with AA amyloidosis. The median age at diagnosis of amyloidosis was 41 years (IQR 29-47). Six patients were treated with the anti-TNF agent etanercept: one was intolerant; one achieved a PR; four had only a transient response and were subsequently successfully switched to anakinra. A further four were successfully treated with anakinra up-front. Four patients have died: two had never received definitive treatment for TRAPS - one died of progressive amyloidosis aged 49, and the other died of sepsis following a second renal transplant; one patient died on dialysis of disseminated

TB after receiving long term immunosuppression with anti-cytokine agents; the fourth patient died of cervical cancer aged 43 on a background of a longstanding renal transplant. Follow-up data is available for the eight patients who received anti-IL-1 agents. Median treatment duration was 23 months; two had renal transplants having reached ESRF before switching to anakinra. Of the others, three patients experienced improvement in proteinuria and stable or improved CKD and three had worsening of CKD over a median follow-up period of 65 months, although only one progressed to dialysis.

4.4.4 CAPS patients

Four of the six patients were Northern European from separate kindreds and two were South Asian cousins. Half the patients had a family history of CAPS, and only the South Asian cousins had amyloidosis in the family. The median age at diagnosis with AAA was 31 years (IQR 25- 42). Four patients have been treated with anti-IL-1 agents and responded dramatically both biochemically and symptomatically. The two other patients died before the role of IL-1 was recognised in CAPS; one was treated with colchicine and steroids without benefit and the other was untreated, the diagnosis of CAPS having been made posthumously. Of the four patients treated with anti-IL-1 agents one had a renal transplant prior to initiation of this treatment. Of the remaining three patients, all have had resolution of proteinuria over the follow-up period. Three of the four patients have had stable CKD and one went from CKD stage 1 to 2 over the 11 year follow-up period, although his SAP scan showed amyloid regression.

4.4.5 MKD patients

Only one of the four patients had a family history with a sibling subsequently diagnosed and none had a family history of AA amyloidosis. The median age at diagnosis of amyloidosis was 25 years (IQR 22-32). Of note, in all four patients the diagnosis of MKD was made at our centre after presentation with AA amyloidosis and none had had

previous treatment. Three had suggestive symptoms with onset in early childhood but the diagnosis of SAID had never been considered; the fourth patient complained of non-specific malaise only. Three cases presented with incipient ESRF and the fourth had severe nephrotic syndrome and developed dialysis-dependent renal failure within months of being diagnosed with AA amyloidosis. Prior to diagnosis one patient underwent cadaveric renal transplant and two others subsequently received renal grafts from their mothers. Three patients were treated with various anti- cytokine agents over the follow-up period, and one received renal transplant associated immunosuppression only, with modest benefit.

Of the patients who received anti-cytokine treatment one appears to have a sustained seven-year PR to etanercept; one achieved PR with IL-1 blockade but developed an anaphylactic reaction - he failed to respond to etanercept but a therapeutic trial of the synthetic anti-IL-6 monoclonal antibody tocilizumab has been successful. The third patient failed to respond to anakinra, but had a sustained 14-month CR to tocilizumab, and showed regression of amyloid on SAP scan (Figure 4.1). One patient died aged 45 having lost her transplant from chronic allograft nephropathy after seven years. Another patient lost their graft after six years due to recurrent amyloid.

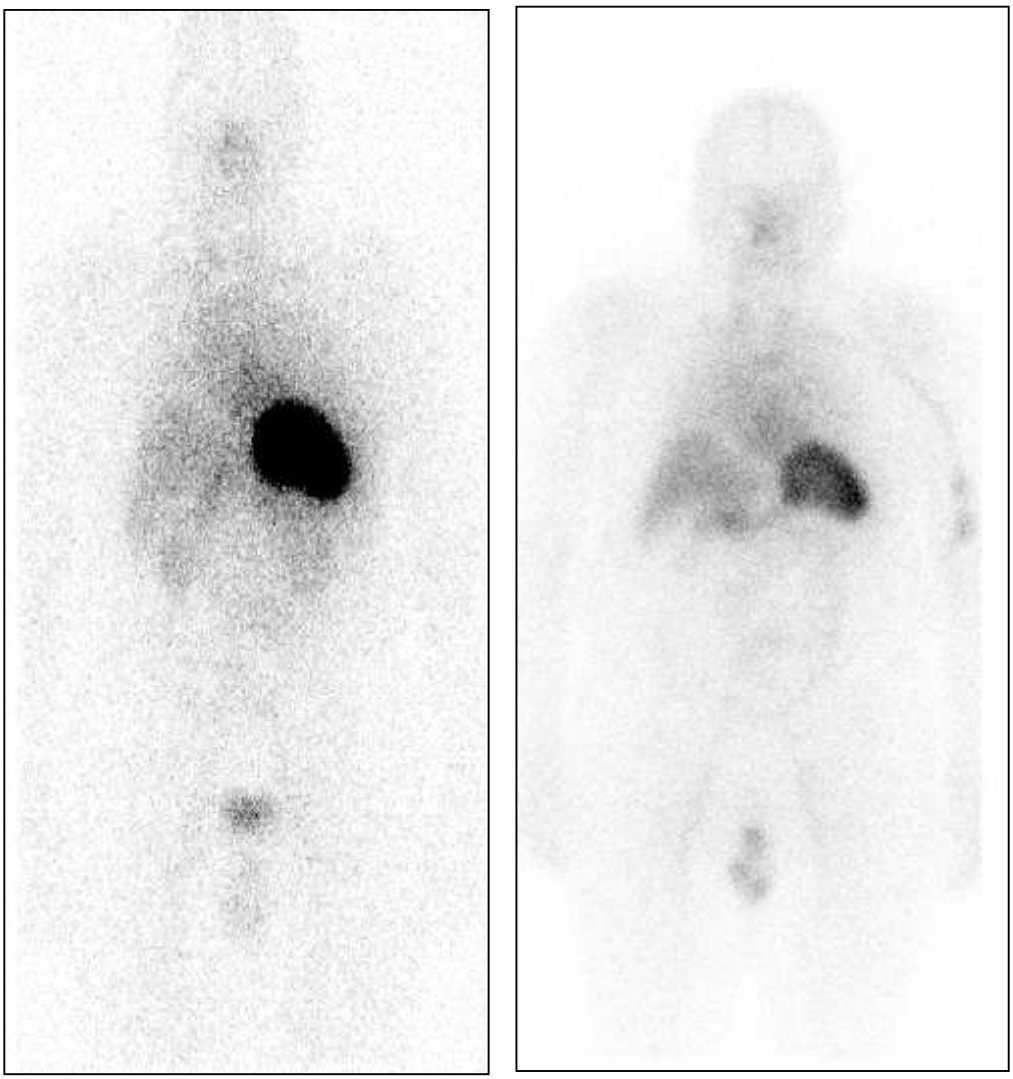


Figure 4.1. Serial ^{123}I-SAP scans demonstrating amyloid regression following successful treatment of mevalonate kinase deficiency with the anti-IL-6 agent tocilizumab.

4.4.6 Whole cohort renal function

Twenty one patients reached ESRF. Eleven (23%) were in ESRF at presentation, and of these three had been transplanted prior to referral to our centre. A further 13 progressed to ESRF over the follow-up period, with 10 undergoing transplantation. The median time to progression to ESRF from onset of AA amyloidosis was 3.3 years (IQR 2-8), with a median time to transplant of four years (IQR 4-6). Table 4.2 summarises the characteristics of the kidney-transplanted patients. Of the entire cohort, 11 patients (23%) experienced improvement in proteinuria, as defined by consensus criteria (116), with a median decrease in urine protein of 4.8g/24hr (IQR 2.9-5.7) over the follow-up period. Five of these patients showed regression of amyloid on SAP scintigraphy, and six showed a stable amyloid load. Nine experienced CR to treatment of the SAID, and two were untreated.

4.4.7 Whole cohort survival

Eleven patients (24%) died (one CAPS, four TRAPS, one MKD and five FMF). Median survival by Kaplan-Meier analysis of the entire cohort was 19 years from diagnosis of AA amyloidosis (IQR 4.8-12.4); the median age at death was 67 years with no deaths occurring before 43.

4.5 Discussion

The risk of developing AA amyloidosis in these SAIDs is difficult to characterise based on this cohort for several reasons. Firstly, much of this data is historical, dating back to when many of the effective treatments in current use were unavailable. Secondly, increasing age is a known risk factor for AA amyloidosis; presentation in childhood is exceedingly rare and so including paediatric patients will result in a prevalence underestimation. Furthermore, as the SAIDs are generally newly described entities, many

adults were never diagnosed in earlier decades and are now lost to follow-up so current cohorts are heavily biased towards children.

In this case series of adults (aged over 18), the 24 FMF patients represented 6%, the 12 TRAPS patients, 18%, the 6 CAPS patients, 10%, and the 4 MKD patients represented 18%, of all adult FMF, TRAPS, CAPS and MKD patients under our care. Whilst there is the possibility of selection bias associated with a national referral centre, the proportions of CAPS, TRAPS and MKD patients who develop AA amyloidosis are not dissimilar from those published by other groups, including international consortia (130-132), although there appears to be much variability in reported proportions of FMF patients developing AA amyloidosis (132-134).

Early diagnosis and initiation of treatment of the SAID is important in preventing the development of AA amyloidosis. Early diagnosis can be problematic, as these diseases are rare and a high index of suspicion is required. Furthermore, clinical features of the SAID overlap with other more common diseases. Broad and inconstant phenotypes combined with variable penetrance add further diagnostic challenges. Finally, recognition of asymptomatic patients with subclinical inflammation before they develop devastating complications is difficult as it relies on serendipitous detection of abnormal inflammatory markers and recognition of its importance. In this cohort eight patients (17%) were asymptomatic despite biochemical inflammation at the time of diagnosis with AA amyloidosis. These included patients who had been previously described as ‘phenotype II FMF’ in which there are no overt symptoms but a response to colchicine (135), as well as some in whom no pathogenic variants were found on Sanger sequencing of known autoinflammation-associated genes, i.e. patients with AA amyloidosis of uncertain aetiology.

Patient	IPFS	Treatment	Treatment response	ESRF at presentation	Time to ESRF from onset of IPFS (y)	Time to ESRF from amyloid diagnosis (y)	Time to transplant from amyloid diagnosis (y)	Graft survival (y)	Reason for graft failure	Cause of patient death
7	FMF	Colchicine	Complete	No	26	2.62	6.78	8.13	NA	NA
12	FMF	Colchicine	Complete	No	41	11.87	15.03	0.04	Patient death	CMV sepsis post-transplant
17	FMF	Colchicine	Partial	No	17	4	6.00	4.96	NA	NA
24	FMF	Colchicine	Complete	Yes	22	-3.59[#]	-2.31[#]	3.61	NA	NA
27	TRAPS	Untreated	NA	Rtx prior	15	2	4.00	20.9	Patient death	Cervical cancer
28	TRAPS	Untreated	NA	Yes	Unknown	0	2.00	17	Patient death	Renal failure secondary to recurrent amyloid in graft
30	TRAPS	Anti-TNF	Complete	Rtx prior	26	0	4.00	16.25	NA	NA
31	TRAPS	Anti-IL-1	Complete	No	29	5.68	5.92	6.38	NA	NA
37	TRAPS	Anti-TNF	Intolerant	No	47	1.66	2.83	8.13	Patient death	Sepsis post second renal transplant
41	CAPS	Anti-IL-1	Complete	Rtx prior	18.25	0	3.38	4.73	NA	NA
45	MKD	Anti-TNF	Complete	Yes	24.71	1.87	3.87	5.54	NA	NA
46	MKD	Anti-IL-1	Partial	No	16.12	0	2.05	6.08	Recurrent amyloid	NA
47	MKD	Untreated	NA	Yes	28	2	7.00	7	Patient death	Unknown

Table 4.2. Characteristics and outcome of 13 patients who underwent renal transplantation. [#] Patient progressed to ESRF and transplant prior to diagnosis of AA amyloidosis; Rtx=renal transplant; NA=not applicable.

Further difficulties are the expense and time associated with sequencing multiple genes and exons. Where this facility is unavailable, attempts to narrow the differential diagnosis clinically can be worthwhile. We routinely monitor disease activity by a combination of patient diaries and serial serum inflammatory markers. In those with suggestive symptoms accompanied by elevated CRP and SAA a trial of therapy can be very revealing. Responses to colchicine, corticosteroids or biologics can help to establish a definitive diagnosis. In addition a successful therapeutic trial can allow effective management of both symptoms and complications in cases where the disease aetiology remains unknown even after extensive genetic investigation.

This series demonstrates that even in patients with established AA amyloidosis effective treatment of the underlying SAID can lead to improved renal function and regression of amyloid as long as the renal impairment is not too advanced at diagnosis. Outcome of renal transplantation in these patients is good, and so for those who reach ESRF, this should certainly be considered as an option. However, the aim of treatment has to be the complete prevention of this devastating complication, which has negative impacts on quality and length of life.

Chapter Five:

Safety and Efficacy of Inhibition of Interleukin-6 in Chronic Inflammatory Diseases and Autoinflammation

5.1 Background

AA amyloidosis is the most serious potential complication of disorders associated with chronic inflammation and results in progressive renal insufficiency, poor quality of life (QoL) and substantial mortality (92, 136). The most important aim of treatment is to suppress inflammation thereby inhibiting production of the acute phase reactant, SAA, which is the precursor of AA amyloid fibrils. Sustained suppression of SAA not only prevents the risk of developing AA amyloidosis, but it is also known to be associated with regression of AA amyloid deposits, improvement in amyloidotic organ function and prolonged survival (91).

Treatment of the inflammatory arthritides, inflammatory bowel disease and SAIDs has been revolutionised by the availability of biological agents that block specific cytokines. TNF blockade is now very widely used as an effective treatment for rheumatoid arthritis (RA) and some SAIDs; IL-1 blockade has dramatically improved management of several previously nigh on untreatable SAIDs, notably cryopyrin -associated periodic syndrome (CAPS), tumor necrosis factor (TNF) receptor-associated periodic syndrome (TRAPS), colchicine-refractory familial Mediterranean fever (crFMF) and many cases of mevalonate kinase deficiency (MKD), also known as hyper-immunoglobulin D and periodic fever syndrome (HIDS) (137, 138). IL-1 inhibition by means of anakinra or canakinumab has also been effective in patients with AA amyloidosis complicating CAPS and TRAPS and, as a result, can reduce proteinuria and salvage renal function (136, 139). Blockade of IL-6 is frequently effective in RA and systemic onset juvenile idiopathic arthritis (SJIA), the anti-IL-6 receptor monoclonal antibody tocilizumab (TCZ) (RoActemra®) is now licensed treatment for both conditions. There are very few published data on the use of TCZ in AA amyloidosis or the SAIDs or chronic inflammatory.

The short- and long-term efficacy of TCZ has been established by means of several clinical trials in RA (140-154) as well as in a real life cohort (155), and in SJIA clinical trials (156-159). TCZ has also been successfully used within clinical trials in other diseases such as multicentric Castleman's disease (MCD) (160) and Crohn's disease (161).

Current knowledge of the safety profile of TCZ in adults with RA is derived from clinical trials involving 3577 patients who received treatment for at least six months, 3296 who were treated for at least one year, 2806 who received treatment for at least two years and 1222 treated for three years. The commonest reported adverse drug reactions (ADRs), i.e. those occurring in ≥ 5% of patients treated with TCZ monotherapy or in combination with sDMARDs were upper respiratory tract infections, nasopharyngitis, headache, hypertension and increased serum alanine aminotransferase. Preliminary safety data on TCZ in children with SJIA has been reported from an international phase 3 trial (TENDER) in 112 children, clinical trials in Japan involving 149 patients, and the Japanese post-marketing monitoring program which includes 366 cases. The authors concluded that TCZ was not associated with an increased risk of macrophage activation syndrome (MAS) (162). In TENDER 57% of patients had at least one episode of neutropenia. This was associated with an increased risk of infection compared to periods when the children were not neutropenic. MXT use and younger age were associated with increased risk for more severe neutropenia, but TCZ exposure was not (163).

The safety and efficacy of TCZ in renal impairment has been supported by a number of publications; three case reports document the safe and effective use of TCZ in patients with end stage renal failure and underlying RA (164) and MCD (165, 166). A Japanese registry study has reported on 102 patients with RA and renal insufficiency showing that the safety and efficacy profile is comparable with the group with independent renal function (n=279) (167). In a case series of five patients with AA amyloidosis secondary

to RA, improvement in renal function was seen at one year (168) with no safety issues. To our knowledge there are no reports on the use of TCZ after renal transplantation.

5.2 Aims

We assessed clinical and serological responses and adverse reactions to TCZ in 20 adult patients with a variety of inflammatory disorders refractory to other treatments, 70% of whom had developed AA amyloidosis, including four patients who underwent renal transplantation.

5.3 Methods

5.3.1 Patients

Three patients (13, 14 and 15) were commenced on 4 weekly infusions of TCZ within the specialist nurse fever clinic, and were also monitored and followed up here. Between 2010 and 2014, in a further 17 patients, treatment with TCZ was recommended by the NAC, but treatment was given locally to minimise unnecessary cost and inconvenience of travel. These patients were monitored locally but also attended the NAC for further follow up. All patients were asked to keep symptom diaries, and to send in blood samples every two to four weeks for monitoring of acute phase reactants until treatment was established; thereafter SAA and CRP would be monitored at routine clinic visits locally, and results were sent from the local team to the NAC. Fourteen of the 20 patients had AA amyloidosis. Disease activity and treatment response were monitored by patient-reported symptoms and serial SAA and CRP measurements. Amyloid load was evaluated by whole body ^{123}I-SAP scintigraphy. Renal function was measured by means of serum and urine chemistry. QoL was measured in 12 patients using the SF36v2® questionnaire.

Patients gave written informed consent for retrospective analysis and publication of their clinical data (REC reference number 06/Q0501/42).

5.3.2 Other methods

Other methods are described in Chapter Two: Methods.

5.4 Results

5.4.1 Whole cohort characteristics

Table 5.1 summarises the patient characteristics. Twelve (60%) were male. Six patients had unclassified inflammatory disorders, two had MKD, seven had RA, four SJIA and one unicentric Castleman's disease. All patients in the cohort had received at least one previous line of treatment with an anti-cytokine therapy or disease-modifying anti-rheumatic drug (DMARD), which had proved ineffective (including anakinra in two patients with MKD and one SJIA patient) or had intolerable side effects. Many patients were extensively pre-treated, one having tried more than ten different therapies over her disease course. Fourteen patients (70%) had developed AA amyloidosis. The total median follow-up time for the whole cohort was 56 months, with an interquartile range (IQR) of 18 to 86 months. Median follow-up of patients on TCZ was 23 months. All results are shown as median (IQR) unless otherwise stated. Details of TCZ therapy are summarised in Table 5.2.

5.4.2 Serial monitoring of the acute phase response

The whole cohort median pre-treatment SAA (the median of all available SAA measurements in the year prior to commencement of TCZ) was 70 mg/L (38 – 158). The first post-treatment SAA measurements were undertaken at 10 days (7 – 13) after the first dose of TCZ, and were significantly improved to a median of 4 mg/L (3 – 7); this excellent serological response has been maintained at median 5 mg/L (3 – 8) over an on-

treatment follow-up period of 23 months (13 – 35) (Mann Whitney p <0.0001) (Figure 5.1).

5.4.3 Change in renal function in AA amyloid cases

Of 14 patients with AA amyloidosis, 12 had renal impairment. Four of the 12 had renal transplants and a further two were dialysis-dependent. The six patients with measurable native renal function showed a mean reduction in proteinuria of 3.4g/24 hours over the on-treatment period when compared with the pre-treatment 24 hour urine collection.

5.4.4 Monitoring of amyloid deposits

Thirteen of 14 patients with AA amyloidosis have had pre- and on-treatment serial SAP scans. Four patients showed stable amyloid deposits whilst nine showed regression of amyloid; none showed worsening amyloid deposition whilst on TCZ. The interval between commencement of TCZ therapy and the first improved SAP scan was 10 months (7 – 13).

Patient ID	Sex	Ethnicity	Inflammatory disorder	Age at onset (y)	Previous unsuccessful therapy	Renal impairment	Renal transplant	AA amy-loidosis	Follow-up (y)
1	M	N. European	RA	36	MTX, SSZ, ETC, RTX, G, LEF	1	1	1	7
2	F	N. European	RA	28	MTX, SSZ, ETC, RTX	1	0	1	4
3	F	N. European	RA	49	MTX, SSZ, ETC	1	0	1	2
4	M	N. European	RA	19	MTX, ETC, CMB	1	0	1	7
5	M	N. European	RA	22	MTX, ETC, RTX	1	Dialysis	1	2
6	F	N. European	RA	40	MTX, RTX, ADA, ETC	1	0	1	8
7	F	N. European	RA	35	MTX, RTX	0	0	1	6
8	M	N. European	SJIA	12	CMB, ETC, INF, ADA, RTX	1	1	1	22
9	F	N. European	SJIA	14	CSP, RTX, LEF, MMF, TAC, ANA, ABT, CMB, CSP, AZA, MTX	1	1	1	6
10	F	N. European	SJIA	6	MTX, ETC	0	0	1	3
11	F	N. European	SJIA	19	MTX, ETC, ADA	1	0	1	5
12	M	N. European	Castleman's	38	MTX	1	0	1	4
13	M	N. European	MKD	<0.5	ANA, ETC	1	1	1	11
14	M	N. European	MKD	<0.5	ANA	0	0	0	2
15	M	S. Asian	Unclassified	41	CSP, AZA, MTX, CAM, RTX, INF, ANA	1	Dialysis	1	8
16	M	S. European	Unclassified	10	ADA, ETC, ANA, MMF	0	0	0	8
17	M	S. Asian	Unclassified	15	MTX, SSZ, PCM, CSP, COL, INF	0	0	0	18
18	F	N. European	Unclassified	16	MTX, ANA	0	0	0	4
19	M	N. European	Unclassified	55	AZA, ANA, ETC	0	0	0	1
20	M	N. European	Unclassified	19	ANA	0	0	0	2

Table 5.1. Characteristics of patients treated with TCZ. Patients are described in terms of demographics, underlying disease, previous therapies and total follow-up period. ANA=anakinra, ADA=adalimumab, ETC=etanercept, MMF=mycophenolate mofetil, MTX=methotrexate, SSZ=sulphasalazine, PCM=penicillamine, CSP=cyclosporine, COL=colchicine, INF=infliximab, RTX=rituximab, G=gold, LEF=leflunamide, CMB=chlorambucil, CAM=CAMPATH, TAC=tacrolimus, ABT=abatacept.

Figure 5.2 shows serial SAP scans of patient 4 who had regression of amyloid.

5.3.5 QoL before and after treatment

QoL was measured in 10 patients whilst treatment with TCZ was ongoing, and in three of these QoL was also measured prior to commencing therapy. Results are shown in Figure 5.3. A comparison of the mean scores in each domain before and after treatment were statistically significant (Mann Whitney $p=0.0354$) whilst clinically significant changes were seen in all domains apart from mental health, in which the change was 9 points. The bodily pain domain showed the greatest improvement of 24 points, followed closely by the vitality and social functioning domains at 22 points each, and the physical functioning and role physical domains which showed a post-treatment improvement of 20 points each.

5.3.6 Adverse events

Adverse events (AEs) were reported in eight patients. Patient six developed two urinary tract infections (UTIs) and a respiratory tract infection within the first year of TCZ therapy; however, this patient had a long history of recurrent UTIs and was on a long term rotational antibiotic regimen of cephalexin and nitrofurantoin. Patient nine developed gallstone pancreatitis which resolved following endoscopic retrograde cholangiopancreatography. Neither of these patients had their TCZ therapy interrupted or stopped as a consequence of the AE.

Patient 20 has had an infusion delayed due to a transient drop in his white cell count. Patient one developed a subclinical Klebsiella ozaenae UTI, discovered coincidentally at a routine clinical review. Treatment was not stopped at that time; however, TCZ was temporarily discontinued just prior to renal transplantation.

Patient ID	Age at Therapy Onset (y)	TCZ dose (mg/kg), frequency, route	Serological response to TCZ	Clinical response to TCZ	Duration of therapy (m)	Monotherapy/ with other agent	Continuing	Reason, if not continuing
1	56	162 mg q2w sc	Complete	Partial	3	Pred 15 mg daily	Yes	
2	65	8 mg/kg q4w iv	Complete	Partial	20	Pred 10 mg daily	Yes	
3	54	8 mg/kg q4w iv	Complete	Partial	12	Monotherapy	Yes	
4	56	8 mg/kg q6w iv	Complete	Partial	23	Monotherapy	Yes	
5	57	8 mg/kg q4w iv	Complete	Complete	14	Pred 10 mg	Yes	
6	53	8 mg/kg q4w iv	Complete	Partial	34	Pred 5 mg daily	Yes	
7	64	8 mg/kg q4w iv	Complete	Partial	30	Monotherapy	Yes	
8	35	8 mg/kg q6w iv	Complete	Partial	28	Pred 5 mg	Yes	
9	39	8 mg/kg q4w iv	Complete	Partial	44	Monotherapy	Yes	
10	6	8 mg/kg q3w iv	Complete	Partial	9	ETC 25 mg bi-weekly; MTX 20 mg weekly; Pred 4 mg daily	Yes	
11	26	8 mg/kg q4w iv	Complete	Partial	24	Pred, LEF	Yes	
12	51	8 mg/kg q4w iv	Complete	Complete	38	Monotherapy	Yes	
13	28	8 mg/kg q4w iv	Complete	Complete	24	Pred 0.5 mg daily	Yes	
14	24	8 mg/kg q4w iv	Complete	Complete	13	Monotherapy	Yes	
15	52	8 mg/kg q4w iv	Complete	Partial	39	Monotherapy	No	Deceased
16	15	8 mg/kg q4w iv	Complete	Complete	18	COL 1.5 mg daily	Yes	
17	50	8 mg/kg q4w iv	Complete	Complete	22	Monotherapy	Yes	
18	25	8 mg/kg q4w iv	Complete	Partial	19	Pred 5 mg daily; ANA 50 mg weekly	Yes	
19	59	8 mg/kg q4w iv	Complete	Partial	6	Pred 15 mg daily	Yes	
20	49	8 mg/kg q4w iv	Partial	Partial	4	COL 1.5 mg daily	Yes	

Table 5.2. Details of tocilizumab treatment. Complete response is defined as complete resolution of symptoms or complete normalisation of serological inflammatory markers SAA and CRP (<10 mg/L); Partial response is defined as good but incomplete resolution of symptoms or reduction by > 50% but not complete normalisation of serological inflammatory markers SAA and CRP. Pred=prednisolone.

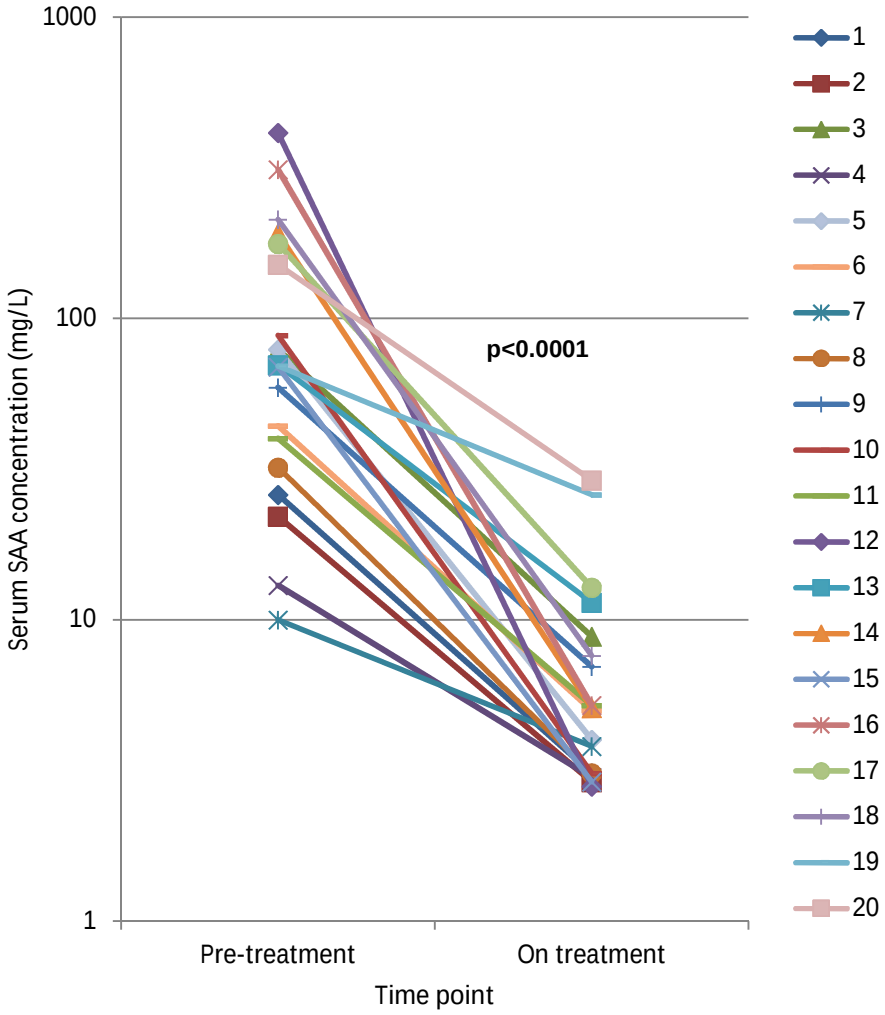


Figure 5.1. Sustained suppression of serum SAA after IL-6 blockade by tocilizumab. Pre-treatment values are shown as the median of all pre-treatment SAA measurement up to 1 year prior to commencement of tocilizumab therapy. On-treatment values are shown as the median of all on-treatment SAA measurements to date. Mann-Whitney test revealed a statistically significant difference between the pre- and post-treatment measurements ($p<0.0001$). The on-treatment follow-up period (duration of treatment) was 23 months (13 - 35).

In view of ongoing inflammatory activity post-transplant TCZ was recommenced with excellent and dramatic anti-inflammatory effect, but was unfortunately complicated by a subcutaneous abscess around a peritoneal dialysis catheter cuff. TCZ treatment was temporarily halted whilst he was being treated for the infection, and has now recommenced. Patient eight had a four month break in treatment due to elevated LFTs; these have now normalised and he has resumed therapy at the same dose.

Patient 19 developed shingles two months after starting treatment, and bacterial pneumonia four months later; both episodes resulted in hospitalisation. Patient 13 developed EBV septicaemia after his renal transplant, detected on routine monitoring. His dose of TCZ was halved for six months until this resolved. Patient 10 was hospitalised for MAS; treatment with TCZ was temporarily halted during the acute period. Despite these complications, all three patients elected to continue with TCZ therapy.

Patient 14 has elected to have a 'treatment holiday'; however having had severe disease flares in the four months off TCZ he has restarted treatment. Patient 15 had a sudden cardiac death after over three years of successful treatment with TCZ; he had been on dialysis for six years at the time of his death

5.4 Discussion

The IL-6 gene is located on chromosome 7 (169). IL-6 is a 26-kDa glycopeptide produced by a variety of cell types including fibroblasts, osteoblasts, endothelial cells, monocytes, keratinocytes, T cells and B cells. IL-6 acts via two known signalling pathways; classic signalling through the membrane-bound IL-6 receptor (mIL-6R) via activation of glycoprotein gp130, or trans-signalling via soluble receptors (sIL-6R) (170).

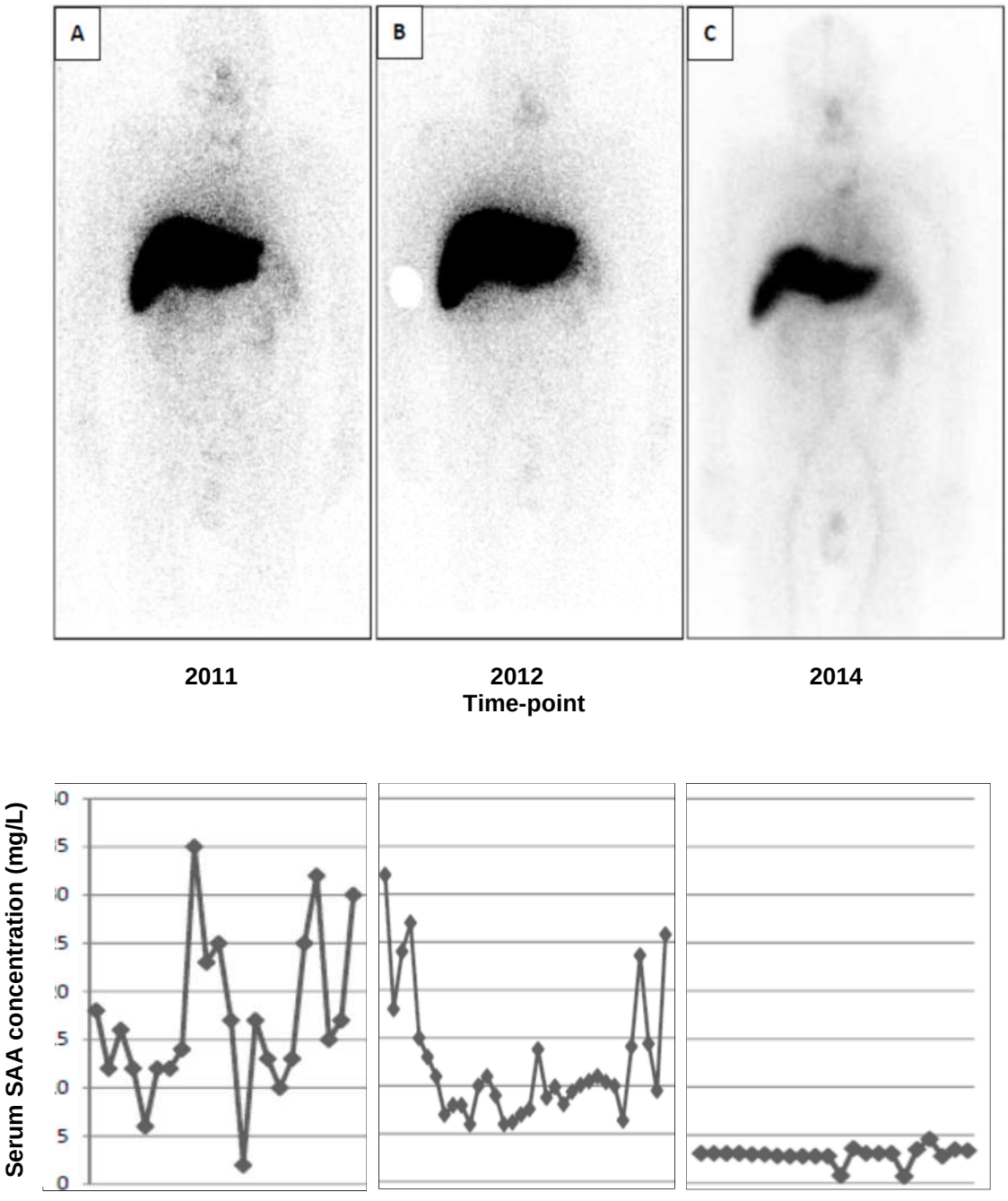


Figure 5.2. Serial SAP scans showing regression of amyloid from the liver of patient 4 with corresponding serum SAA measurements over the time period. Previous treatment with MTX, ETC and CMB had little effect on the underlying inflammatory disease resulting in further accumulation of amyloid in the liver shown on the anterior SAP scan in 2012 (panel B) compared with the baseline scan in 2011 (panel A). Subsequent successful treatment with tocilizumab for two years resulted in regression of amyloid as shown in the 2014 SAP scan (panel C).

There is increasing evidence that classic signalling plays a role in tissue regeneration and trans-signalling is responsible for the majority of pro-inflammatory responses. Novel agents that specifically target soluble IL-6R are currently under investigation and may be advantageous in human disease (171). TCZ is a humanised anti-human IL-6R monoclonal antibody which binds both sIL-6R and mIL-6R, thereby inhibiting both signalling pathways (172). The recommended dosing for adults with RA is 8 mg/kg infused once every four weeks. Dose adjustments are recommended in cases of liver enzyme abnormalities, low absolute neutrophil count and low platelet count. Successful dose reduction in patients with low disease activity is possible, with dose escalation to treat occasional flare (173).

This small series shows that in patients with treatment-refractory chronic inflammatory conditions which have either already resulted in AA amyloidosis or which are a high risk of being complicated by it, TCZ can be effective in suppressing the hepatic acute phase response. In patients with AA amyloidosis suppression of SAA levels is the major goal of treatment as this is known to dramatically improve outcome. We had been optimistic that TCZ would reduce SAA levels as IL-6 is the most important cytokine driving the hepatic acute phase response. This property raises the theoretical possibility that IL-6 blockade could work at the level of hepatocytes to dissociate on-going symptomatic inflammatory disease from a measurable hepatic acute phase response.

In AA amyloidosis such an outcome could effectively prevent progressive amyloid deposition without providing any relief from inflammatory symptoms, particularly in those patients with chronic joint damage from inflammatory arthritides. Somewhat to our surprise whilst not all patients reported complete symptom resolution, all patients reported feeling at least somewhat better. Furthermore, two patients with MKD showed complete clinical and serological response to TCZ; this has previously been described

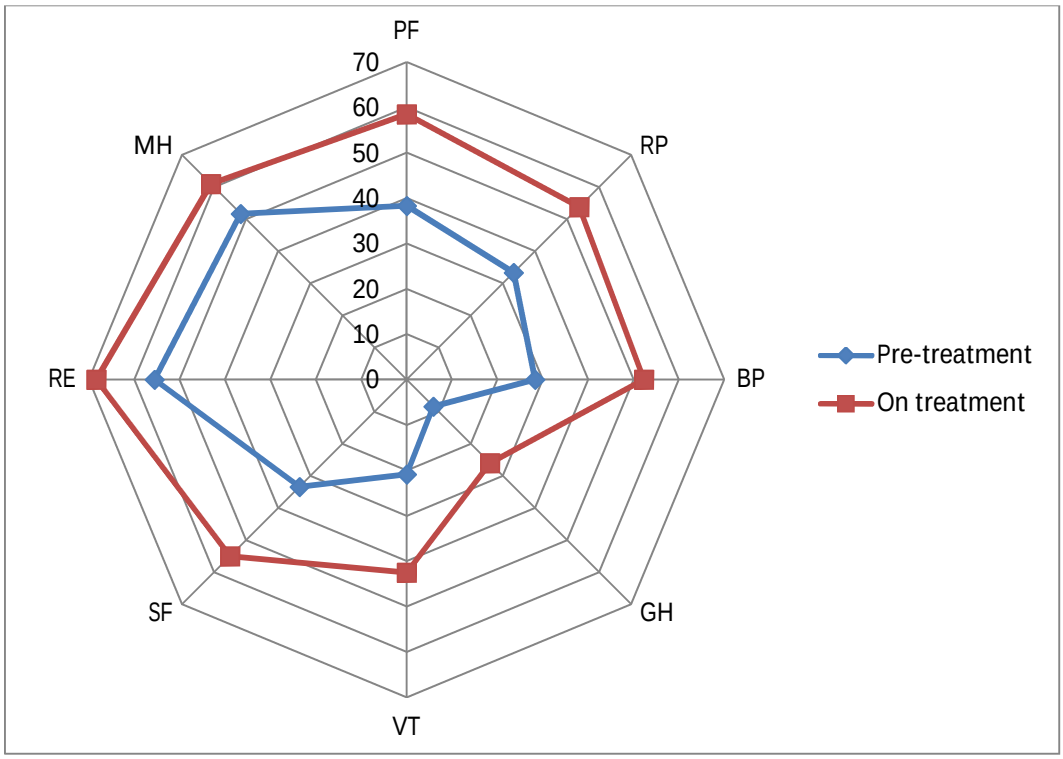


Figure 5.3. Quality of life before and during treatment with tocilizumab. Patients were surveyed before and whilst on treatment. A comparison of the mean scores in each domain before and on-treatment were statistically significant (Mann Whitney p=0.0354) whilst clinically significant changes were seen in all domains apart from mental health, in which the change was 9 points (a change of 10 points or more is considered clinically significant). PF=physical function, RP=role physical, BP=bodily pain, GH=general health, VT=vitality, SF=social function, RE=role emotional, MH=mental health.

only in one other patient (174). The response has been sustained to date in all patients who remain on TCZ (17, 85%); none have discontinued treatment due to loss of efficacy, however, longer follow-up is required. Treatment-related AEs were largely infections. Despite three patients requiring hospitalisation for AEs, all elected to continue treatment feeling that their clinical improvement justified the risks. Six patients temporarily stopped treatment due to AEs, and none yet have permanently discontinued TCZ therapy due to AEs.

At present IL-1 inhibition remains the first-line therapeutic option in patients with confirmed or suspected systemic autoinflammatory disorders as they have a proven safety and efficacy profile (175, 176) and are generally well tolerated. However, this small series with an on-treatment follow-up of 23 months (13 – 35) demonstrates that TCZ appears both relatively safe and effective even in the situations of renal failure, solid organ transplantation and extensively pre-treated diseases, and has resulted in stabilisation or reduction of amyloid deposits in all patients with AA amyloidosis. A therapeutic trial of TCZ is therefore reasonable in patients who fail to respond to other treatments, even in those in whom a firm diagnosis has not yet been made.

Chapter Six:

Safety and Efficacy of Inhibition of Interleukin-1 in Inflammatory Disorders and AA Amyloidosis

In 1962 the first report of the familial syndrome of urticaria, deafness and 'aguey bouts', with and without amyloidosis, was published by TJ Muckle and M Wells (177). Thereafter followed several similar reports and 40 years later, mutations in the gene *NLRP3* (previously known as *NALP3*, *CIAS1* and *PYPAF1*) were identified by Hal Hoffman as the cause of Muckle-Wells syndrome (MWS) and familial cold autoinflammatory syndrome (FCAS) (178), as well as chronic, infantile, neurologic, cutaneous and articular (CINCA) syndrome described by Anne-Marie Prieur (179), also known as the neonatal-onset multisystem inflammatory disease concurrently described by Ivona Aksentijevich (180). Another year or two hence and the team of Jurg Tschopp described how the interaction of the pyrin domain of NLRP3 with ASC, stimulated NF-κB signalling and increased interleukin (IL)-1β secretion (14); increased IL-1β secretion had previously been seen in the unstimulated PBMCs of a child with CINCA syndrome/NOMID (180).

The identification of IL-1 as the key pro-inflammatory cytokine in these diseases heralded a breakthrough, and in 2003 Philip Hawkins and Helen Lachmann successfully administered the first anti-IL-1 therapeutic agent (the recombinant IL-1 receptor antagonist, anakinra) to two patients with MWS and AA amyloidosis here at the UK NAC (181). In the decade-or-so since, there have been advances such that there are now two additional therapeutic agents, canakinumab and rilonacept, licensed for the treatment of cryopyrin-associated periodic syndrome (CAPS), the all-encompassing term now used to describe the continuum of FCAS, MWS and CINCA/NOMID. The success of these therapies in modulating disease activity in CAPS has stimulated the empirical use of some of these drugs in other inflammatory conditions.

CHAPTER SIX

Chapter 6 is composed of two parts. Part one describes the use of anti-IL-1 therapies in CAPS. Part two describes the empirical use of anakinra in AA amyloidosis of unknown aetiology.

Chapter Six Part I:

Interleukin-1 Inhibition in Cryopyrin-Associated Periodic Syndrome – A 13-Year, Single-Centre Experience of Safety and Efficacy

6.1.1 Background

To our knowledge the first case of CAPS to present at our Centre was a 36 year old Australian man of Jewish descent in 1993. He presented with renal failure secondary to AA amyloidosis. MWS was suspected due to the history of urticarial rash since birth, progressive deafness and AA amyloidosis. Sadly he died of calciphylaxis in the year 2000, before the identification of the genetic mutation which causes the disease. Since discovery of the gene in 2001, about 150 cases of mutations in NLRP3 have been identified by our genetic screening service, and since then, 116 patients have been reviewed in the clinic, with 113 treated with the anti-IL-1 drugs anakinra or canakinumab. Anakinra had been used off-license for CAPS and other autoinflammatory diseases for well over a decade until authorisation for the CAPS indication was obtained in 2013/4 by means of retrospective safety and efficacy data. Canakinumab went through the usual phases of clinical trials and achieved license for CAPS in the UK in 2009; this sparked the initiation of the NHS National CAPS Treatment Service at the UK National Amyloidosis Centre (NAC) in London.

The NHS National CAPS Treatment Service was set up as a canakinumab treatment and monitoring service; a few patients are now being treated with anakinra within the service. Funded directly by NHS England, the aim of the service was to safely reconstitute and administer this high cost drug to patients in a controlled environment, as well as to closely monitor drug efficacy and any adverse effects. It is composed of a nurse-led and medical consultant-led clinic running in parallel. Patients undergo initial assessment and diagnosis in the consultant clinic, and are assessed, monitored and treated with canakinumab every eight to 12 weeks in the nurse clinic. They are reviewed by a consultant annually, unless there are complex issues requiring more frequent medical review or intervention. Patients treated with anakinra are reviewed every three to six months.

6.1.2. Aims

We sought to describe a single-centre experience of over a decade of IL-1 inhibition using anakinra and canakinumab in patients with CAPS.

6.1.3 Patients and Methods

6.1.3.1 Patients and assessments

One hundred and seventeen patients with a clinical and/or genetic diagnosis of CAPS were seen at our Centre between 1993 and 2015, and these patients make up the 'whole' cohort. Seventy three patients treated with an anti-IL-1 agent and who attended regular follow-up at the NAC were included in the 'analysis' cohort. Patients attended clinic approximately every eight weeks if they were taking canakinumab and every three to twelve months if they were on anakinra. Some patients were untreated and attended annually for review. Patients underwent the following assessments at each visit:

- Vital signs measurements (blood pressure, heart rate and weight)
- Blood sampling for biochemistry including renal and liver function and full blood count; and anti-nuclear antibodies annually
- Urinalysis
- Physical examination where indicated
- Quality of life assessment (annually)

And additional tests for patients treated with canakinumab only:

- CAPS disease activity assessment (symptom score)
- Ophthalmology, audiometry, neurological assessment and brain MRI (where indicated) annually.

More detail about the clinic protocol may be found in Chapter Two: Methods.

Patients gave written informed consent for retrospective analysis and publication of their clinical data (REC reference number 06/Q0501/42).

6.1.3.2 Other methods

Further methods are described in Chapter Two: Methods.

6.1.4 Results

6.1.4.1 Whole cohort characteristics

Of the 117 patients, half were female. Twenty two were children who were followed-up at a partner paediatric hospital, although their anti-IL-1 therapies were prescribed as part of the UK NAC National CAPS Treatment Service. A variety of genetic mutations were found among the cohort (Figure 6.1.1), and in 13 no variant was detected on Sanger sequencing, although patients exhibited clinical features of the disease. Age at diagnosis ranged from 10 months to 79 years. Three patients went untreated; two died before identification of the pathogenic role of IL-1, and one had very mild disease. Five patients developed AA amyloidosis as a complication.

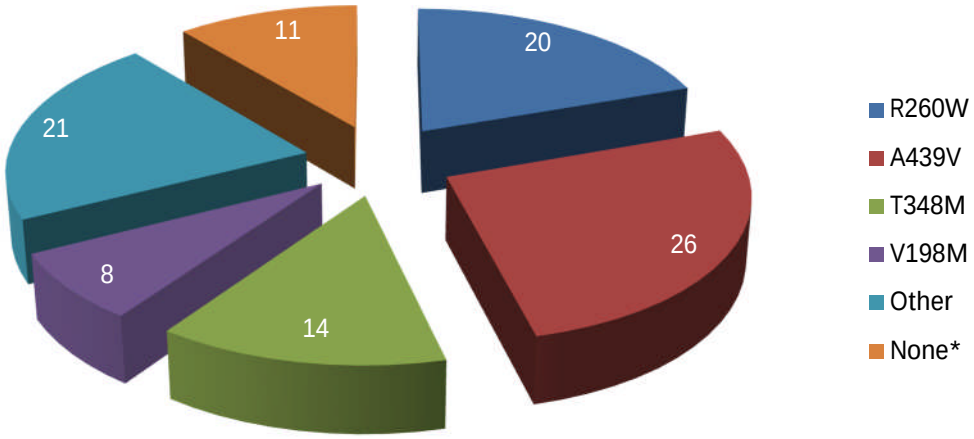


Figure 6.1.1. Distribution (%) of variants within the whole cohort. *Conventional Sanger sequencing revealed no variant in 11% of patients.

6.1.4.2 General characteristics of the analysis cohort

Characteristics of individual patients in the analysis cohort are shown in Table 6.1.1. Seventy three patients were included. Forty (55%) were female. Patients were predominantly Caucasian (89%). Median age of the cohort at the first clinic visit (this was the diagnostic visit in the overwhelming majority of patients) was 32 years (IQR 23 to 48). Ten were children under 16 years of age and four were between 16 and 18 years old. The youngest child to commence therapy was 8 years old and was treated with canakinumab (patient 36 in Table 6.1.1). The oldest patient at diagnosis was 78 and was treated with anakinra (patient 72).

A variety of mutations are represented: 24 A439V (33%), 15 R260W (21%), 10 T348M (14%), 5 V198M (7%), 4 patients with somatic mosaic mutations (5%) and 15 patients with a variety of other mutations (21%). Onset of symptoms occurred predominantly in the neonatal period, infancy or childhood (86% of patients), but 5 patients had onset in the teenage years (patients 15, 27, 32, 54, and 56), and 5 had adult onset (patient 51 with V198M and the 4 somatic mosaics, patients 70 to 73). Family history of disease was present in 42 patients (58%). Twelve kindreds are represented within this cohort.

Sixteen patients (22%) are currently being treated with anakinra and 57 (78%) with canakinumab. Six patients currently on anakinra have switched from canakinumab due to inadequate response. Twenty one patients currently on canakinumab have switched from anakinra, in preference of the eight-weekly (rather than daily) injection regimen; many also cited painful injections as secondary reason for switching from anakinra. Two female patients swapped from canakinumab to anakinra during pregnancy and resumed canakinumab therapy in the post-partum period. The median duration of anti-IL-1 therapy within the analysis cohort was 5 years (IQR 2.5 to 7.8).

Patient No.	Sex	Ethnicity	Age at V1 (y)	Variant	Clinical manifestations	Disease pattern	Family history	Current therapy	Response	Previous therapy	Duration of IL-1 blockade (y)
1 ∝	F	Caucasian	25.3	A439V	S, F, MS, E	CE	Yes	Anakinra	Complete		8.09
2 ∝	F	Caucasian	26.52	A439V	S, F, C, MS, E, O	CE	Yes	Anakinra	Complete		7.62
3 ∝	F	Caucasian	61.34	A439V	S, F, C, MS, N, E, O	CE	Yes	Canakinumab	Complete	Anakinra	7.76
4 ∝	F	Caucasian	58.02	A439V	S, C, MS, N, E, O	CE	Yes	Canakinumab	Complete	Anakinra	7.02
5 β	M	Caucasian	68.44	A439V	S, F, C, MS, E	R	Yes	Canakinumab	Partial		2.30
6 β	M	Caucasian	29.10	A439V	S, F, C, MS, O	R	Yes	Canakinumab	Complete		6.91
7 β	F	Caucasian	54.63	A439V	S, F, C, E, O	CE	Yes	Anakinra	Complete		7.14
8 γ	F	Caucasian	28.67	A439V	S, F, C, MS, N, E, O	CE	Yes	Canakinumab	Complete	Anakinra (p)	3.33
9 γ	F	Caucasian	27.29	A439V	S, C, MS, E, O	CE	Yes	Canakinumab	Complete		3.94
10 γ	F	Caucasian	53.22	A439V	S, F, C, MS, E*, O	CE	Yes	Canakinumab	Complete		3.94
11 γ	F	Caucasian	50.33	A439V	S, C, MS, E, O	CE	Yes	Canakinumab	Complete		3.94
12 €	M	Caucasian	47.07	A439V	S, F, C, MS, E, O	CE	Yes	Anakinra	Partial (I)	Canakinumab	3.76
13 €	F	Caucasian	14.25	A439V	S, F, C, MS, N, E, O	CE	Yes	Canakinumab	Complete		3.76
14 ∞	M	Caucasian	20.01	A439V	S, F, C, MS, E	R	Yes	Canakinumab	Complete	Anakinra	7.77
15 ∞	M	Caucasian	48.27	A439V	S, F, C, MS, E	CE	Yes	Canakinumab	Complete	Anakinra	7.77
16 ∞	F	Caucasian	51.22	A439V	S, C, MS, N,E, O	C	Yes	Canakinumab	Complete	Anakinra	8.33
17 £	F	Caucasian	60.56	A439V	S, MS, E	C	Yes	Canakinumab	Complete		1.63
18 £	F	Caucasian	27.00	A439V	S, F, C, MS	CE	Yes	Canakinumab	Complete		1.63
19 ¥	M	Caucasian	46.43	A439V	S, F, C, MS, N, E	CE	Yes	Canakinumab	Partial		1.73
20 ¥	M	Caucasian	50.60	A439V	S, F, C, MS, E	C	Yes	Canakinumab	Complete		1.00
21 ¥	M	Caucasian	23.72	A439V	S, F, C, MS, N, E, O	C	Yes	Canakinumab	Complete		0.53
22 ¥	F	Caucasian	49.61	A439V	S, F, C, MS, N, E	CE	Yes	Canakinumab	Partial		2.53
23	M	Caucasian	28.36	A439V	S, F, C, MS, E	R	Yes	Canakinumab	Complete.		0.21
24	F	Caucasian	21.04	A439V	S, F, C, MS, N, E, O	CE	No	Anakinra	Complete		2.78

Table 6.1.1. Characteristics of individual patients in the analysis cohort. Symbols next to patient numbers indicate membership of the same kindred. * indicates severe clinical manifestations. V1 = first clinic visit; S = skin, F = fever, C= other constitutional symptoms, MS = musculoskeletal, N = neurological, E = eye symptoms, H = hearing loss, O = other. Anakinra (p) indicates use of anakinra only during pregnancy. (I) indicates inadequate responder to one or both anti-IL-1 therapies.

Patient No.	Sex	Ethnicity	Age at V1 (y)	Variant	Clinical manifestations	Disease pattern	Family history	Current therapy	Response	Previous therapy	Duration of IL-1 blockade (y)
25 ×	F	Asian	12.91	R260W	S, MS, E	CE	Yes	Canakinumab	Complete		1.08
26 ×	F	Asian	10.10	R260W	S, C, MS, O	CE	Yes	Canakinumab	Complete		5.26
27 ×	M	Asian	45.58	R260W	S, F, C, MS, O	C	No	Canakinumab	Complete		5.26
28 ×	M	Asian	8.56	R260W	S, F, MS, O	R	Yes	Canakinumab	Complete		4.14
29 μ	M	Caucasian	39.17	R260W	S, MS, E	CE	Yes	Anakinra	Complete		10.95
30 μ	M	Caucasian	11.50	R260W	S, C, MS, N, E	CE	Yes	Canakinumab	Complete	Anakinra	10.66
31 μ	M	Caucasian	5.12	R260W	S, MS, E	CE	Yes	Canakinumab	Complete		6.25
32 μ	F	Caucasian	48.94	R260W	S, F, C, MS, E	CE	Yes	Canakinumab	Complete		9.40
33 π	F	Caucasian	32.25	R260W	S, C, MS, E	C	Yes	Canakinumab	Complete		5.42
34 π	F	Caucasian	57.50	R260W	S, C, N, E	CE	Yes	Anakinra	Complete		6.97
35	M	Caucasian	42.81	R260W	S, F, C, MS*, N*, E, O	CE	No	Canakinumab	Partial	Anakinra	3.92
36	F	Caucasian	40.44	R260W	S, F, MS, E	CE	No	Canakinumab	Complete		7.43
37	M	Caucasian	51.00	R260W	S, F, C, MS, E, H	CE	No	Canakinumab	Complete		4.89
38	F	Caucasian	43.29	R260W	S, F, C, MS, N, E, H, O	R	No	Anakinra	Partial (I)	Canakinumab	2.24
39	M	Mixed	23.43	R260W	S, F, C, MS, E, H, O	CE	No	Canakinumab	Complete	Anakinra	13.12
40 Ω	M	Caucasian	14.40	T348M	S, MS, N*, E*, H	C	Yes	Canakinumab	Complete	Anakinra	8.03
41 Ω	M	Caucasian	11.12	T348M	S, N, E, H	C	Yes	Canakinumab	Complete		5.08
42 Ω	F	Caucasian	42.64	T348M	S, F, MS, N, E, H	CE	No	Canakinumab	Partial	Anakinra	8.03
43 Σ	M	Caucasian	30.29	T348M	S, C, MS, N*, E, H	CE	Yes	Canakinumab	Complete		10.70
44 Σ	F	Caucasian	33.27	T348M	S, F, C, MS, N, E, H	CE	Yes	Canakinumab	Complete	Anakinra	11.04
45	F	Caucasian	18.72	T348M	S, C, MS, N, E, H, O	CE	No	Canakinumab	Complete	Anakinra (p)	8.57
46	M	Caucasian	41.53	T348M	S, F, C, MS, N*, E, H, O	C	No	Anakinra	Partial (I)	Canakinumab	3.11
47	M	Caucasian	23.47	T348M	S, F, C, MS, N*, E, H, O	C	No	Canakinumab	Partial (I)		7.48
48	M	Caucasian	34.57	T348M	S, F, MS, N, E, H	CE	No	Canakinumab	Complete	Anakinra	10.26
49	M	Caucasian	35.23	T348M	S, F, C, MS, N*, E, H, O	C	No	Canakinumab	Complete		10.50

Table 6.1.1 cont. Characteristics of individual patients in the analysis cohort. Symbols next to patient numbers indicate membership of the same kindred. * indicates severe clinical manifestations. V1 = first clinic visit; S = skin, F = fever, C= other constitutional symptoms, MS = musculoskeletal, N = neurological, E = eye symptoms, H = hearing loss, O = other. Anakinra (p) indicates use of anakinra only during pregnancy. (I) indicates inadequate responder to one or both anti-IL-1 therapies.

Patient No.	Sex	Ethnicity	Age at V1 (y)	Variant	Clinical manifestations	Disease pattern	Family history	Current therapy	Response	Previous therapy	Duration of IL-1 blockade (y)
50 ᐞ	F	Caucasian	56.63	V198M	S, F, C, MS, N, E, O	R	Yes	Canakinumab	Complete		2.03
51 ᐞ	F	Caucasian	26.32	V198M	S, F, C, MS, E, O	CE	Yes	Canakinumab	Complete		2.49
52	F	Caucasian	14.70	V198M	S, MS	R	No	Canakinumab	Complete		5.27
53	F	Caucasian	29.49	V198M	S, F, CMS, E	R	No	Anakinra	Complete		1.67
54	F	Caucasian	33.40	V198M	S, F, C, MS, O	R	Yes	Anakinra	Partial		2.20
55 Ψ	M	African	35.08	Y859C	S, F, C, MS, N*, E, H, O	CE	Yes	Canakinumab	Complete		2.93
56 Ψ	F	African	59.98	Y859C	S, MS, H, E, H, O	CE	Yes	Canakinumab	Partial		0.67
57	F	Caucasian	14.61	Y570F	S, F, MS, N*, E, H, O	CE	No	Canakinumab	Partial (I)	Anakinra	10.54
58	F	Caucasian	43.11	A352P	S, E	R	Yes	Canakinumab	Complete		1.34
59	F	Caucasian	24.55	A352T	S, C, MS, N*, E, H, O	C	No	Canakinumab	Partial (I)	Anakinra	2.86
60	M	Caucasian	40.43	T346I	S, F, C, MS, N, E	C	No	Anakinra	Complete (I)	Canakinumab	4.81
61	M	Caucasian	25.81	D303N	S, C, MS, N, E, H, O	C	No	Canakinumab	Complete		5.71
62	M	Caucasian	26.69	F523C	S, F, C, MS, N, E, H, O	CE	No	Canakinumab	Partial		6.08
63	F	Caucasian	32.15	G569R	S, C, MS, E*, H, O	CE	No	Anakinra	Complete		8.13
64	M	Caucasian	16.71	G755R	S, MS, N, E	C	No	Canakinumab	Partial	Anakinra	8.45
65	F	Caucasian	23.10	L353P	S, MS	CE	No	Canakinumab	Complete		3.36
66	F	Caucasian	18.10	L632F	S, F, C, MS, N, E, H, O	CE	No	Canakinumab	Complete	Anakinra	10.62
67	M	Mixed	16.34	S547C	S, F, C, MS, N	CE	No	None	Partial (I)	Both	0.44
68	F	Caucasian	22.59	V351M	S, F, C, N, E*, H	C	No	Canakinumab	Complete	Anakinra	6.45
69	M	Caucasian	22.68	DelT438-A439	F, MS, N, E*, H	C	No	Canakinumab	Partial		1.95
70	F	Caucasian	66.45	Y563C mosaic	F, C, N, E, H	C	No	Anakinra	Partial (I)	Canakinumab	5.09
71	M	Caucasian	47.39	E567K mosaic	S, F, C, N*, H, O	C	No	Anakinra	Partial (I)	Canakinumab	5.60
72	M	Caucasian	78.86	G569V mosaic	S, C, MS, E	CE	No	Anakinra	Complete		0.96
73	F	Caucasian	61.43	Y563C mosaic	S, F, C, MS, H	CE	No	Canakinumab	Partial	Anakinra	1.17

Table 6.1.1 cont. Characteristics of individual patients in the analysis cohort. Symbols next to patient numbers indicate membership of the same kindred. * indicates severe clinical manifestations. V1 = first clinic visit; S = skin, F = fever, C= other constitutional symptoms, MS = musculoskeletal, N = neurological, E = eye symptoms, H = hearing loss, O = other. Anakinra (p) indicates use of anakinra only during pregnancy. (I) indicates inadequate responder to one or both anti-IL-1 therapies.

6.1.4.3 Clinical characteristics of the analysis cohort

Nineteen patients (26%) exhibited a chronic disease pattern with symptoms every day or most days prior to treatment. Forty three (59%) reported having a chronic pattern with intermittent exacerbations prior to commencing therapy. Eleven patients (15%) had a recurrent pattern with asymptomatic periods between flares. Disease manifestations are summarised for each patient in Table 6.1.1 above and described in more detail for the entire analysis cohort in Table 6.1.2 below. Description and categorisation of manifestations are based on those reported/developed by PRINTO and Eurofever (182).

	n	%
Fever	47	64
Other constitutional symptoms[+]	15	21
No fever or other constitutional symptoms	11	15
Skin manifestations	71	97
Urticarial rash	68	93
Maculopapular rash	3	4
No rash	2	3
Musculoskeletal manifestations	66	90
Myalgia	22	30
Arthralgia	57	78
Arthritis	25	34
Severe musculoskeletal manifestations*	4	5
Neurological involvement	39	53
Headache	39	53
Papilloedema	9	12
Meningitis	8	11
Severe neurological manifestations[$]	10	14
Eye involvement	62	85
Conjunctivitis	61	84
Uveitis/Iritis	14	19
Severe eye manifestations[#]	5	7
Hearing impairment	25	34
Other	38	52
Oral ulcers	29	40
Gastrointestinal manifestations	3	4
Pleurisy	2	3
Pericarditis	1	1
Growth stunting	2	3
AA amyloidosis	1	1

Table 6.1.2. Clinical characteristics and disease manifestations in the analysis cohort. [+]Other constitutional symptoms defined as fatigue, malaise or mood disturbances. [*]Severe musculoskeletal symptoms were joint contractures, bone deformities or patellar overgrowth. [$]Severe neurological symptoms were evidence of raised intracranial pressure or cerebral atrophy. [#]Severe eye manifestations were optic nerve atrophy, glaucoma or impaired vision.

6.1.4.4 Efficacy of anti-IL-1 therapy

Table 6.1.1 identifies individuals who were complete and partial responders. Fifty nine patients (81) were considered complete responders to anti-IL-1 therapy. We defined a complete response as normalisation of serum acute phase reactants (SAA $\leq$ 10 mg/L and CRP $\leq$ 5 mg/L) and resolution of chronic disease symptoms and flares/exacerbations. Mild recurrent symptoms such as oral ulcers, or occasional mild headaches or rash or joint aches, which result in symptom scores of 1 or 2, and which were not bothersome to patients, were not considered to be in conflict with the classification of complete response. A partial response was defined as reduction in, but not normalisation of, SAA and CRP measurements and/or an improvement in disease symptoms, but not complete resolution as defined above. Non-responders or inadequate responders were patients who had no significant improvement in either or both domains.

Figure 6.1.2 shows typical CAPS urticaria-like rash covering the back of patient 61 prior to initiation of canakinumab therapy, and with the back rash resolved after the first dose of canakinumab. Figure 6.1.3 shows the pre-treatment and post-treatment SAA concentrations within the analysis cohort. Pre-treatment plots are a single SAA measurement taken before initiation of treatment, as it was often the case that only a single measurement was available prior to starting treatment. A few patients showed pre-treatment SAA concentrations within the normal range on the single pre-treatment assay. Untreated, CAPS has a relapsing-remitting disease course and there is therefore a natural variability in SAA concentrations. On treatment plots are the medians of all available SAA measurements in the past year. Figure 6.1.4 show the pre- and on-treatment symptom scores.

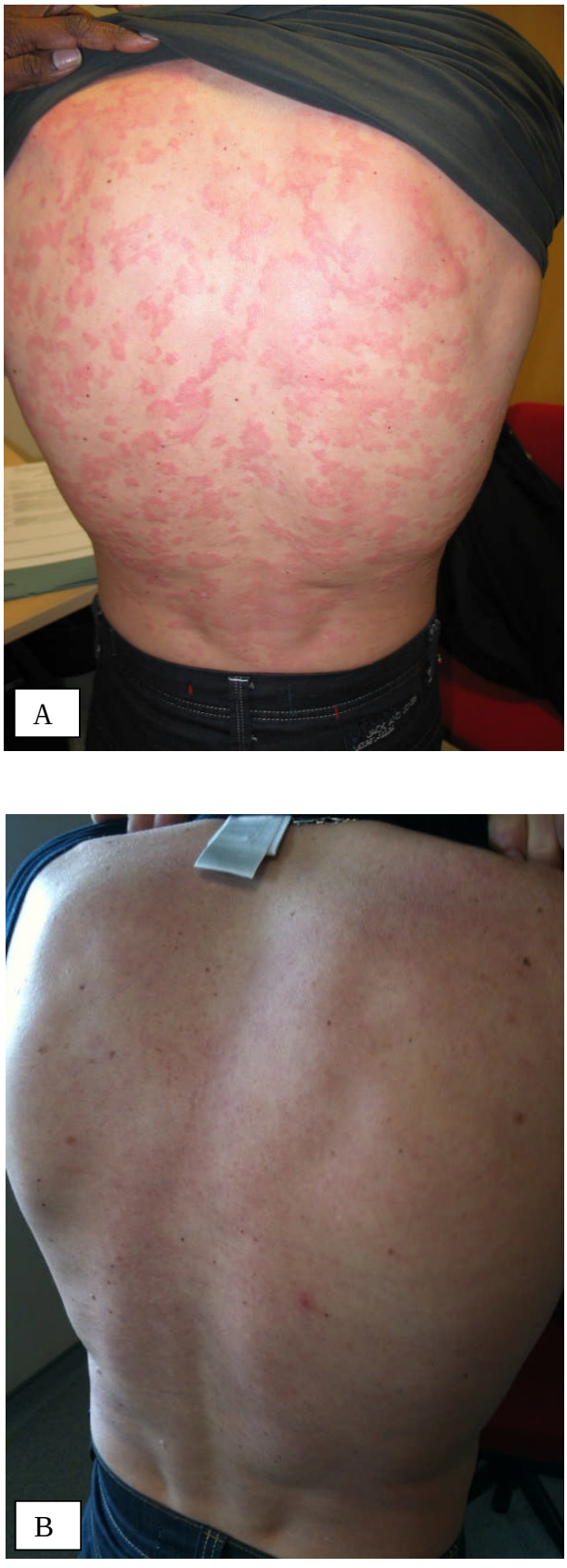


Figure 6.1.2. Patient 61 before and after first dose of canakinumab. Panel A shows typical CAPS urticaria-like rash covering the back of patient 61 prior to initiation of canakinumab therapy. Panel B shows the back rash resolved after the first dose of canakinumab.

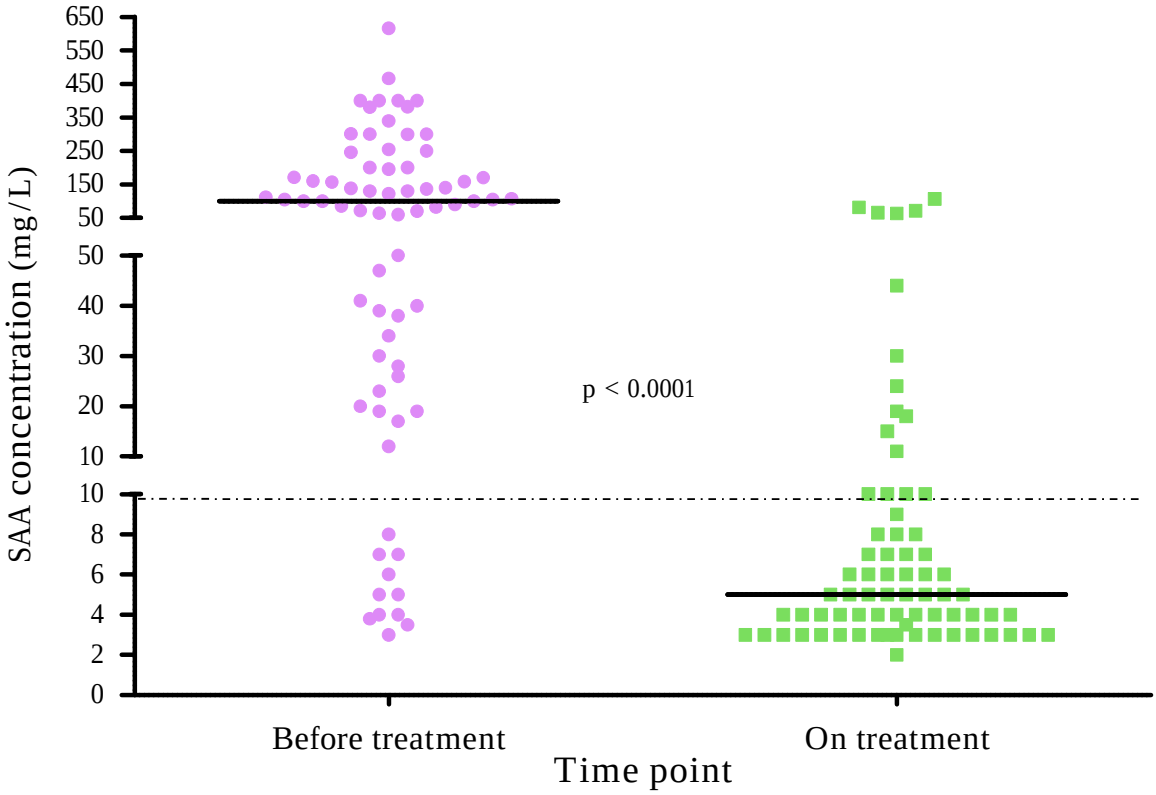


Figure 6.1.3. Pre- and on- treatment SAA concentration for individuals in the analysis cohort. Pre-treatment plots are single SAA measurement taken before initiation of treatment. On treatment plots are the medians of all available SAA measurements in the past year. Solid lines indicate medians which were significantly different ($p < 0.0001$). Broken line indicates upper limit of normal range (10 mg/L).

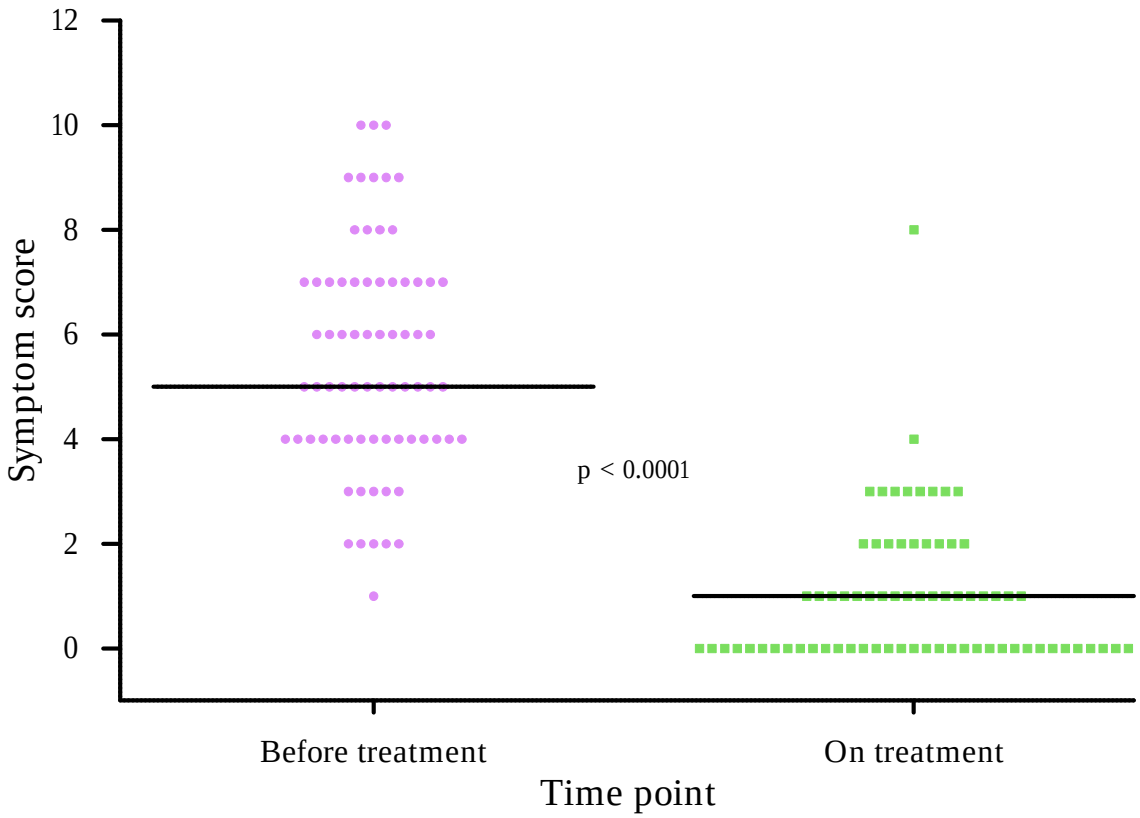


Figure 6.1.4. Pre- and on- treatment symptom scores for individual patients in the analysis cohort. Pre-treatment plots are single symptom scores taken before initiation of treatment. On treatment plots are the medians of all available symptom scores in the past year. Solid lines indicate medians which were significantly different ($p < 0.0001$).

6.1.4.5 Non-responders or inadequate responders

Amongst the cohort we identified 10 patients who had a sub-optimal response to canakinumab or anakinra, or to both anti-IL-1 therapies.

Patients 38 and 60

Patient 38, with a MWS phenotype, and patient 60, with a CINCA phenotype experienced only modest and very transient reductions in both serum inflammatory markers and symptoms when treated with canakinumab. Both patients developed marked inflammation with morphea-like (Figure 6.1.5) rash bilaterally on their lower limbs shortly afterwards. Skin biopsy showed only morphea. Patient 60 also developed a diffuse, tender, erythematous rash over his torso laterally on the left (Figure 6.1.6). Skin biopsy and chest CT scan were both inconclusive. Both patients were switched to anakinra therapy with excellent effect, and rash resolved within a few weeks. Figure 6.1.7 shows the treatment course of patient 60.

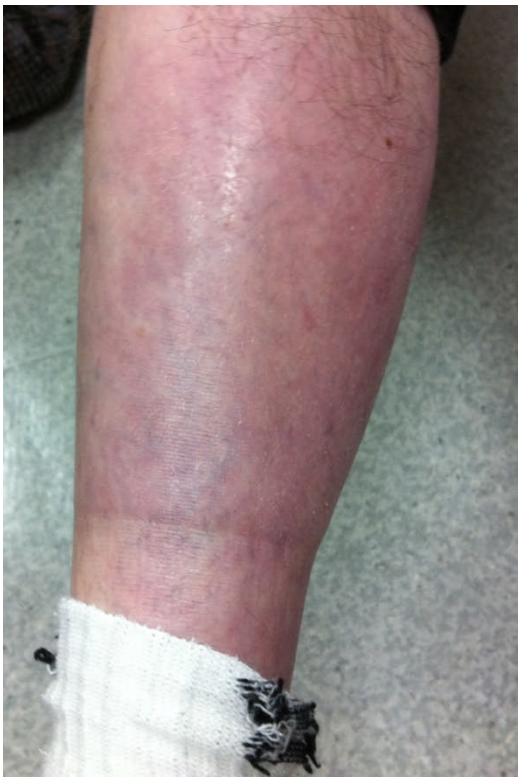


Figure 6.1.5. Morphea-like rash on the lower limb of patient 60.

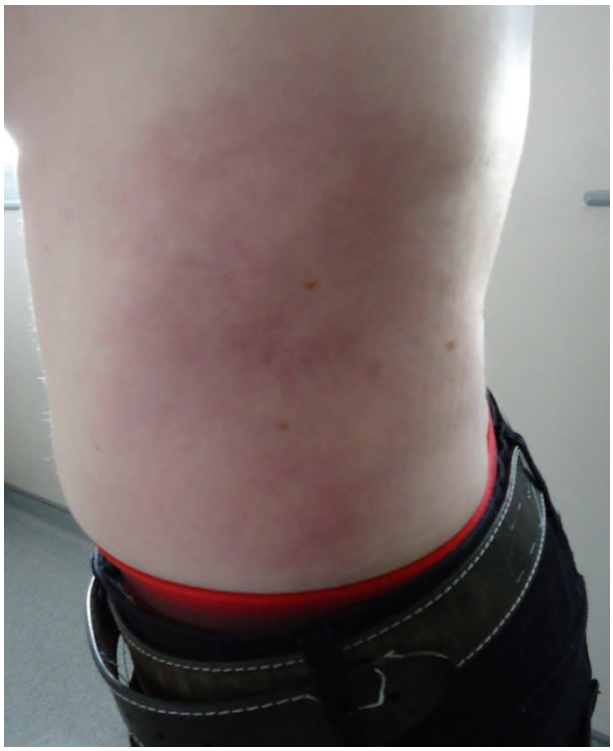


Figure 6.1.6. Left lateral torso rash of patient 60.

Patients 13, 57, 67 and 70

Patient 13, with a FCAS phenotype, weighed 130 kg. He had a partial response to 300 mg canakinumab q8w (a dose concentration of < 3mg/kg). He was switched to anakinra, and remains well on a partial response to 200 mg anakinra daily. Patients 57 and 67 with CINCA phenotypes, and patient 70 with the Y547C mosaic genotype and atypical presentation had severe disease. Patient 57, experienced partial response to canakinumab and anakinra, although subjectively feels better on 300 mg canakinumab (10 mg/kg) q8w. Patient 67 has had no biochemical or symptomatic response to 300 mg canakinumab (10 mg/kg) q8w, and has elected to come off treatment. He does not wish to have a trial of anakinra just yet. Patient 70 has been treated with both canakinumab and anakinra over a 10-year period, at times concurrently. She experienced a severe flare with aseptic meningitis after attempted conversion to canakinumab.

She is currently on anakinra 400 mg daily and biochemical inflammation and headaches have remained consistent.

Patients 46, 47, 59 and 71

These patients had central nervous system (CNS) inflammation symptomatically, and on lumbar puncture or MRI. Patients 46 and 59 with CINCA had resolution of most CAPS symptoms and SAA normalised on canakinumab 300 mg q8w, however headaches and fatigue continued. Both had previous strokes attributed to CNS inflammation. Patient 46 has had some symptomatic improvement on anakinra.

Patient 71 (E567K mosaic with atypical presentation) weighed 102 kg. He had resolution of most CAPS symptoms and SAA normalised, but headaches, poor balance and fatigue continued on both treatments. Patient 47 with MWS had normalisation of inflammatory markers and peripheral symptoms on 300 mg canakinumab q8w. However, headaches and fatigue remained. Encouragingly, in patients 47 and 71 fatigue and mood have now begun to improve after 5 years or more of treatment.

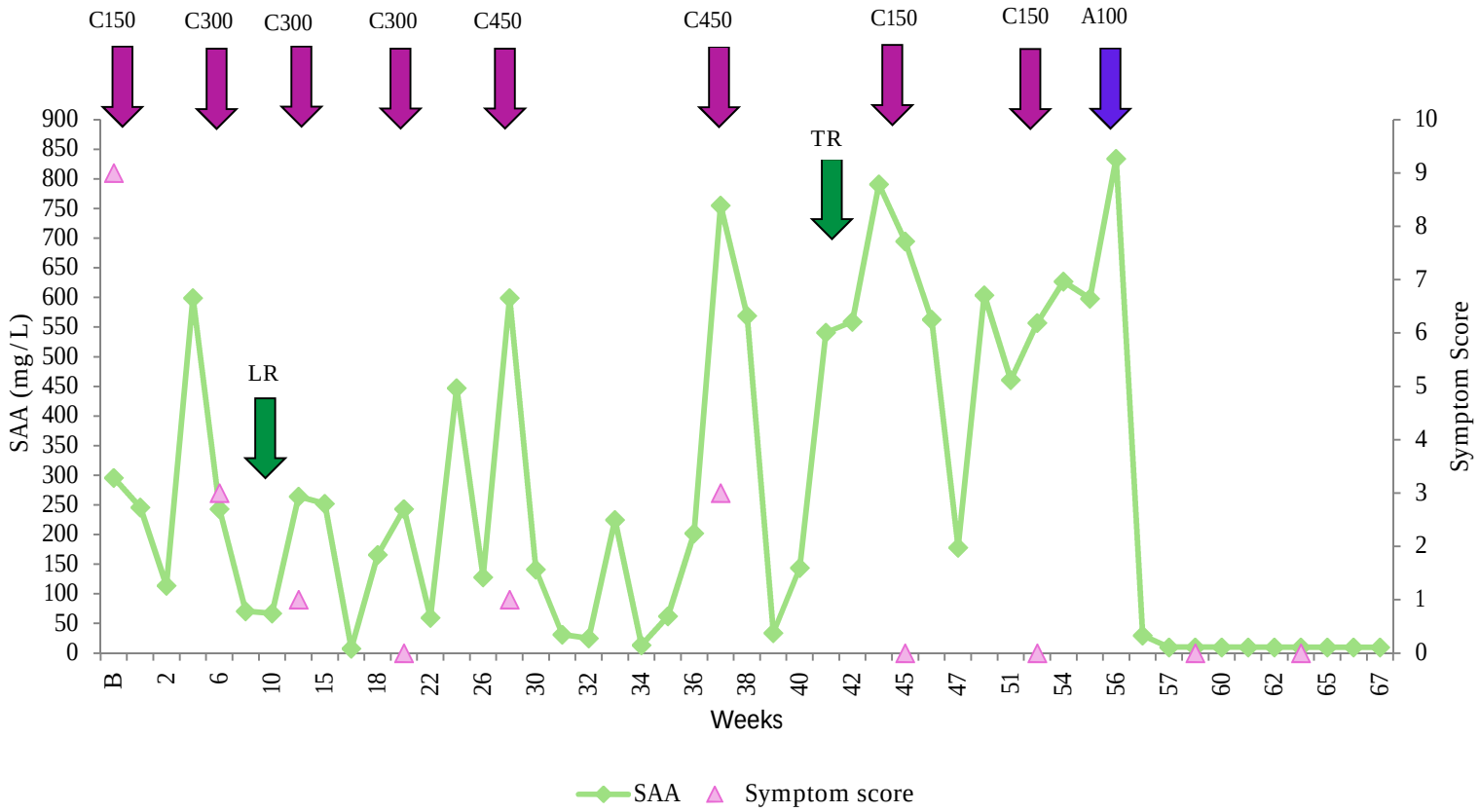


Figure 6.1.7. Disease and treatment course for patient 60, showing drugs and doses administered and corresponding SAA measurement and symptom score. C150/300/400 with pink arrows= canakinumab 150/300/450 mg doses administered; LR and TR with green arrows indicate timepoint at which leg rash and torso rash appeared; A100 with purple arrow = anakinra 100 mg administered.

6.1.4.6. Breakthrough symptoms

Forty two patients (56%) report breakthrough symptoms. These are CAPS symptoms which recur occasionally and usually mildly despite otherwise effective disease suppression, and are distinct from disease flares and from the more severe symptoms experienced by the inadequate responders. These breakthrough symptoms are responsible for symptom scores of one or two when patients are assessed at clinic visits. The commonest are:

- Headache, 16 patients (22% of analysis cohort)
- Musculoskeletal symptoms, 15 patients (21%)
- Fatigue, 14 patients (19%)
- Rash, 12 patients (16%)
- Oral ulcers, 10 patients (14%)
- Eye symptoms, 9 patients (12%).

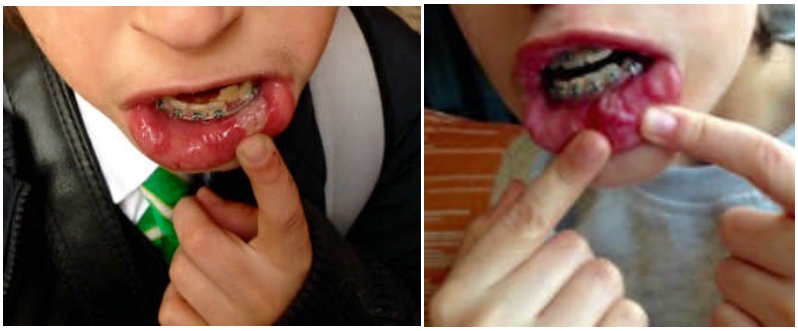


Fig 6.1.8. Severe, large oral ulcers in patient 28. Photos taken on two separate occasions show two large, severe ulcers on the inside of the lower lip of patient 28.

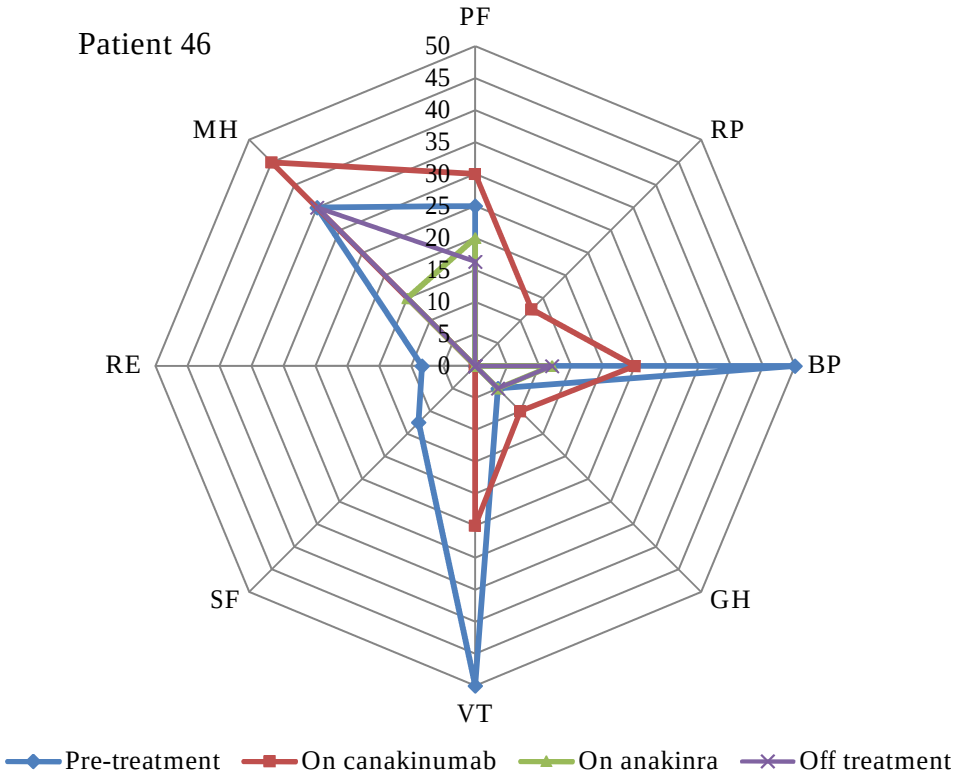
6.1.4.7 Quality of life

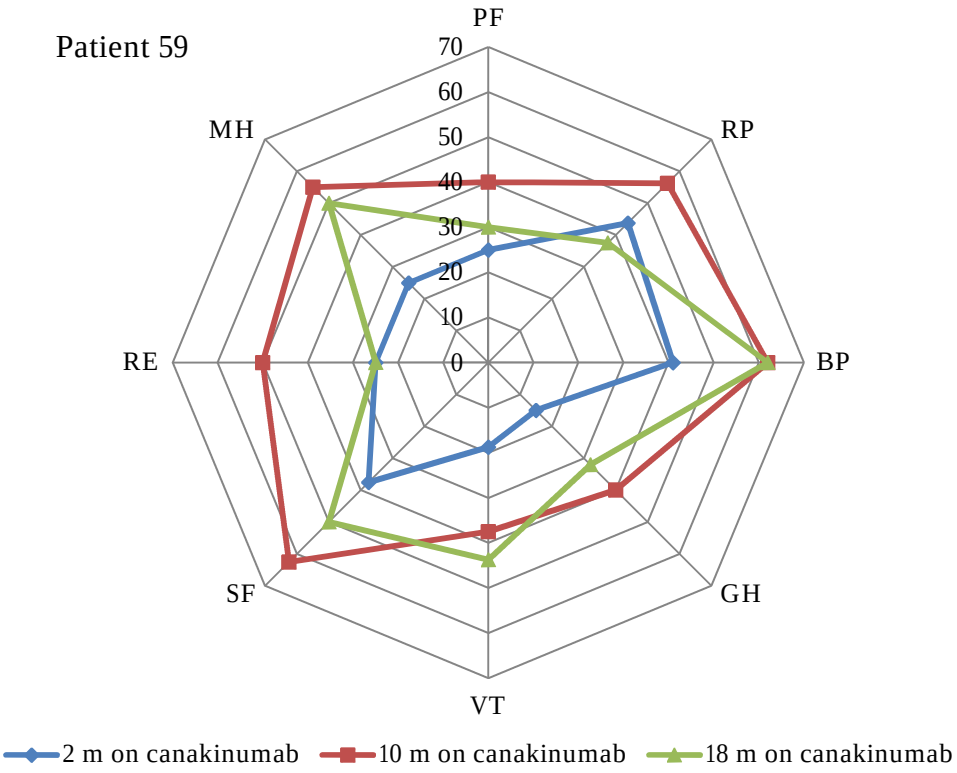
Two hundred and sixty two SF36v2 QoL surveys were completed by 65 patients between 2010 and 2015. Questionnaires from three patients were excluded from the analysis on the grounds of inadequate cognitive capacity to complete questionnaires independently. Eight patients had completed a only single questionnaire at a single time-point and were also excluded. Pre-treatment questionnaires were not available for 22 patients who had commenced anti-IL-1 treatment prior to initiation of the CAPS Service (patients who had been on long term anakinra, or those treated with canakinumab within a clinical trial). Surveys from complete and partial responders were grouped together for analysis and inadequate responders were analysed separately. Pre- and post-treatment questionnaires were completed by 16 complete and partial responders; pre-treatment questionnaires were available for six inadequate responders.

Domain	Mean pre-treatment score	Mean on-treatment score	Difference
PF	69	95	26
RP	57	95	38
BP	49	95	46
GH	44	75	31
VT	35	75	40
SF	60	98	38
RE	87	98	12
MH	70	86	16

Table 6.1.3. Change in QoL domain scores in 16 patients who were considered complete or partial responders to canakinumab after one dose. The survey was administered before commencing treatment and 2 to 6 months after. PF = physical function, RP = role physical, BP = bodily pain, GH = general health, VT = vitality, SF = social function, RE = role emotional and MH = mental health.

After initiation of therapy, QoL scores were seen to improve significantly across all domains in the 16 complete and partial responders, compared to their pre-treatment scores. Mean scores before canakinumab and the first on-treatment scores (two months after first dose) are shown in Table 6.1.3. The biggest improvements were seen in the bodily pain, vitality, and role, social and physical function. In contrast, patients who were considered inadequate responders, scores did not improve in some domains, even over measurement at multiple time-points, and on different treatments (Figure 6.1.9).





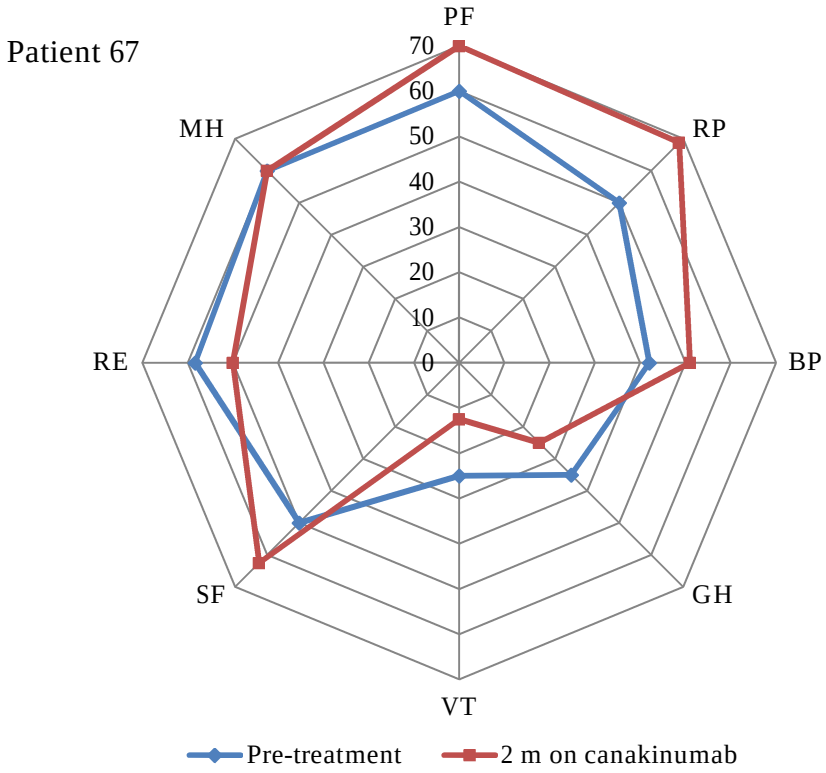


Figure 6.1.9. Change in QoL domain scores in 3 patients who were considered inadequate responders to anti-IL-1 therapies. Scores are plotted for each time-point in the on-treatment follow-up period as indicated in the legend of each plot. PF= physical function, RP=role physical, BP=bodily pain, GH=general health, VT=vitality, SF = social function, RE = role emotional and MH=mental health. 2m=2 months etc. on treatment.

One hundred and forty eight surveys taken by complete and partial responders were available for comparative evaluation ranging from two months to 10 years on treatment medium- to longer term QoL). Pre-treatment scores of 21 patients are shown in Figure 6.1.10. For comparison over the follow-up period scores in each domain are shown by box plots at each of the following post-treatment time-points in Figure 6.1.11: two months, n = 15 patients; six months, n = 14; 12 months, n = 14; 24 months, n = 14; 36 months, n = 14; 48 months, n = 15; 60 months, n = 18; 72 months, n = 14; 84 months, n = 15; 96 months, n = 8; 108 months, n = 5 and 120 months, n = 2. Mean scores in all domains are improved on treatment as compared to baseline, and the improvement is sustained over the longer term follow-up period.

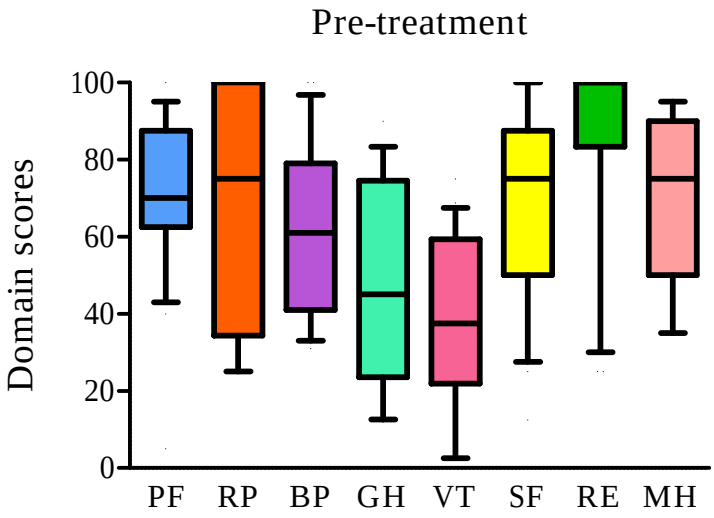


Figure 6.1.10. Pre-treatment QoL scores by domain. Pre-treatment scores of n = 21 patients (complete and partial responders) are shown as box plot with whiskers indicating 10^{th} – 90^{th} percentile. PF = physical function, RP = role physical, BP = bodily pain, GH = general health, VT = vitality, SF = social function, RE = role emotional and MH = mental health.

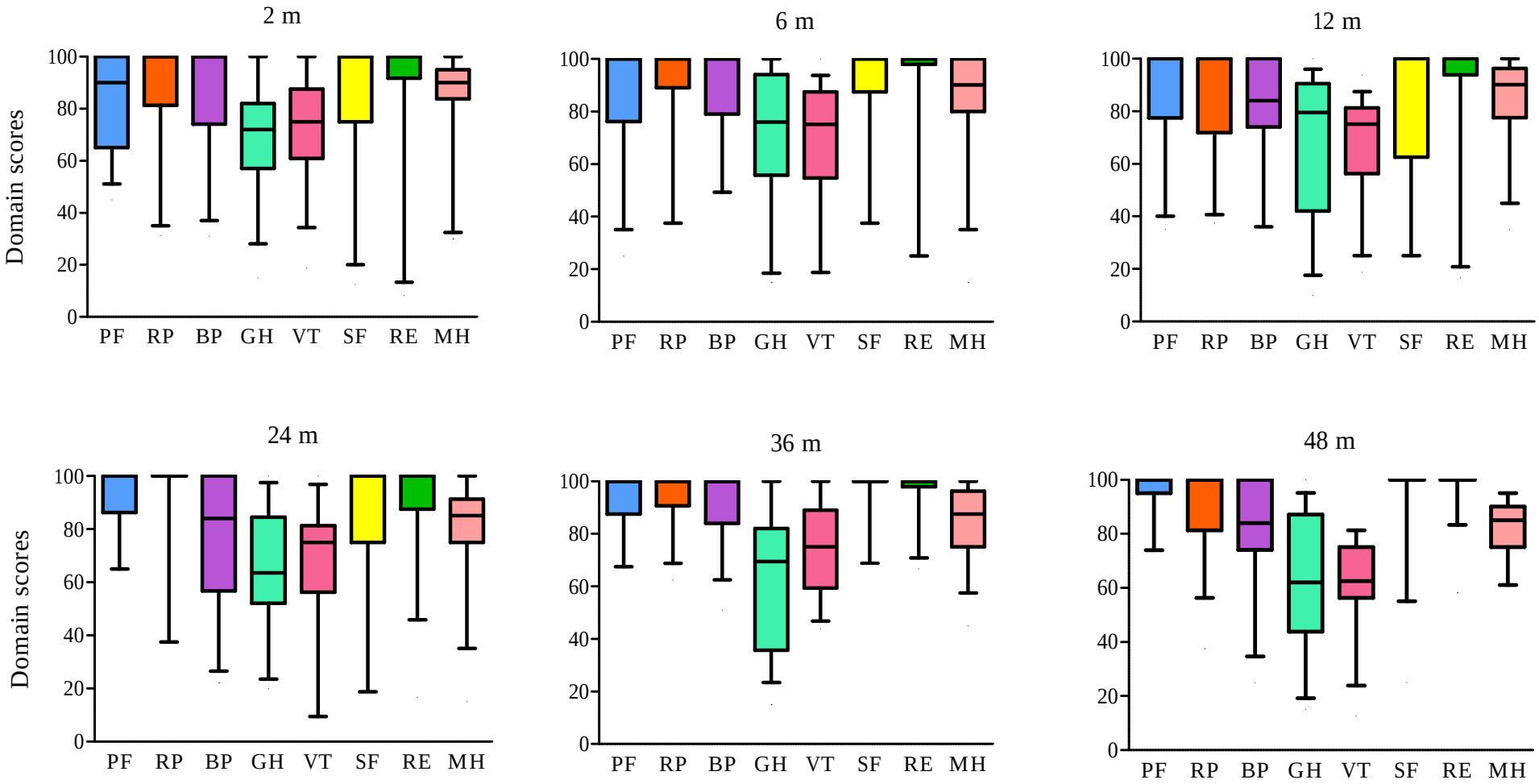


Figure 6.1.11. On-treatment QoL scores by domain. On-treatment scores of patients (complete and partial responders) are shown at various time-points over the follow-up period (2 m = 2 months on treatment, 6 m = 6 months on treatment etc.). Domain scores are shown as box plots with whiskers indicating 10^{th} – 90^{th} percentile. PF = physical function, RP = role physical, BP = bodily pain, GH = general health, VT = vitality, SF = social function, RE = role emotional and MH = mental health.

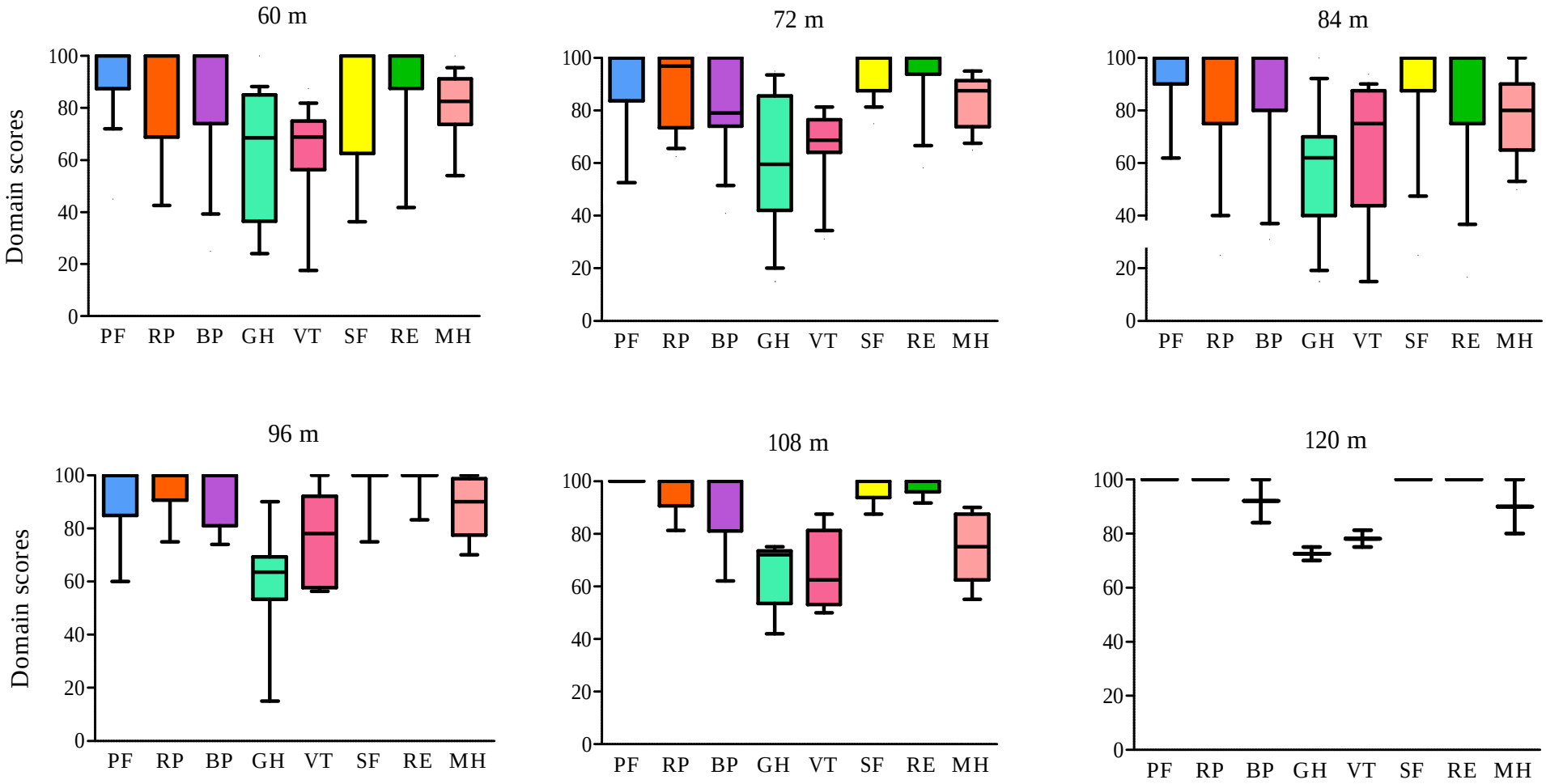


Figure 6.1.11 continued. On-treatment QoL scores by domain. On-treatment scores of patients (complete and partial responders) are shown at various time-points over the follow-up period (60 m = 60 months on treatment, 72 m = 72 months on treatment etc.). Domain scores are shown as box plots with whiskers indicating 10^{th} – 90^{th} percentile. PF = physical function, RP = role physical, BP = bodily pain, GH = general health, VT = vitality, SF = social function, RE = role emotional and MH = mental health.

6.1.4.8 Safety

Both anakinra and canakinumab are associated with increased rates of infections.

In the analysis cohort 289 adverse events (AEs) were reported by 56 patients treated with canakinumab. Most (n = 231, 80%) were not suspected to be related to canakinumab. Of the 57 (20%) which were suspected to have a relationship to canakinumab, 28 (10%) were infections:

- 12 episodes of chest infections among 10 patients
- Nine episodes of tonsillitis or throat infections among five patients
- Recurrent urinary tract infections – seven episodes between two patients.

Among patients treated with anakinra, 13 AEs possibly related to anakinra were reported by five patients. Four (31%) were infections suspected to be related to anakinra: recurrent urinary tract infections (two to three per year) were reported by one patient; recurrent tonsillitis in one patient; and two chest infections in another.

6.1.5 Discussion

Wide genotypic and phenotypic ranges are represented within this cohort. Most patients have first appearance of CAPS symptoms in early life, apart from the patients with somatic mosaic mutations who have an atypical presentation in adulthood and later life. Contrary to accepted definitions of SAIDs as hereditary periodic fever syndromes, in this cohort nearly half had no family history of CAPS, 85% had a chronic disease pattern as opposed to a periodic or recurrent one, and 15% experienced no fever or other constitutional symptoms – this points to a broadening of the phenotype and our understanding of the disease as more patients are diagnosed and follow up time increases.

The majority of patients experienced an excellent and life-changing response to anti-IL-1 therapies, and this effect has been sustained over a median on-treatment follow-up

period of five years. A small number of patients, however, did not respond as well as the majority.

Patients 38 and 60 had an incomplete response to canakinumab but a complete response to anakinra. Canakinumab is a fully humanised monoclonal antibody against IL-1β. It acts by binding free IL-1β, thereby inhibiting binding to the IL1RI and subsequent activation of the NLRP3 inflammasome. Measurement of circulating IL-1β levels has proved very disappointing with near normal values even in CAPS patients. IL-1β concentration is most appropriately assessed by looking at generation of IL-1β, and mathematical modelling has allowed this in canakinumab-treated patients (183), demonstrating that IL-1β is generated at increased but highly variable rates in CAPS, broadly corresponding with phenotypic severity. It is therefore possible that in some patients with high levels of IL-1β production this is not completely neutralised, leaving some free IL-1β to bind to the IL-1RI and activate the inflammasome. Anakinra, on the other hand, is a synthetic IL-1 receptor antagonist. It acts by binding to the IL-1RI on the cell surface. Once these are adequately saturated IL-1β signalling is blocked regardless of the rate of generation of IL-1β. This provides a possible explanation as to the superior efficacy of anakinra over canakinumab in providing IL-1β blockade in some patients.

The treatment course of patients 13, 57, 67 and 70 suggest incomplete blockade of IL-1 activity with the maximum canakinumab dose available on the NHS (300 mg). Patient 13 is obese and even at the maximum available canakinumab dose was being under-treated at a dose concentration of < 3 mg/kg; he remains a partial responder on 200 mg anakinra daily, and does not wish to introduce a third daily injection.

Patients 57 and 67 patients have the CINCA phenotype; patients with CINCA are known to require higher dose concentrations for efficacy, however both of these patients are

being treated at the maximum available dose level. Patient 70 is a complex patient with somatic mosaicism and atypical phenotype. The proportion of somatic cells displaying the variant genotype in this patient is increasing over time; this may provide an explanation as to the need for increased doses of canakinumab and anakinra in this patient over time.

Patients 46, 47, 59 and 71 showed signs and symptoms of severe central nervous system (CNS) inflammation (headaches, papilloedema, sensorineural hearing loss), with inflammation and aseptic meningitis confirmed on lumbar puncture and/or brain MRI. All four had raised intracranial pressure. Patients 46 and 59 both had previous strokes attributed to CNS inflammation. The cause of headaches in patients with CAPS remains unclear. Data from our cohort show that some patients do have chronic aseptic meningitis and/or chronic intracranial hypertension, and/or papilledema eventually leading to optic atrophy (Parker et al, in press). However, not all patients who suffer headaches display this severe phenotype. In some patients headaches are not as debilitating as in others and it is not clear whether mild headaches represent a milder phenotype or a different pathogenic mechanism (Parker et al, in press). For example, in a study involving 25 patients suffering migrainous headaches, circulating levels of IL-1β were significantly higher during migraine attacks than between attacks (184). Further investigation is warranted, but the persistent headaches in these patients despite improvement in some systemic symptoms and/or improvement or normalisation of serum acute phase proteins suggest that some effects of CNS inflammation may be irreversible.

Breakthrough symptoms are reported by more than half the patients. This could be due to inadequate IL-1 blockade on the current dosing regimen, or to activity of other inflammatory molecules or pathways, and again, further investigation is warranted.

Health-related quality of life of those patients considered complete or partial responders is seen to improve in all domains after commencing treatment. The greatest improvements are seen in the bodily pain, vitality, and role, social and physical function scales. This is consistent with improvement in, or complete resolution of, the physically and psychologically debilitating symptoms of fever, rash, arthralgia, myalgia and fatigue, once successfully treated, and is corroborated by the improvement in clinical symptoms scores. The improvements in the complete and partial responders are maintained over the longer term, up to 10 years in two patients. Many have been able to return to education and employment and have formed relationships and social groups. Patient testimonials (see Supplementary Information section) provide confirmation of the life-transforming result of successful therapy. Those considered non-responders or inadequate responders, as expected, did not enjoy the same level of improvement in health-related QoL, with some patients actually faring worse on increased doses or after switching to a different agent. These unfortunate patients require further though with regards to treatments and other diagnoses.

Both treatments appear to have favourable safety profiles with favourable risk:benefit ratios within the median five year follow-up period. Longer follow up is required to confirm these findings.

Chapter Six Part II:

Safety and Efficacy of Empirical Interleukin-1 Inhibition in AA Amyloidosis of Uncertain Aetiology

6.2.1 Background

AA amyloidosis is a serious complication of persistent inflammation, which, untreated will progress to renal failure and death (92). Effective suppression of the underlying inflammatory condition can halt organ damage or even lead to improved organ function (91), and this is therefore the focus of treatment. However, in approximately 20% of cases the underlying inflammatory disease is uncertain at the time of diagnosis of amyloidosis, and in about 7% remains uncharacterised after extensive investigation. This creates a dilemma as to the choice of empirical treatment.

The use of corticosteroids in these cases is problematic; the presence of AA amyloidosis points to a chronic, rather than an episodic, inflammatory disorder and chronic immunosuppression in this already vulnerable patient group is not only unlikely to address the underlying inflammation, but is likely to contribute significant morbidity due to side effects. Colchicine works well in in a minority of patients and may provide an effective and low-cost alternative to steroids. However, a high proportion of patients fail to benefit from a therapeutic trial and some experience intolerable gastrointestinal side effects thereby precluding effective long term treatment. The goal of treatment in AA amyloidosis remains to find a safe, non-toxic long term treatment which rapidly and effectively suppresses the underlying inflammatory disease.

Interleukin (IL)-1α and IL-1β are potent pro-inflammatory cytokines implicated in a variety of chronic inflammatory and autoinflammatory disorders. Both are ligands for the IL-1 receptor; the activity of both cytokines is inhibited by IL-1Ra, the naturally occurring IL-1 receptor antagonist. Anakinra (Kineret®) is a recombinant, non-glycosylated analogue of the interleukin IL-1 receptor antagonist. It is a licensed treatment for rheumatoid arthritis and Cryopyrin-associated periodic syndromes (185), but is widely used off-license in several other inflammatory diseases such as TRAPS, MKD, colchicine-

resistant familial Mediterranean fever (crFMF) and Schnitzler's syndrome with encouraging medium term safety and efficacy (52). Anakinra has a short half-life of 4 to 6 hours, meaning that dosing could be paused or stopped with a quick wash-out period should an adverse event arise.

6.2.2 Aims

We report the result of empirical treatment with anakinra in a cohort of 11 patients with AA amyloidosis of unknown aetiology who were all referred to a national specialist referral centre for diagnosis and management advice.

6.2.3 Patients and Methods

6.2.3.1 Patients

We identified 11 patients with AA amyloidosis of unknown aetiology who were under the care of the UK NAC and who were given a therapeutic trial of anakinra. Table 6.2.1 summarises the patient characteristics, but further clinical information is provided here.

- Patient 1 underwent screening tests after her brother was diagnosed with colonic cancer. Serum liver function tests revealed mild hepatic dysfunction and liver biopsy showed hepatic steatosis and mild chronic inflammation. Colonic biopsies were clear. One year later she developed ankle oedema. Investigation revealed renal dysfunction and renal biopsy was diagnostic of AA amyloidosis. The biopsy showed no evidence of vasculitis. Her ANA screen was positive at 1 in 1000 but repeated autoantibody screens showed no other evidence of autoimmune disease. Tuberculosis (TB) screen was negative. She had a polyclonal rise in immunoglobulin (Ig) M with only reactive changes on bone marrow biopsy. Upper gastrointestinal (GI) tract biopsy was normal. Coeliac screen was normal.

Echocardiogram was unremarkable, as was PET-CT scan of chest, abdomen and pelvis.

- Patient 2 had a history of Stage IAE Classical Hodgkin's Lymphoma of the tonsils with an associated para-vertebral mass. Due to the limited disease, he was treated with four cycles of ABVD chemotherapy to a complete remission. His remission was ongoing for three years when the diagnosis of AA amyloidosis was made on a renal biopsy after he was noted to have elevated creatinine on routine follow-up blood tests. The biopsy showed no evidence of vasculitis. At this time the patient was clinically very well, without any constitutional B symptoms or any other inflammatory symptoms. PET-CT scans repeated at three to six month intervals did not reveal recurrent disease. Echocardiography was normal. Serial auto antibody profiles remained negative. Sanger sequencing of known autoinflammatory genes revealed only the *MEFV* E148Q polymorphism of unknown significance.
- Patient 3 was diagnosed with human immunodeficiency virus (HIV) and started antiretroviral therapy (ART) two years prior to the diagnosis of AA amyloidosis. A year after the HIV diagnosis she was found to have a disseminated TB which was treated with two courses of anti-TB therapy. She developed systemic immune reconstitution inflammatory syndrome after starting ART, and responded well to steroids. Diagnosis of AA amyloidosis was made on renal biopsy after she was found to have developed nephrotic range proteinuria during routine follow-up. The renal biopsy showed no evidence of vasculitis. PET-CT scans have ruled out Castleman's tumour and other malignancy. The patient had a normal echocardiogram and autoantibody screen.
- Patient 4 had a five year history of seronegative polyarthritis, when he presents with renal dysfunction. Renal biopsy confirmed AA amyloidosis but nil else.

Echocardiogram was unremarkable. The patient showed a pattern of marked variability in his acute phase response raising the possibility of a periodic fever syndrome, although Sanger sequencing of known autoinflammatory genes was negative. An eventual tentative clinical diagnosis of urate arthropathy was made, although this has never been confirmed.

- Patient 5 gave a ten-year history of bowel symptoms but had been diagnosed with ulcerative colitis two years prior to the diagnosis of AA amyloidosis, and treated with steroids. Over a nine month period he developed oedema, and on investigation was found to have proteinuria and elevated serum creatinine. Renal biopsy confirmed AA amyloidosis. The biopsy showed no evidence of vasculitis. Repeat gut biopsies (colonic, duodenal and gastric) showed only mild reactive changes and barium meal follow-through was reported as normal. Echocardiogram was normal. The patient was obese, but gave no other medical history of note.
- Patient 6 was well until about four months prior to being diagnosed with AA amyloidosis when he developed intermittent bone pain in his knees and tibias, intermittent diffuse abdominal pain, a diffuse scaly skin rash and ankle oedema. Renal biopsy confirmed AA amyloidosis but nil else. He had a mild left lower lobe cystic bronchiectasis, little sputum production. There was no medical history of note apart from a left lower lobectomy for a possible cyst-forming infection in childhood, and no family history. OGD and sigmoid biopsy revealed only amyloid and an echocardiogram was normal. High resolution CT scan of his chest, abdomen and pelvis showed widespread lymphadenopathy but biopsy found only reactive changes. HIV, hepatitis and HHV6 serology were negative, as was a TB screen and blood cultures. Bone marrow biopsy was unremarkable.

- Patient 7 had a complex medical history including mastoidectomy at 18 months for chronic otitis media and craniopharyngioma at the age of six for which she underwent surgical excision and radiotherapy. She was also diagnosed with panhypopituitarism for which she received growth hormone. She had type I diabetes mellitus. She had chronic diarrhoea but multiple level GI biopsies ruled out Crohn's disease or any other specific abnormality. Echocardiogram was unremarkable. The original renal biopsy showed no evidence of vasculitis.
- Patient 8 had only a five year history of an inflammatory lung mass in the right middle lobe. CT-guided biopsy and bronchoscopy demonstrated non-specific inflammation and rigid bronchoscopy yielded no specific diagnosis. A PET scan showed only low grade uptake suggesting a benign process rather than a malignant one. Active TB was ruled out. Echocardiogram was normal. The biopsy showed no evidence of vasculitis. After 2 years treatment with anakinra the lung mass had entirely resolved on imaging, but the inflammation persists.
- Patient 9 was fit and well until he presented with ankle oedema in acute renal failure. He had no medical history of note apart from mild asthma and an IgA monoclonal gammopathy of undetermined significance. Chest x-ray and bronchoscopy were normal as were CT chest and abdomen. PET-CT and bone marrow biopsy showed no significant abnormalities. The original renal biopsy showed no evidence of vasculitis.
- Patient 10 was diagnosed with low grade Non-Hodgkin's lymphoma (NHL) twelve years prior to being diagnosed with AA amyloidosis. As he was asymptomatic he had no therapy and follow-up CT scans showed that the nodes were decreasing in size over time. He presented with oedema and nephrotic syndrome and renal biopsy confirmed AA amyloidosis with no evidence of vasculitis. He then underwent extensive investigation to restage the NHL. CT

scan showed some small lymph nodes in the para-aortic region but no other detectable lymphadenopathy. Abdominal ultrasound scan was normal, as was a bone marrow biopsy. Four years later repeat CT scan of chest, abdomen and pelvis and bone marrow biopsy with trephine were all again negative. Cardiac MRI showed hypertensive cardiomyopathy. The patient died of peritoneal dialysis-related peritonitis and post-mortem examination did not reveal the source of the underlying inflammation.

- Patient 11 gave a seven year history of pedal oedema and generalised myalgia before presenting with nephrotic-range proteinuria. CT scan showed a 4 cm density, which when biopsied was consistent with Ig heavy chain deposition. He had a reactive Mantoux test after BCG vaccine but this was reviewed by the chest physicians and repeated chest X-rays ruled out TB infection. Blood-borne virus screens were negative. Repeat CT scan after four years showed reduction of the size of the mass. Sanger sequencing of known autoinflammatory disease genes revealed variants on unknown significance S52N and D386N in *MVK*. The original renal biopsy showed no evidence of vasculitis.

All patients had been investigated locally in order to characterise the underlying inflammatory condition, without success. Sanger genetic sequencing was performed to exclude known monogenic autoinflammatory diseases. Disease activity and treatment response were monitored by serial SAA and CP measurements. Amyloid load was evaluated by whole body ^{123}I-SAP scintigraphy. Renal function was measured by means of serum and urine chemistry. Patients gave written informed consent for retrospective analysis and publication of their clinical data (REC reference number 06/Q0501/42).

6.2.3.2 Other methods

Further methods are described in Chapter Two: Methods.

6.2.4 Results

6.2.4.1 Whole cohort characteristics

Table 6.2.1 summarises the patient characteristics. Eight (73%) were male. Age ranged from 33 to 67 years. Eight (73%) had been pre-treated with corticosteroids or colchicine or both, unsuccessfully. All patients were proteinuric at presentation with AA amyloidosis and three had already progressed to end-stage renal failure (ESRF). The total median follow-up time for this cohort was 7.7 (3.5 – 8.2) years; the median duration of treatment with anakinra was 1.6 (0.6 – 7.4) years. Four patients are deceased (patients 7, 9, 10 and 11), with cause of death unrelated to anakinra use; one patient was lost to follow-up (patient 6) and the rest are alive and under active follow-up.

6.2.4.2 Response to anakinra

Patients 1 to 9 were considered to be either complete responders or partial responders to anakinra, whilst patients 10 and 11 were considered non-responders (Table 6.2.1). Response to treatment was characterised as described in Chapter Two: Methods. All patients tolerated treatment well, with the only adverse events being the transient injection site reactions known to be associated with anakinra (185).

6.2.4.3 Serial monitoring of SAA

SAA was measured serially before and during treatment with anakinra. The median pre-treatment SAA (defined as the median of all available SAA measurements prior to commencing anakinra) for the whole cohort was 116 (39 - 238) mg/L. SAA was measured at a median of 17 (4 – 28) days after commencing anakinra. For the whole cohort, including patients 10 and 11 who were considered non-responders, the median on-

anakinra SAA (defined as the median of all available SAA measurements whilst on anakinra) was 9 (4 – 62) mg/L. In the patients considered responders to anakinra (c or partial), the median pre-treatment SAA was 74 (34 – 190) mg/L, and the median on-treatment SAA was 6 (4 – 16) mg/L. Both comparisons were statistically significant (p = 0.0047). In responders the effect has been maintained for a median on-treatment follow-up period of 1.8 (1 – 7.6) years. Individual median pre- and on-treatment responses are shown in Figure 6.2.1.

6.2.4.4 Renal function

Patients 7, 8 and 9 were in ESRF at first presentation at our Centre. At the time of commencing treatment with anakinra, patients 4, 5, 8, 9 and 11 were in ESRF; patients 1, 3, 6 and 10 had acceptable renal excretory function but were severely nephrotic, all having > 10g urine protein in 24 hours; and patients 2 and 7 were in CKD stage 3, post renal transplant. Over the follow-up period, patients 4, 5 and 8 also underwent renal transplantation. Amyloid recurred in the graft of only one patient (patient 7). Patients 2, 4 and 8 remain stable and well on 100mg daily anakinra post-transplant. Patient 5 stopped anakinra treatment prior to transplant and has not recommenced as his acute phase response is reasonably controlled on post-transplant immunosuppressive drugs. Patient 7 continued anakinra therapy until her sudden cardiac death (in the absence of cardiac amyloidosis). Patients on renal replacement therapy were treated with 100 mg anakinra three times weekly, after dialysis. Patient 3 had a serum creatinine within the normal range before commencing anakinra, but she had a heavy protein leak of 10.5 g in 24 hours. Six months after starting anakinra, the protein leak improved to 7.2 g and one year later had fallen to 1.9 g, with stable CKD.

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Patient	Sex	Ethnicity	Age at presen-tation (y)	ESRF at presen-tation	Renal trans-plant	Previous unsuccessful therapies	Serum creatinine on starting anakinra (mmol/L)	Proteinuria on starting anakinra (g/24 hr)	Response to anakinra	Duration of therapy (y)	Continuing therapy (Yes/No, and reason if not continuing)
1	F	N.European	61	No	No	Cort, MMF, Colch	82	16.9	Partial	7.4	Yes
2	M	S. Asian	36	No	Yes*	Colch	194	0.1	Complete	0.9	Yes
3	F	N. African	36	No	No	Colch, Cort	56	10.5	Complete	1.8	Yes
4	M	N. European	35	No	Yes	Cyclo, Chlor, Etan	ESRF	ESRF	Complete	9	Yes
5	M	N. European	48	No	Yes	Colch	ESFR	ESRF	Partial	3.2	No, stopped for renal transplant
6	M	Turkish	33	No	No	Colch, Cort	248	No result	Partial	5.6	No, stopped when ESRF reached
7	F	N. European	36	Yes	Yes*	Cort	163	2.0	Complete	4.4	No, deceased
8	M	N. European	63	Yes	Yes	None	ESRF	ESRF	Complete	7.9	Yes
9	M	N. European	67	Yes	No	Colch	ESRF	ESRF	Partial	0.6	No, deceased
10	M	S. European	60	No	No	Cort	210	10.5	None	6.9	No, no response
11	M	S. Asian	41	No	No	Cort	ESRF	ESRF	None	6.3	No, no response

Table 6.2.1. Summary of characteristics of the cohort. Cort = corticosteroids; MMF = mycophenolate mofetil; Colch = colchicine; Chlor = chlorambucil; Etan = etanercept. ESRF = end stage renal failure. * Indicates renal transplantation prior to starting anakinra.

6.2.4.5 Monitoring of amyloid deposits

Eight of the nine responders had serial SAP scans over the follow-up period, with five (patients 3, 4, 5, 7 and 8) showing regression and three (patients 1, 2 and 6) showing stabilisation of amyloid load, i.e. no new accumulation. Figure 6.2.2 shows the serial SAP scans of patients 4 and 8, demonstrating amyloid regression from the spleen and liver and spleen, respectively after successful treatment with anakinra.

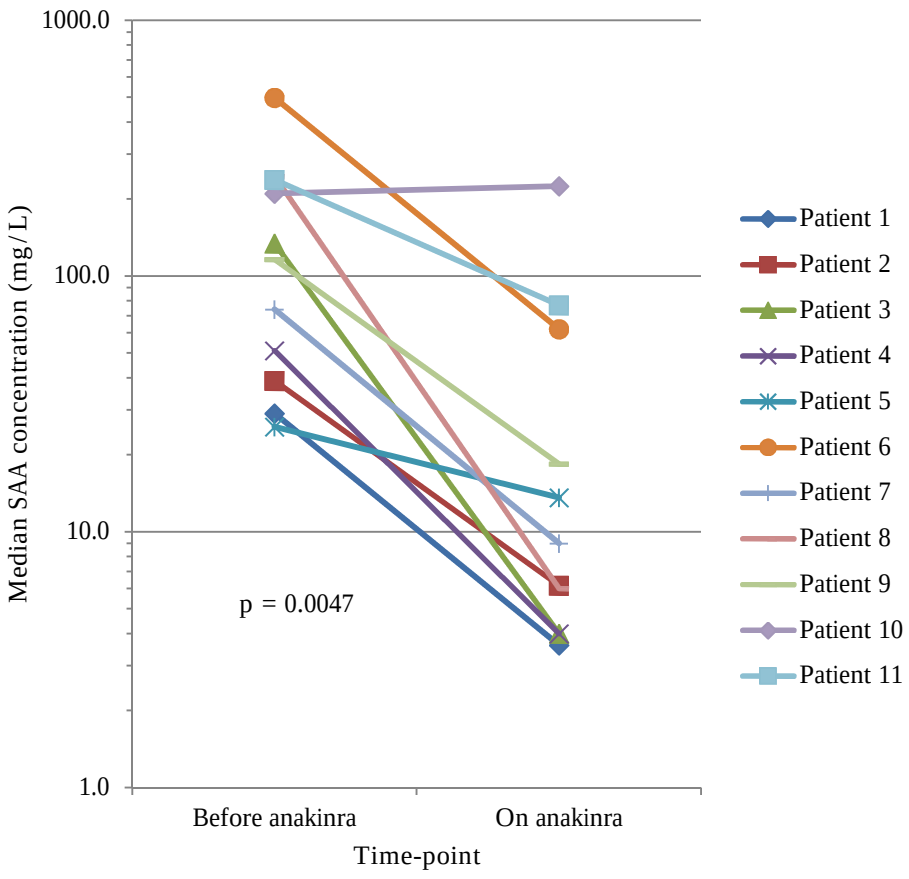

Figure 6.2.1. Serial SAA concentration in 11 patients with AA amyloidosis of uncertain cause. Results are presented as median of all pre-treatment and on-treatment SAA measurements.

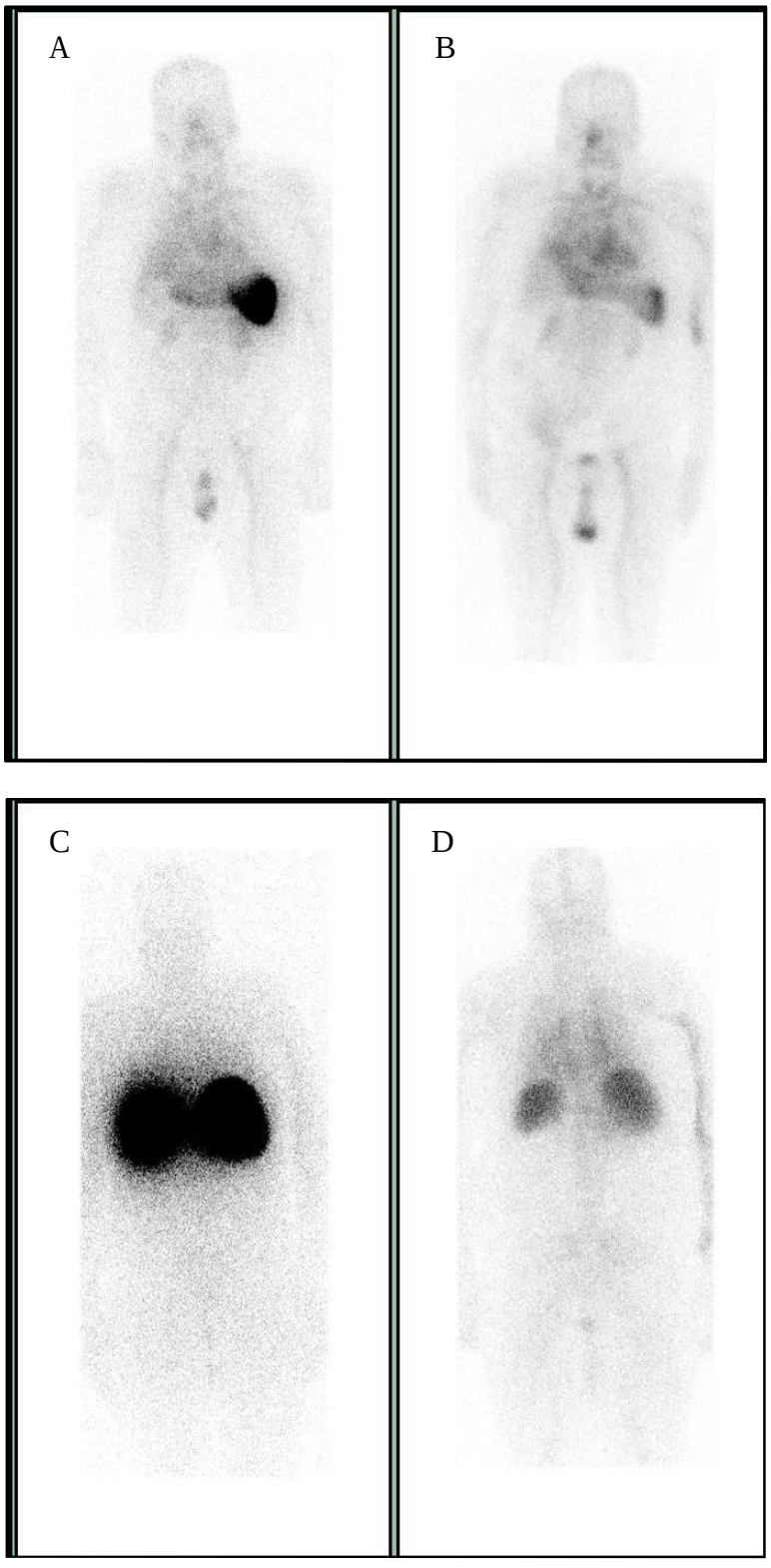

Figure 6.2.2. Serial SAP scans showing regression of amyloid after treatment. Panels A and B are anterior images of patient 4 taken in 2007 and 2013 respectively, showing regression of amyloid from the spleen after successful treatment. Panels C and D are posterior images of patient 8 taken in 2007 and 2012 respectively, showing regression of amyloid from the liver and spleen after successful therapy.

6.2.5 Discussion

AA amyloidosis is a potentially reversible cause of proteinuric renal disease. Historically treatment has been more successful than in other types of systemic amyloidosis but has relied completely on diagnosis of the underlying condition and targeted anti-inflammatory treatments. The advances in antimicrobials, and effective biologics in the inflammatory arthritides, have both reduced the incidence of AA amyloidosis and when it does develop, have improved outcomes in what were until recently the major aetiological sources of AA amyloidosis. However, choice of treatment to suppress inflammation in the absence of a clear underlying disease is difficult, and patients with AA amyloidosis of unknown aetiology remain at considerable risk of succumbing to ESRF or losing their renal grafts if they had already received transplants.

Furthermore, these patients are often at risk of under-treatment as symptoms of systemic inflammation are often non-specific and may be minimised by both patients and their medical carers (NAC, unpublished data). It is well recognised that symptoms particularly those of a non-specific nature such fatigue and anhedonia, and insidious onset, are often minimised by patients. An empirical trial of cytokine blockade can be highly effective in these patients and give clues as to the underlying pathological pathways but outside experienced centres its use is very limited due to financial constraints and concerns about possible side effects of therapies to which relatively few centres have been exposed, and for which they are not licenced. These concerns are even more potent in the context of renal failure, the commonest manifestation of AA amyloidosis.

Nonetheless we have demonstrated here that a therapeutic trial of anakinra is worth trying given its potentially dramatic effect, rapid onset, and excellent safety profile even in the context of organ failure and transplantation. In addition it is more cost-effective than the newer immunomodulators. Current data suggest it has a better long term safety profile

than high dose corticosteroids, other anti-cytokine therapies or immunosuppressive drugs (175, 176). In this series one patient with HIV and previously treated TB tolerated the treatment well. The summary of product characteristics (SPC) for anakinra states that it must not be used in patients with severe renal impairment (defined as creatinine clearance < 30 ml/minute) (185). Nonetheless in six patients with ESRF we have shown that anakinra is well tolerated, and that dosing three times per week post-dialysis provides clinically effective treatment which has not been accompanied by increased serious sepsis, and provides a cost saving. There have been concerns about the additional risks of adding anakinra to the immunosuppression associated with solid organ transplantation but the data shown here on five patients (median age 36, median follow up 4.4 years) suggests a favourable risk benefit profile in these rare cases.

This small cohort shows that even in potentially high risk cases with organ damage secondary to AA amyloidosis, immune deficiency due to previous immunosuppression and/or nephrotic syndrome, anakinra, when used appropriately and carefully monitored, has proved remarkably effective and well tolerated. Although longer term follow-up of this off-label use is clearly required our data suggests that carefully monitored empirical trails of single cytokine suppression can have transformative effects in selected cases.

Chapter Seven:

Changing Epidemiology of AA Amyloidosis – Clinical Observations Over 25 Years at a Single National Referral Centre

7.1 Background

Systemic AA amyloidosis is a serious and life threatening complication of chronic inflammatory disorders. Despite the significant morbidity and mortality caused by AA amyloidosis, and the consequent cost burden to healthcare systems, there are relatively few published data on the incidence of AA amyloidosis.

Finnish studies report mortality from AA amyloidosis complicating rheumatoid arthritis (RA) ranging from 9 to 24% (186) (187). A Spanish 9-year observational study of RA attributed a mortality rate of 17% to AA amyloidosis (188). In other European studies, mortality from AA amyloidosis has been reported as much lower in comparison: 2% to 7% (189, 190). And post-mortem examinations of individuals with RA reveal detection rates ranging from 7% to 61% (191-194). These studies demonstrate varying incidence.

A Turkish study reported a change in incidence of AA amyloid detected on renal biopsy over time. Akse-Onal and colleagues (195) reported incidence of 12.1% in renal biopsies in Turkey between 1978 to 1990 and only 2% between 2000 to 2009 with no difference in age and gender distribution, age at onset, disease severity etc. between the two populations.

In the UK, AA amyloidosis is the third commonest type of systemic amyloidosis diagnosed, and represents approximately 10% of new cases seen annually at the UK NAC.

7.2 Aims

We sought to examine the changing epidemiology of AA amyloidosis over a period of a quarter of a century at a single national referral centre.

7.3 Patients and methods

7.3.1 Patients

All patients were under the care of a single national referral centre in the UK. We performed a database search for all patients first seen between January 1990 and December 2014. 625 patients were identified with confirmed AA amyloidosis. Patients were divided into 3 cohorts for analysis:

- Cohort 1: 153 patients who were first seen between 1990 and 1997
- Cohort 2: 236 patients who were first seen between 1998 and 2006
- Cohort 3: 236 patients who were first seen between 2007 and 2014.

Patients gave written informed consent for retrospective analysis and publication of their clinical data (REC reference number 06/Q0501/42).

7.3.2 Diagnosis

Diagnosis of AA amyloidosis was made either histologically, or scintigraphically where biopsy was not possible or tissue samples were not available for review. Exclusion of other amyloid types was by genetic analysis (hereditary forms) or serum and urine protein electrophoresis (AL amyloidosis). Patients were reviewed six to 12 monthly after diagnosis.

7.3.3 Other methods

Further methods are described in Chapter Two: Methods.

7.4 Results

7.4.1 Whole-cohort characteristics

625 patients are described. 341 (55%) were male. The median age at first presentation at our Centre was 54 years (IQR 39 - 65). The underlying diseases were as follows: rheumatoid arthritis (RA) 28%, chronic sepsis 18%, seronegative arthritis 10%, systemic autoinflammatory diseases (SAIDs) 9%, juvenile idiopathic arthritis (JIA) 8%, inflammatory bowel disease (IBD) 5% and other causes 6%. In 15% of patients the underlying inflammatory disease was uncharacterized at diagnosis of AA amyloidosis. 300 patients reached end stage renal failure (ESRF), with 159 (53%) patients in ESRF at presentation. Median time to ESRF was 26 months. The numbers of new cases referred annually over the study period are shown in Figure 7.1. Referral rates of new AA patients have been stable during the past decade at an average of 30 per year, in contrast with a three-fold increase among other types of amyloidosis (NAC, unpublished data).

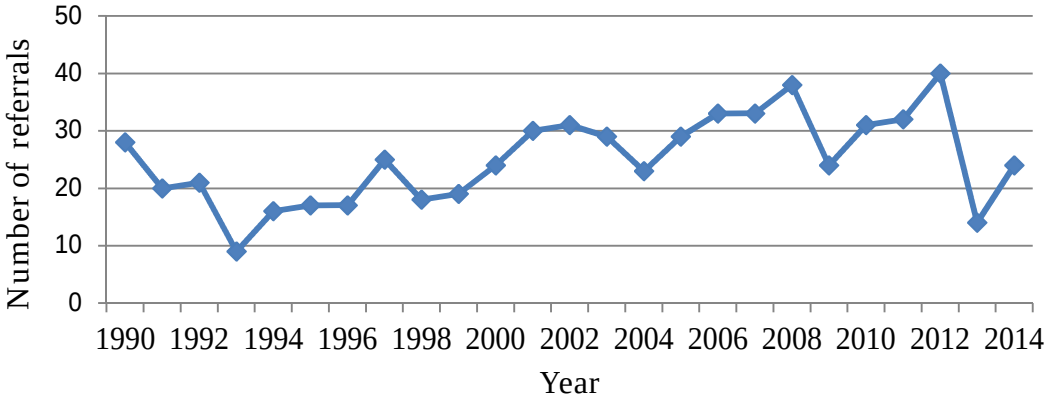

Figure 7.1. Annual new referral numbers over the 25 year study period.

7.4.2 Comparison of cohorts

7.4.2.1 Age at referral, ethnicity and underlying disease

Mean age at presentation has significantly increased from 46 in the earliest cohort (cohort 1) to 56 in the latest cohort (cohort 3) ($p < 0.0001$) (Figure 7.2). Ethnic group representation remains largely unchanged, although referral of Asian patients has increased from 4% in cohort 1 to 17% in cohort 3 ($p = 0.0006$). The overwhelming majority of patients were Caucasian (Figure 7.3). A comparison of the underlying diseases between cohorts 1 and 3 reveals a reduction in patients with juvenile idiopathic arthritis (JIA) from 25% to 2% ($p < 0.0001$). Increased numbers of patients have presented with chronic sepsis due to intravenous recreational drug use (IVDU) – 1% in cohort 1 versus 13% in cohort 3 ($p < 0.0001$). There has also been a rise in AA amyloidosis of unknown aetiology from 5% to 24% ($p <0.0001$).The full comparison of underlying diseases between cohorts is shown graphically in Figure 7.4.

7.4.2.2 Development of end stage renal failure

In comparison with cohort 1, significantly more patients were in established ESRF at presentation in cohort 3 – 15% and 29% respectively ($p = 0.0028$). There was no difference in survival from ESRF between the cohorts.

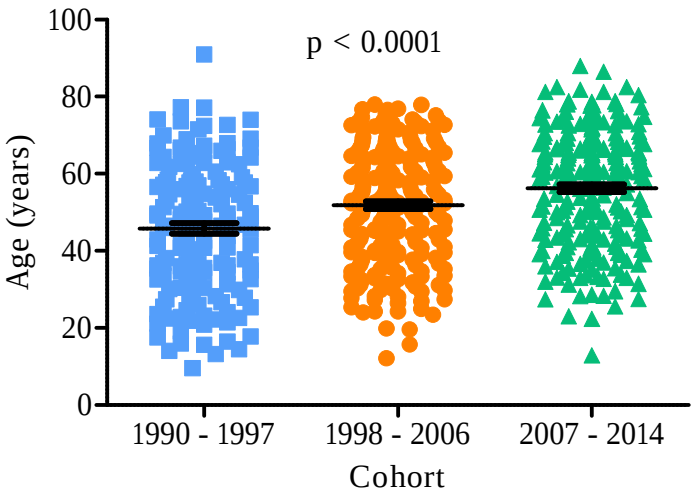

Figure 7.2. Change in mean age at presentation

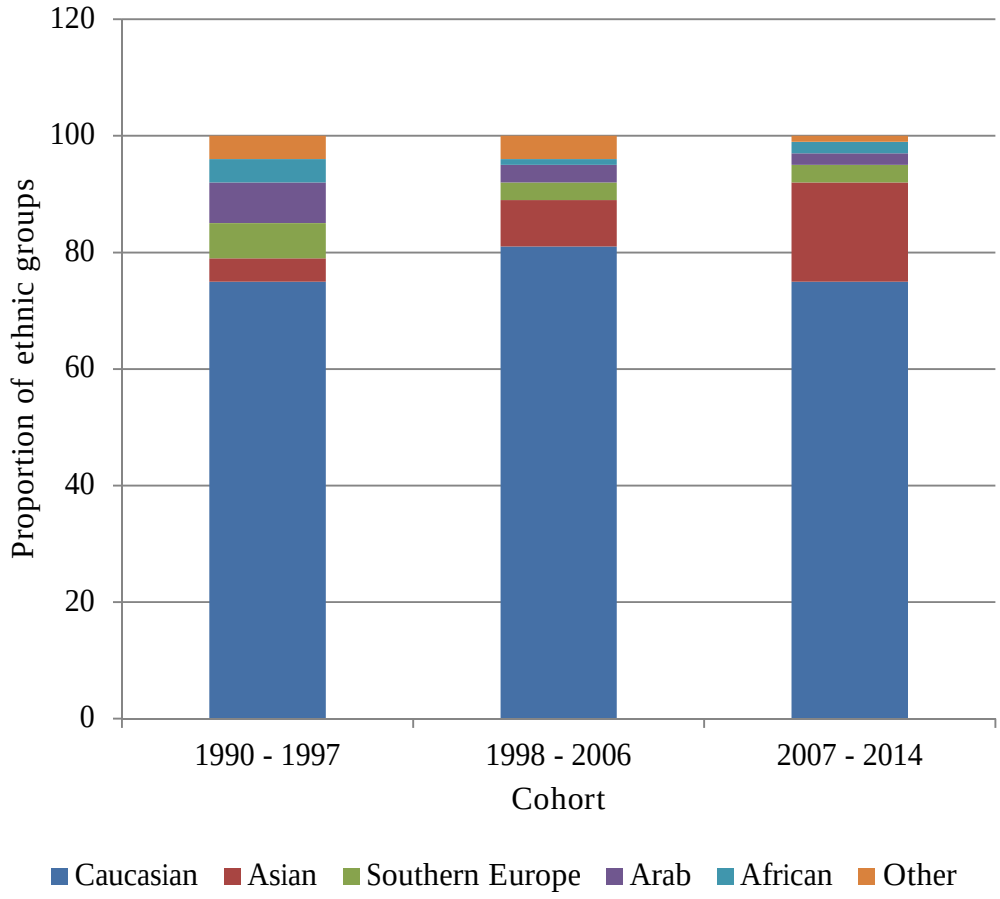

Figure 7.3. Ethnic group proportions by cohort.

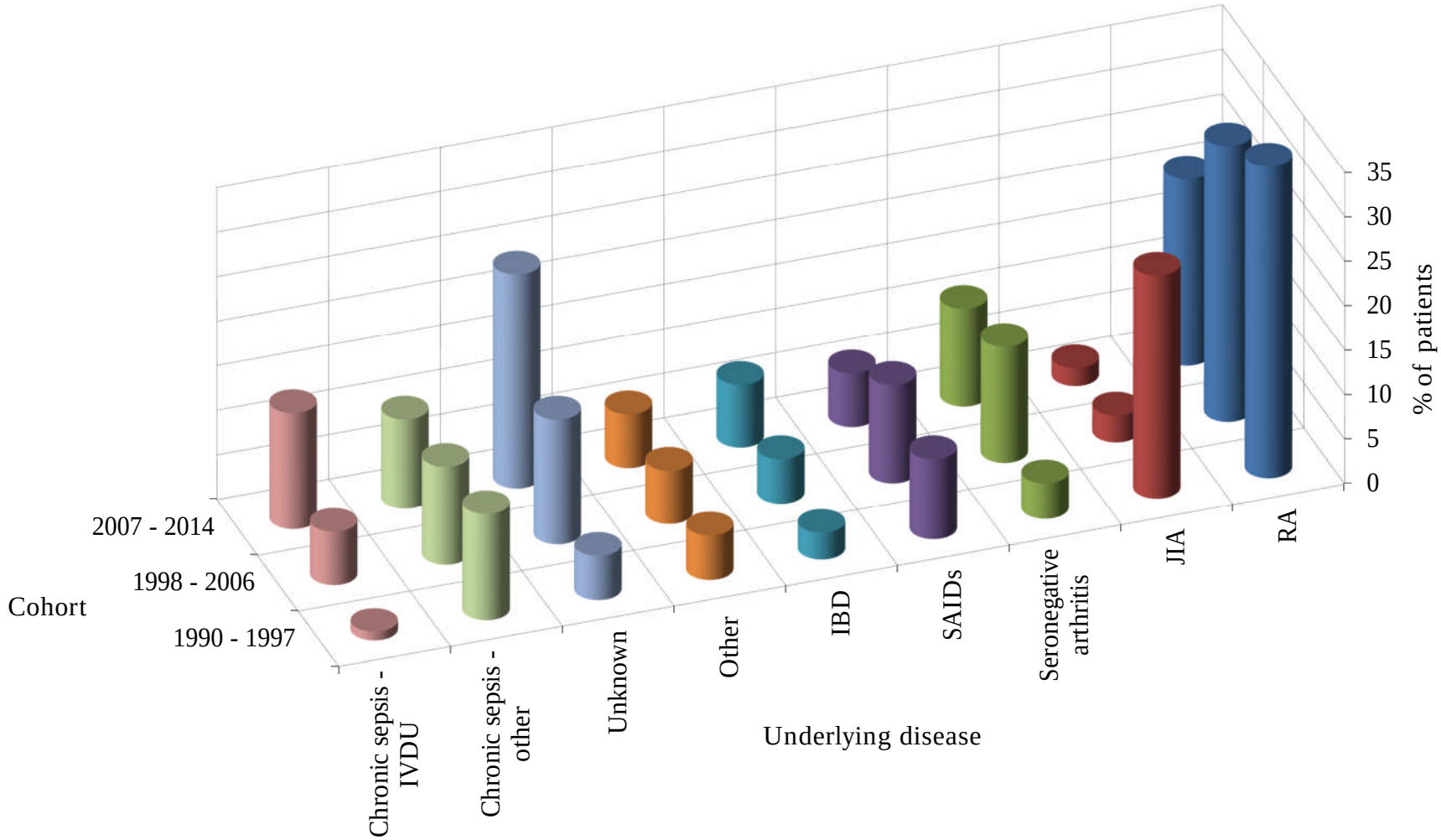

Figure 7.4. Comparison of underlying disease by cohort. RA, rheumatoid arthritis; JIA juvenile idiopathic arthritis; SAIDs, systemic autoinflammatory disease; IBD, inflammatory bowel disease; IVDU, intravenous drug use.

7.4.2.3 Survival

There was no difference in overall survival between the cohorts (Figure 7.5). However, age at death was significantly different at a median of 54 years in cohort 1 and 62 years in cohort 3 (p = 0.0012) (Figure 7.6).

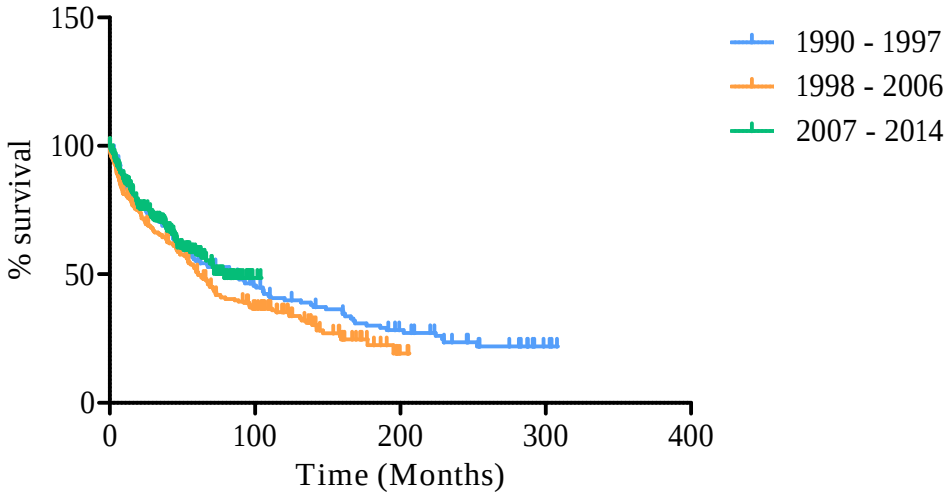

Figure 7.5. Survival by cohort.

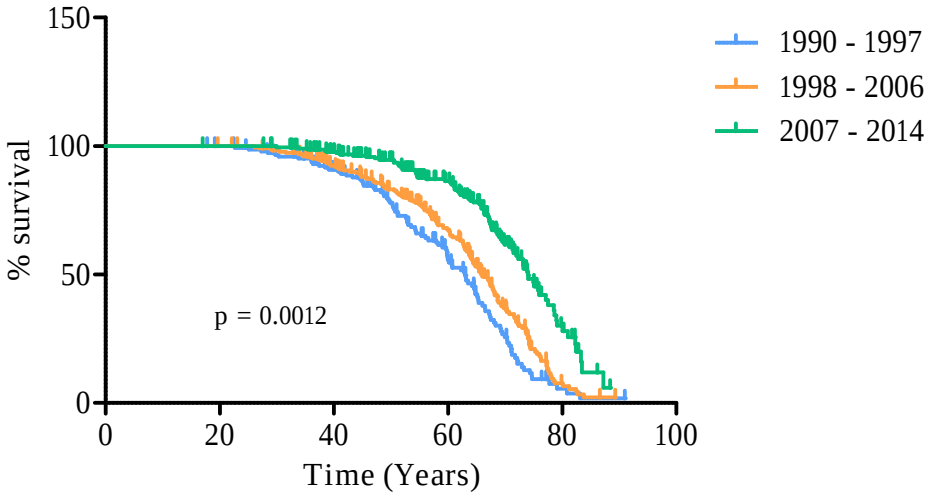

Figure 7.6. Age at death.

7.5 Discussion

In contrast to a threefold increase in referrals to the NAC of AL and other types of amyloidosis during the past decade, referral rates for AA amyloidosis have remained stable. This observation that AA amyloidosis is becoming less common may be a reflection of great advances in the use of biological agents in the effective treatment of the inflammatory arthritides. This has both reduced the incidence of AA amyloidosis and when it does develop, has improved outcomes in what were until recently the commonest pathologies underlying AA amyloidosis. Whilst JIA and RA are no longer the main aetiological sources of AA amyloidosis, greater proportions of recent AA patients have uncharacterised underlying inflammatory disorders or chronic sepsis secondary to IVDU, compared to historical cohorts. Both patient groups pose challenges for clinical management, and it may be that these numbers represent an increase in referrals for management advice due to expertise and experience with off-licence biological treatments at the National Amyloidosis Centre, rather than a true increase in incidence of these diseases. Similarly, the significantly increased proportion of patients in ESRF in the latest cohort compared with the earliest is likely to be due to a referral bias in recent years.

Although ethnic group proportions have largely remained the same, with a preponderance of Caucasian patients, significantly more Asian patients are represented in the recent cohort compared to the oldest. This change coincides with an increase in AA amyloidosis of unknown aetiology and suggests that novel as yet unrecognized pathology is contributing to chronic inflammation in this group. Clearly there may be other potential contributors such as an effect of increased migration over the study period, or perhaps greater use of the health service by a younger generation.

The mean age at presentation with AA amyloidosis in the recent cohort is significantly older than the earliest cohort. This again may be a reflection of the availability of effective

biological treatments for inflammatory conditions. Whilst overall survival does not appear to have changed despite new effective therapies, age at death has increased significantly over the last 25 years, suggesting improved global management and supportive care including renal replacement, as well as more effective management of the underlying pathology.

Overall, these data suggest both falling incidence (corrected for improved diagnostic rates) and better outcome in AA amyloidosis over a period of two-and-a-half decades, reflecting advances in therapeutics and in overall management of complex chronic disease in an aging population. A major problem to have evolved over this time is AA amyloidosis of uncertain aetiology which has proved challenging to manage empirically. We hope that newer techniques such as next generation sequencing and gene expression analysis may aid diagnosis and effective treatment of these 'uncharacterised' inflammatory disorders thereby improving overall survival.

Chapter Eight:

The Classical Acute Phase Protein - The Non-Pathogenic Role of Serum C-Reactive Protein

8.1 Background

A great deal of interest in measurement of CRP in relation to cardiovascular disease occurred following the original observations in patients with known cardiovascular disease (94, 95) and subsequent findings in the general population, that baseline values of CRP are statistically significantly associated with risk of future coronary events. Controversy arose in the field with CRP as a risk marker being promulgated by some groups on the basis of some epidemiological studies; further controversy was contributed by the conflation of epidemiological association with causality, leading to claims that increased baseline CRP concentration is not only a risk marker for cardiovascular disease but also a risk factor contributing to the pathogenesis of atherosclerosis and atherothrombosis.

In 2005 a study was published describing the infusion of purified bacterial recombinant CRP into human volunteers, in all of whom there followed a marked acute phase plasma protein response (196). The authors concluded that the latter represented a pro-inflammatory property of CRP itself, and this has promulgated the notion that CRP contributes to the pathogenesis of cardiovascular disease.

These data suggest two rather serious potential complications for patients with chronic inflammatory conditions. First, that an elevated serum CRP concentration associated with chronic inflammatory conditions puts these patients at risk of developing atherosclerosis and atherothrombosis. Second, that CRP is itself pro-inflammatory and if this is the case then patients with inflammatory conditions would be in a persistent and dangerous state of inflammation despite any therapy.

8.2 Aims

In order to investigate these assertions we administered authentic highly purified current Good Manufacturing Guidelines (cGMP) grade human CRP to seven healthy male volunteers, in sufficient quantity to modestly raise the circulating CRP concentration in recipients, and monitored the effects.

8.3 Subjects and Methods

8.3.1 Subjects

Seven healthy male volunteers were recruited by email advertisement within the University College London student body. The following inclusion criteria applied: Healthy males aged 18-25 years; Non-smoking; Normal body mass index (18.5-24.9); No clinical evidence of active infection or allergy; No significant physical injuries or surgery within preceding 3 months. Volunteers were pre-screened over the telephone and then invited to a formal discussion, and screening, after written informed consent was given. Volunteers were compensated £15 per hour for their time and effort, plus a completion bonus of £50 if they attended all study visits to completion.

8.3.2 Study investigations and procedures

Seven study visits were conducted over a three week period (Table 8.1). Screening involved a full medical history review, vital signs (oral temperature and resting blood pressure and heart rate), physical examination, electrocardiogram (ECG), urinalysis and blood tests including glucose, renal function, liver function, haematology, cardiac biomarkers, acute phase proteins SAA and CRP, erythrocyte sedimentation rate (ESR) and cytokine profiles. The baseline visit took place within one week of the screening visit, subject to a positive outcome of the screening assessments, including a healthy basal serum CRP concentration of $\leq$3 mg/L.

Visit	1	2								3	4	5	6	7
Timepoint	Screening (Day -7 to day -1)	Baseline (Day 0)	0 hours	+ 15 min	+30 min	+1 hour	+2 hours	+4 hours	+8 hours	+24 hours (Day 1)	+48 hours (Day 2)	+72 hours (Day 3)	Day 5 to 7	Day 10 to 12
Informed Consent	x													
Medical history	x													
Concomitant meds	x	x								x	x	x		
Adverse events		x	x	x	x	x	x	x	x	x	x	x	x	x
Physical examination	x											x		
Vital signs	x	x	x	x	x	x	x	x	x	x	x	x	x	x
ECG	x			x						x	x	x		
Urinalysis	x													
Blood samples	x	x			x			x	x	x	x	x	x	x
CRP administration			x											

Table 8.1. Schedule of procedures and assessments.

The CRP dose was administered at the baseline visit, after the pre-dose assessments (vital signs and blood sampling) had been completed.

8.3.3 Regulatory and Ethical Aspects

The study was approved by the Central London REC 1 Research Ethics Committee (REC reference number 10/H0718/86)). The UK Medicines and Healthcare Products Regulatory Agency (MHRA) confirmed that this mechanistic study protocol fell outside the scope of the Medicines for Human Use (Clinical Trials) Regulations, 2004, the prevailing UK law covering such studies. The study was Sponsored by University College London, and approved by the host institution, the Royal Free Hospital. All participants gave fully informed written consent.

8.3.4 Other methods

Further methods are described in Chapter Two: Methods.

8.4 Results

Following the bolus infusion of CRP, there was a clear dose dependent increase in circulating CRP concentration, peaking in the 30-minute sample in all subjects and reaching 36 and 44 mg/L respectively in the two individuals who received the top dose of 2 mg/kg. After 30 minutes the circulating CRP concentrations fell steadily in all subjects, at a rate consistent with the known approximately 19-hour plasma half-life of human CRP in humans (Figure 8.1)(121). There was no evidence that infusion of human CRP triggered any new production of CRP. Similarly, none of the seven subjects showed any increase beyond the reference range in their circulating concentrations of SAA, or any significant change in neutrophil or platelet counts (Figure 8.2).

The plasma concentrations of the pro-inflammatory cytokines, IL-1β, TNFα, and IL-6, also confirmed that the infusion of CRP had no significant effects (Table 8.2). Only in subject 1, who received the lowest dose of CRP was there any significant apparent rise in just a single cytokine, TNFα, and at just a single time point. However the raw optical density data from the four replicates of the ELISA assay on this sample were widely variant and included one effectively zero value consistent with the zero values in the three other samples from this individual, and suggests that the other readings reflect a technical aberration. Crucially the circulating SAA values were within the reference range in all samples from all subjects. There was thus no evidence that CRP infusion affected production of any of the cytokines assayed immunochemically or any sign of increased functional pro-inflammatory cytokine activity in vivo. Values for the anti-inflammatory cytokine, IL-10, were variable but there was no evidence of any consistent or dose-dependent effect of CRP infusion.

There were no clinically significant changes in any other physical, biochemical or haematological parameters monitored in the study (Table 8.3), and the complete set of results for each individual subject may be found in the Supplementary Information section.

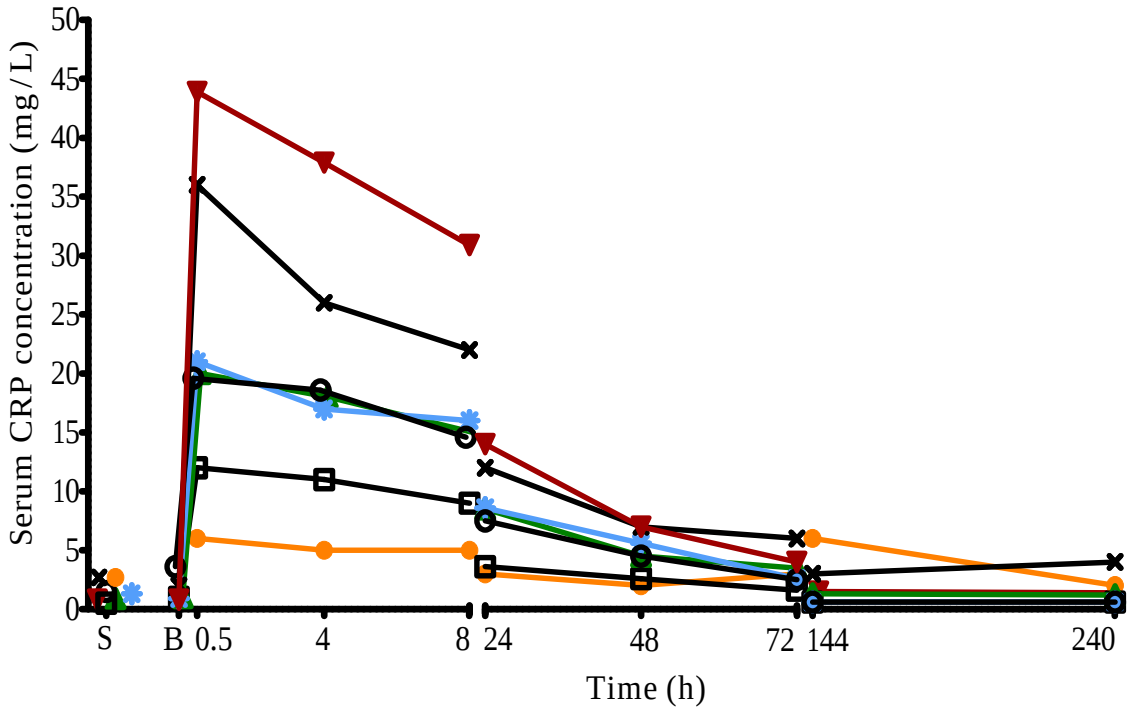

Figure 8.1. Serum concentration of CRP before and after infusion. Values are shown for each individual subject at screening (S), at baseline (B) immediately before infusion of human CRP, and at the times shown after infusion. Subject 1, CRP dose 0.25 mg/kg; Subject 2, 0.5 mg/kg; Subject 3, 1.0 mg/kg; Subject 4; 1.25 mg/kg; Subject 5; 1.25 mg/kg; Subject 6, 2.0 mg/kg; Subject 7, 2.0 mg/kg.

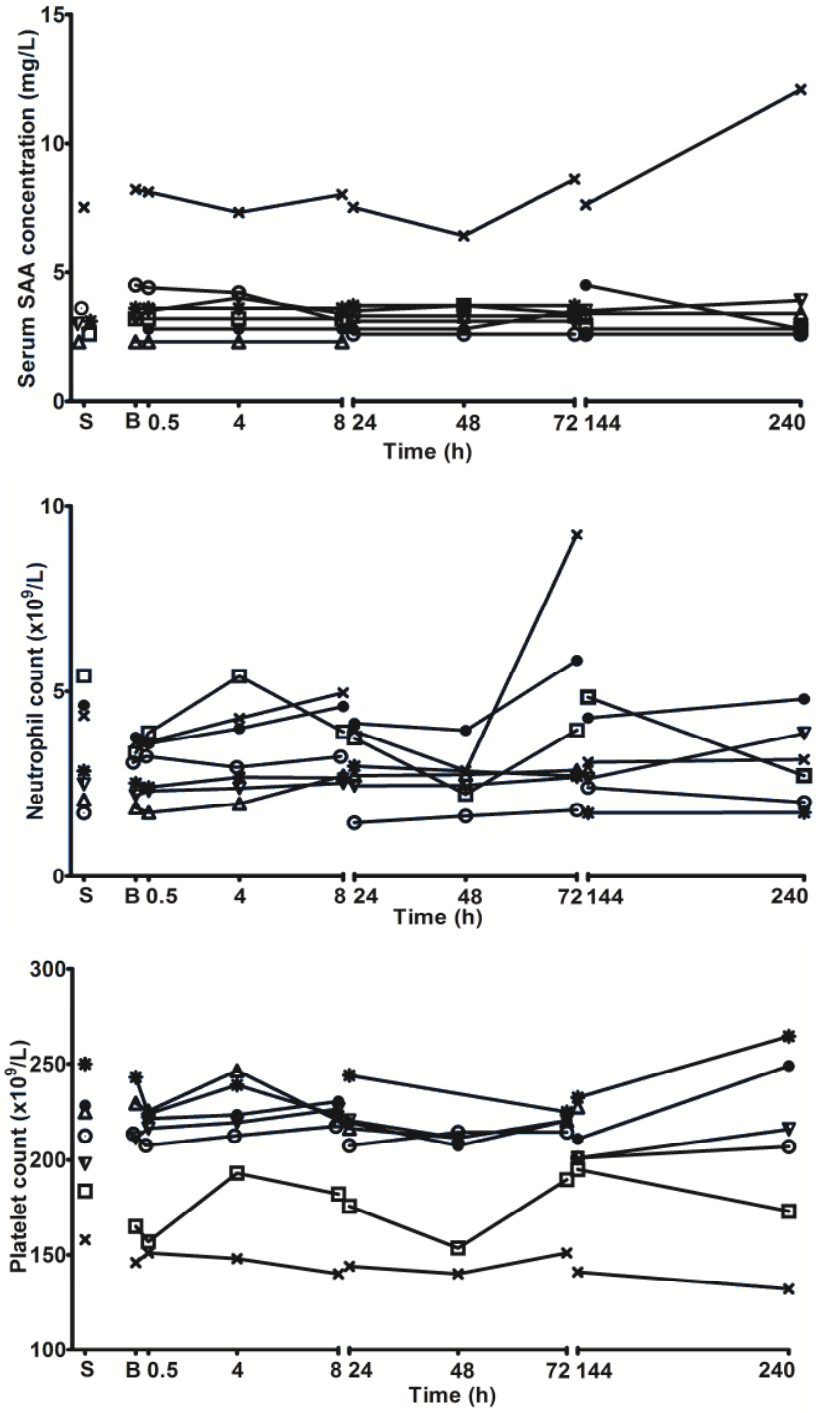

Figure 8.2. Circulating SAA, neutrophils and platelets before and after CRP infusion. Values are shown for each individual subject at screening (S), at baseline (B) immediately before infusion of human CRP, and at the times shown after infusion. ●— Subject 1, CRP dose 0.25 mg/kg; ■— Subject 2, 0.5 mg/kg; △— Subject 3, 1.0 mg/kg; ✱— Subject 4; 1.25 mg/kg; ○— Subject 5; 1.25 mg/kg; ✱— Subject 6, 2.0 mg/kg; ▽— Subject 7, 2.0 mg/kg.

Subject (CRP dose)	Time after CRP infusion	Plasma cytokine concentration mean pg/mL			
		IL-1β	IL-6	IL-10	**TNFα**
1 (0.25 mg/kg)	Baseline	<16	<16	<16	<16
	4 h	<16	<16	18	145
	8 h	<16	<16	<16	<16
	24 h	<16	<16	17	<16
2 (0.50 mg/kg)	Baseline	<16	<16	41	28
	4 h	<16	<16	<16	17
	8 h	<16	<16	<16	19
	24 h	<16	<16	<16	<16
3 (1.00 mg/kg)	Baseline	<16	<16	<16	<16
	4 h	<16	<16	19	<16
	8 h	<16	<16	44	<16
	24 h	<16	<16	<16	<16
4 (1.25 mg/kg)	Baseline	<16	<16	<16	<16
	4 h	<16	<16	<16	<16
	8 h	<16	<16	<16	<16
	24 h	<16	<16	<16	18
5 (1.25 mg/kg)	Baseline	<16	<16	22	21
	4 h	<16	16	<16	26
	8 h	20	<16	<16	34
	24 h	<16	<16	88	28
6 (2.00 mg/kg)	Baseline	<16	60	<16	23
	4 h	<16	67	<16	<16
	8 h	<16	69	<16	<16
	24 h	<16	59	<16	<16
7 (2.00 mg/kg)	Baseline	<16	<16	<16	46
	4 h	<16	<16	<16	<16
	8 h	<16	<16	<16	<16
	24 h	<16	<16	18	<16

Table 8.2. Plasma cytokines monitored during and after infusion of human CRP.

Parameter	Clinically significant change
C-reactive protein	Dose dependent increase followed by clearance with expected $t_{½}$
Serum amyloid A protein	None
Serum albumin	None
IL-1β	None
IL-6	None
TNFα	None
IL-10	None
Systolic blood pressure	None
Diastolic blood pressure	None
Heart rate	None
Temperature	None
Electrocardiogram	None
Medications	None
Total white blood cell count	None
Neutrophils	None
Eosinophils	None
Basophils	None
Lymphocytes	None
Monocytes	None
Platelets	None
Hemoglobin	None
Hematocrit	None
Red cell count	None
MCV	None
MCH	None
MCHC	None
Erythrocyte sedimentation rate	None
Sodium	None
Potassium	None
Urea	None
Creatinine	None
Total Bilirubin	None
Alanine transaminase	None
Aspartate transaminase	None
Alkaline Phosphatase	None
Glucose	None
Cardiac Troponin T	None
NT Pro-BNP	None
Von Willebrand Factor antigen	None
Fibrinogen D-dimer	None
Prothrombin fragment 1+2	None
Plasminogen activator inhibitor 1 antigen	None

Table 8.3. Other parameters monitored during and after infusion of human CRP. Apart from the concentration of CRP which increased in a predictable dose-dependent manner, no other parameters showed any clinically significant change.

8.5 Discussion

Only very limited quantities of 'normal' human CRP are available because of the extremely low concentration of CRP in health, and the CRP which has been used in other work on this protein, whether isolated by researchers themselves or bought commercially, is derived either from serum or effusion fluids of patients with malignancy or other diseases, or is produced by recombinant technology. The human CRP produced by recombinant *E. coli* bacteria is intended only for use as an in vitro immunoassay standard, although it has been widely used for in vitro functional studies on cells (98, 197). The Pepys group demonstrated that this source of CRP contains highly active pro-inflammatory bacterial contaminants (198), leading one to the conclusion that the widespread use of this recombinant CRP has been somewhat misleading.

The commercially manufactured CRP is produced at low concentrations of approximately 5 mg/L, in the culture fluid of *E. coli* fermenters and isolated by a single non-specific affinity chromatography fractionation. It is therefore unlikely that it would not be contaminated with bacterial products, including LPS; when tested by the Pepys group, after publication of the paper, this proved to be the case. It is also unlikely that gel filtration could possibly remove all endotoxin and other potential bacterial products from the CRP preparation. Contamination with bacterial substances is not relevant to the intended use of the recombinant protein as an immunoassay standard but inevitably has major consequences when such preparations are used for in vitro functional studies with cells or injected into human subjects.

Another potential explanation for the induction of inflammation in the experiment involving the commercially manufactured CRP could be structural changes in the protein. Native CRP exists as a stable pentamer, however, a dissociation mechanism of pentameric CRP into monomeric CRP has been identified on activated platelets and damaged or

apoptotic cells (199) and the monomers appear to be strongly pro-inflammatory. Conditions under which the native protein structure may be destabilised causing it to dissociate into monomeric CRP include heat exposure, an acid microenvironment or immobilisation on tissue culture flasks, or even during preparation of material for immunohistochemistry (200). Monomeric CRP has been identified in mice but it is not yet established whether dissociation occurs *in vivo* in man.

In contrast to the persistent and increased CRP production observed by Bisoendial et al (196, 201-204), the CRP values in the subjects in this study declined at a rate consistent with the normal clearance of human CRP. Infusion of 1.25 mg/kg of bacterial recombinant CRP produced initial peak CRP values of 20.5 to 28.1 mg/L, which fell before rising again to 29 mg/L. Here, the infusion of authentic human CRP itself triggered no new production of CRP, and the concept that autologous human CRP triggers production of more CRP, is not supported by the results of this study. Furthermore none of the seven recipients of a range of doses of purified human CRP showed any increase in their circulating concentrations of SAA, the most sensitive human acute phase protein. Once again this contrasts sharply with the Bisoendial results in which infusion of bacterial recombinant CRP induced massive acute phase responses of SAA, reaching more than 2,000 mg/L in some subjects (196), values typically seen in acute sepsis or major trauma.

Our present subjects also showed no meaningful increase in cytokine production and there was no evidence for abnormally raised pro-inflammatory cytokine activity at any stage, with both SAA concentrations and the levels of all other acute phase reactants within the reference range throughout. Indeed there was no significant change in any of the clinical, physical, biochemical, coagulation, or hematological parameters which were

assessed, despite administration of sufficient human CRP to achieve typical circulating acute phase response values.

These findings thus demonstrate that purified, pharmaceutical grade human CRP itself does not have any detectable pro-inflammatory effects in normal healthy adult subjects. This critical experiment will extend to humans the well-established absence in animals of any proinflammatory or other adverse effects of purified human CRP in vivo, and thereby address a crucially important scientific question.

This result is consistent with the compelling evidence from very large scale observational (205, 206) and genetic epidemiology studies (207) showing no evidence to support a causative role of CRP in human atherosclerosis. Similarly, robust experimental animal studies also show that CRP does not contribute to atherogenesis (208-210).

It is of the greatest clinical importance to distinguish clearly between the fact that CRP has no inherent pro-inflammatory effects itself and its potential pro-inflammatory or opsonising role of human CRP in individuals with pre-existing tissue damage. Within its role as an acute phase reactant, useful in the diagnosis of inflammatory conditions and the monitoring of effectiveness of treatments, CRP has thus far not been proven to be either pathogenic or pro-inflammatory.

Chapter Nine:

General Conclusions

The many benefits of specialist nurse-led clinical services are well documented in the published literature. Specialist nurse-led clinical services can be found in many different contexts and disease areas such as heart failure, multiple sclerosis, chronic pain, rheumatology, cardiac rehabilitation and diabetes, to name a few. In the context of this body of work, the nurse-led clinic has made possible the provision of very specialist, perhaps even niche, clinical care to patients with complex inflammatory conditions requiring treatment with biologic therapies, and careful and close monitoring. It has also made possible nurse-led clinical and academic research. Combining research with clinical work, especially in such rare diseases where patient numbers are small, is the gold standard. However, in order to make this possible, optimal conditions must exist – the combination of adequate funding, infrastructure, human resources and resourceful humans. The result when these conditions are met is benefit to the patients, the multidisciplinary team of healthcare professionals and to the NHS as a whole.

Chronic inflammatory disorders such as RA and JIA, in the absence of effective treatments, are known to progress inexorably to joint damage and destruction, leaving patients debilitated and in need of social intervention such as mobility aids and disability benefits, and medical intervention for associated co-morbidities. Autoinflammatory diseases, although less frequent than the commoner chronic inflammatory disorders discussed, also have a significant adverse effects on patients' lives. As these diseases manifest in early life normal growth and development are disturbed, as is schooling and social development. Inflammatory diseases can therefore impact negatively not only on patients and their families, but on the wider health service and social care system too.

Systemic AA amyloidosis is a rare but most feared complication of chronic inflammatory disorders. The amyloid fibrils in AA amyloidosis are derived from serum amyloid A protein, SAA, an exquisitely sensitive hepatic acute phase protein synthesized under the

transcriptional regulation of pro-inflammatory cytokines such as IL-1, IL-6 and TNFα. Clinical presentation of AA amyloidosis is almost always due to the consequences of renal dysfunction, with proteinuria and progressive renal decline, and progression to end stage renal failure is common. Management of AA amyloidosis is centred on reducing supply of the precursor protein by effectively treating the underlying inflammatory condition, whilst supporting the amyloidotic organs. Where AA amyloidosis has not yet manifest as a complication, management of the underlying inflammatory disorder remains the focus of therapy in order to prevent the development of amyloidosis, and thereby prevent renal failure which is not only costly, but is associated with poor quality of life in many patients.

The risk of developing of AA amyloidosis is difficult to characterise based on the cohorts of patients who are the subject of this thesis, and there are several reasons for this. Firstly, increasing age is a known risk factor for the development of AA amyloidosis with childhood presentation a very rare occurrence, thus, the inclusion of paediatric patient's results in underestimation of both prevalence and incidence. Furthermore, as the SAIDs are generally newly described entities, many adults were never diagnosed in earlier decades and are now lost to follow-up and so current cohorts are heavily biased towards children. Lastly, much of the available data are historical, dating back to when many of the effective treatments in current use were unavailable.

Early diagnosis and initiation of treatment of inflammatory conditions is important in order to prevent the development of organ damage including AA amyloidosis. Early diagnosis can be problematic with the SAIDs in cases where there is no family history, as these diseases are rare and a high index of clinical suspicion is required for successful diagnosis. Furthermore, clinical features of the SAIDs overlap with other more common diseases. Broad and atypical phenotypes add further diagnostic challenges. Also, recognition of asymptomatic patients with subclinical inflammation before they develop

renal AA amyloidosis is difficult as it is reliant upon serendipitous detection of deranged inflammatory markers and recognition of the importance of further investigation in these cases. Increasing knowledge and awareness of these diseases is crucial.

Another patient group presenting a diagnostic and therapeutic dilemma are those with AA amyloidosis of uncertain or unknown cause. Basic genetic diagnostic services may be unavailable, or unhelpful; next generation sequencing may be expensive or unavailable; and biopsy or advanced imaging techniques may also prove non-diagnostic. At our Centre disease activity is monitored by a combination of patient symptom diaries and serial serum inflammatory markers. In those with suggestive symptoms with accompanying acute phase activation a trial of therapy can be very revealing. Responses to colchicine, corticosteroids or biologics such as anti-IL1 or anti-IL6 drugs can help to establish a definitive diagnosis. In addition a successful therapeutic trial can allow effective management of both symptoms and prevention of devastating complications in cases where the disease aetiology remains unknown even after extensive investigation.

In monogenic diseases such as CAPS, huge strides have been made in terms of therapies, and the disease has been effectively switched off in most cases. However, in turn, pharmacological treatments, specifically unsuccessful pharmacological therapies have revealed broader and more unusual disease phenotypes. Close and intensive follow up of these patients is extremely important in revealing new insights into extending phenotypes and trends and new therapeutic possibilities. Furthermore, close, intensive and long term follow up are crucial in evaluating the overall safety and efficacy of long term treatment with biological agents, and to determine their long term effects on quality and length of life in these patient groups. Where the treatments have been effective, they have been life-transforming.

The observation that AA amyloidosis is becoming less common may be a reflection of great advances in the use of biological agents in the effective treatment of the inflammatory arthritides. Overall, these data suggest both falling incidence (corrected for improved diagnostic rates) and better outcome in AA amyloidosis over a period of two-and-a-half decades, reflecting advances in therapeutics and in overall management of complex chronic disease in an aging population. This has both reduced the incidence of AA amyloidosis and when it does develop, has improved outcomes in what were until recently the commonest pathologies underlying AA amyloidosis. The mean age at presentation with AA amyloidosis is now significantly older than in historical cohorts. This again may be a reflection of the availability of effective biological treatments for inflammatory conditions. Whilst overall survival does not appear to have changed despite new effective therapies, age at death has increased significantly over the last 25 years, suggesting improved global management and supportive care including renal replacement, as well as more effective management of the underlying pathology. It is hoped that newer techniques such as next generation sequencing and gene expression analysis may aid diagnosis and effective treatment of these 'uncharacterised' inflammatory disorders thereby improving overall survival.

Monitoring of the acute phase proteins SAA and CRP is crucial to assess whether the underlying disease is adequately suppressed; in their roles as markers of the acute phase, neither are pathogenic and both are extremely clinically useful. However, both SAA and CRP also have pathogenic effects. The pathogenic effect of SAA is, as previously mentioned, that it is the precursor for AA amyloidosis. The circulating concentration of SAA is a powerful predictor of both patient survival and renal outcome. In patients in whom SAA production is adequately suppressed amyloid deposits can be seen to regress

and renal function can be stabilised or even improve. This can be seen in Chapters 2, 5 and 6.2.

The pathogenic effect of CRP lies in its role as an opsonin; in cardiovascular disease, CRP is attracted to the lesion or injury site, and attracts further pro-inflammatory cells and molecules which have the undesirable effects of increasing the size and extent of the lesion. In patients with known cardiovascular disease elevated levels of CRP are statistically significantly associated with risk of future coronary events. Unfortunately inappropriate conflation of epidemiological association with causality, has led to claims that increased baseline CRP concentration is not only a risk marker for cardiovascular disease but also a risk factor contributing to the pathogenesis of atherosclerosis and atherothrombosis. This has been compounded by the use of (presumably) contaminated recombinant CRP in in vitro and in vivo studies, which produced acute phase reactions, thereby implying that CRP of itself produces inflammation. This is clearly of concern to all clinicians, and so we sought to explore the validity of this assertion by administering highly purified human CRP to seven heathy volunteers. This experiment showed that there were no pro-inflammatory effects from this pure CRP, thereby answering a very important clinical question - CRP is used worldwide as the main measure of inflammation, injury and infection, and response to therapy. It was therefore crucial to distinguish its role as a simple acute phase reactant, from its role as a ‘pro-inflammatory’ agent in specific circumstances, i.e. its role as an opsonin.

Limitations and future directions

It remains unclear why some patients develop AA amyloidosis and others do not. Although prolonged elevation of SAA concentration is known to be a prerequisite, some patients with only low level inflammation for a relative short time may develop it whilst others with very high SAA concentrations over a much longer period of time do not. Associations have been found by other groups between particular *SAA1* alleles and the development of AA amyloidosis, and future work should include a genotypic comparison between patients with inflammatory conditions who do and do not develop amyloidosis.

Other factors involved may be environmental or epigenetic. In Chapter seven we see that the proportion of Asian patients diagnosed with amyloidosis has increased over the 25 year period has doubled from 4% between 1990 and 1997 to 8% between 1998 and 2006, and quadrupled between 2007 and 2014 – this is an interesting phenomenon and warrants further exploration.

Chapter seven also reveals a reduction in the incidence of JIA and SAIDs as diseases underlying AA amyloidosis, whist there has been a huge rise in AA amyloidosis of uncertain aetiology. Future work with the patient group in whom the underlying cause of the AA amyloidosis is unknown could involve next generation sequencing (NGS), and perhaps further imaging (CT, PET etc.) although patients may find this unnecessarily invasive or inconvenient.

Pathogenic mechanisms in autoinflammation are complex and incompletely understood. The genotypic and phenotypic spectra are ever expanding, even in monogenic disease such as CAPS. Patient with CAPS who respond inadequately to treatment or who have

atypical presentation could be investigated further with NGS. Clinically, in these patients, dual cytokine therapy, e.g. an anti-IL-1 agent combined with anti-TNF or anti-IL-6 agent, could be trialled although the increased risk of developing serious infections must be carefully weighed against the potential benefit.

The SAIDs involve cradle to grave care, therefore any safety data presented herein may only be viewed as preliminary. Longer follow up is required to reveal the true safety and efficacy of the treatments studied within this thesis.

Publications Arising From Thesis Work

Lane T and Lachmann HJ. **The emerging role of interleukin 1β in autoinflammatory** diseases. Current Allergy & Asthma Report. 2011 Oct; 11(5):361-8.

Lane T, Loeffler JM, Rowczenio DM, Gilbertson JA, Bybee A, Russell TL, Gillmore JD, Wechalekar AD, Hawkins PN, Lachmann HJ. AA amyloidosis complicating the inherited periodic fever syndromes. Arthritis & Rheumatism 2013; 65 (4): 1116 – 1121.

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Lane T, Gillmore JD, Wechalekar AD, Hawkins PN, Lachmann HJ: Therapeutic blockade of interleukin-6 by tocilizumab in the management of AA amyloidosis and chronic inflammatory disorders: a case series and review of the literature. Clinical and Experimental Rheumatology. 2015, 33(6 Suppl 94): 46-53.

Please see appendix for full articles.

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Supplementary Information

Appendices

NATIONAL AMYLOIDOSIS CENTRE
UCL DIVISION OF MEDICINE

Date: 22 July 2015
Our ref: TL[redacted]
NHS no: [redacted]
Nurse Clinic: 16 July 2015

[redacted]

Dr [redacted]

Dear Dr [redacted],

Re: [redacted]

Diagnoses:

1. Muckle-Wells Syndrome (CAPS)
   a. *NLRP3* mutation T348M
   b. Classical clinical symptoms of neonatal onset rash, myalgia, aseptic meningitis, papilloedema, conjunctivitis and bi-lateral hearing loss
   c. Bleeding gums – now resolved
   d. Commenced canakinumab (monoclonal anti-IL-1β antibody) as part of Novartis trial June 2008; now treated as part of the NHS-funded National CAPS Treatment Service
   e. Dose increased to 300 mg, January 2011
   f. Headaches - ongoing
2. Chronic inflammatory sinusitis affecting the left frontal sinus, requiring rhinoplasty and sinus washout July 2012
3. **Severe fatigue and drowsiness – unlikely to be related to CAPS, possibly related to nortriptyline**

It was a pleasure to see [redacted] in the CAPS clinic today. From a CAPS point of view his headaches continue but remain stable, and he had no other symptoms.

He did however complain of severe tiredness/sleepiness. He says he is getting enough sleep – more than 8 hours per night – but wakes up tired and remains sleepy and tired all day. He has been late for work on more than one occasion due to being unable to get out of bed in the morning. Objectively he looked tired, even a bit drowsy, and kept rubbing his eyes; he also had quite a flat affect, markedly changed from when I last saw him a few months ago. I am rather concerned about this and **I wonder if you wouldn't mind reviewing him (and perhaps his prescription of nortriptyline) as a matter of urgency please?** I could not ascertain by questioning him, any other cause of his tiredness/drowsiness. Bloods today were completely normal apart from slightly low bicarbonate (enclosed).

Today I have administered the usual 300mg canakinumab and will see him again in September 2015, when he will also be reviewed in the consultant clinic.

Yours sincerely,

Checked and signed off electronically

Thirusha Lane
Lead Nurse

Results: serial free light chains or serum amyloid A levels from posted blood samples should be sent out automatically - If you do not receive them please contact Onana on o[redacted] or 020 7433 2812.

Enc: Cumulative Results

Cc:

National Amyloidosis Centre, UCL Division of Medicine, Royal Free Hospital, Rowland Hill Street, London NW3 2PF. UK
[redacted]
Royal Free London NHS

NATIONAL AMYLOIDOSIS CENTRE
UCL DIVISION OF MEDICINE

Date: 04 November 2015
Our ref: RW/[redacted]
NHS No: [redacted]
Nurse Clinic: 22 October 2015

[redacted]

Dr S [redacted]

Dear Dr [redacted],

Re: [redacted]

Diagnoses:

1. Cryopyrin Associated Periodic Syndrome (CAPS)
   a. *NLRP3* mutation A439V
   b. Commenced canakinumab as part of Novartis trial in 29 December 2008; now treated as part of the NCG-funded National CAPS Treatment Service.
2. Extremely marked contact dermatitis from his motorcycling gloves and helmet- now resolved
3. Fractured clavicle sustained in bicycle accident –March 2011, now healed
4. Eczema – treated with Fucibet cream

It was a pleasure to see [redacted] in the CAPS clinic today. He remains well in general and continues to be asymptomatic from a CAPS perspective apart from having one mouth ulcer. His CAPS assessment score for today is 1/20, indicating very mild disease activity.

He remains on no other regular medication.

His SAA today is 3.4mg/L and CRP <1mg/L. Cumulative results are enclosed.

We have dosed him with a further 150mg canakinumab and will see him next in December 2015.

Yours sincerely,

Checked and signed off electronically

Rene Williams
CAPS Clinic Nurse

Thirusha Lane
Lead Nurse

Results: serial free light chains or serum amyloid A levels from posted blood samples should be sent out automatically - if you do not receive them please contact Onana o[redacted]r 020 7433 2812.

Enc: Cumulative Results
Cc:
Dr D [redacted]

PRE-TREATMENT CAPS CLINICAL SCORE WORKSHEET FOR PATIENT 8 (CHAPTER 6.1)

NATIONAL AMYLOIDOSIS CENTRE
DIVISION OF MEDICINE
UCL MEDICAL SCHOOL

UCL

NAME [redacted] HOSP No. [redacted] DOB [redacted]

CANAKINUMAB CLINICAL ASSESSMENTS

1. PHYSICIAN'S CLINICAL ASSESSMENT OF AUTOINFLAMMATORY DISEASE ACTIVITY

Symptom	Absent (0)	Mild/Moderate (1)	Severe (2)
1. Skin disease (urticarial rash)	□	☑	□
2. Arthralgia	□	☑	□
3. Myalgia/limb pain	□	☑	□
4. Headache/migraine	☑	□	□
5. Conjunctivitis	□	☑	□
6. Fatigue/malaise	□	☑	□
7. Fever/chills	□	☑	□
8. Cold-induced symptoms	□	□	☑
9. Abdominal pain	□	☑	□
10. Oral ulcers	☑	□	□
SCORE (out of 20)			9.

2. PHYSICIAN'S GLOBAL ASSESSMENT OF CURRENT CAPS DISEASE ACTIVITY

Absent	Mild/Moderate	Severe
□	☑	□

3. PHYSICIAN'S OVERALL GLOBAL ASSESSMENT OF CAPS ACTIVITY SINCE LAST VISIT

BASELINE

Much improved	Slightly improved	Stable	Slightly worsened	Much worsened
□	□	□	□	□

Date of assessment:	Signature of Physician:
19/12/11	[redacted]

National Amyloidosis Centre, UCL Division of Medicine, Royal Free Hospital, Rowland Hill Street, London NW3 2PF, UK
www.ucl.ac.uk/medicine/amyloidosis
Royal Free Hampstead NHS NHS Trust

CAPS CLINICAL SCORE WORKSHEET FOR PATIENT 8 AFTER FIRST DOSE OF CANAKINUMAB (CHAPTER 6.1)

NATIONAL AMYLOIDOSIS CENTRE
DIVISION OF MEDICINE
UCL MEDICAL SCHOOL

UCL

NAME [redacted] HOSP No. [redacted] DOB [redacted]

CAPS DISEASE ACTIVITY ASSESSMENT

1. CLINICIAN'S ASSESSMENT OF AUTOINFLAMMATORY DISEASE ACTIVITY SINCE LAST VISIT

Symptom	Absent (0)	Mild/Moderate (1)	Severe (2)
1. Skin disease (urticarial rash)	☑	☐	☐
2. Arthralgia	☑	☐	☐
3. Myalgia/limb pain	☑	☐	☐
4. Headache/migraine	☑	☐	☐
5. Red eyes	☑	☐	☐
6. Fatigue/malaise	☑	☐	☐
7. Fever/chills	☑	☐	☐
8. Cold-induced symptoms	☑	☐	☐
9. Abdominal pain	☑	☐	☐
10. Oral ulcers	☑	☐	☐
SCORE (out of 20)			0

2. CLINICIAN'S GLOBAL ASSESSMENT OF CURRENT CAPS DISEASE ACTIVITY

Absent	Mild/Moderate	Severe
☑	☐	☐

3. CLINICIAN'S OVERALL GLOBAL ASSESSMENT OF CAPS ACTIVITY SINCE LAST VISIT

Much improved	Slightly improved	Stable	Slightly worsened	Much worsened
☑	☐	☐	☐	☐

4. DID THE PATIENT EXPERIENCE ANY SERIOUS INFECTIONS AND/OR ANY EVENTS REQUIRING HOSPITALISATION?

Yes	No
☐ → *Enter event details on AE log in medical notes*	☑

Date of assessment:	Signature of Clinician:
13/02/2012	[redacted]

National Amyloidosis Centre, UCL Division of Medicine, Royal Free Hospital, Rowland Hill Street, London NW3 2PF, UK
www.ucl.ac.uk/medicine/amyloidosis
Royal Free London NHS NHS Foundation Trust

Since starting on Canakinumab my life has certainly changed for the better.

I have not had any of the painful swollen joints that have stopped doing me doing my job,, as prior to the treatment my wrists, fingers and elbows were sometimes immobile.

In addition, I haven't had the bad eyes, or uncontrollable fever that was a regular occurrence prior to the treatment.

Sometimes if a fever came on at night I couldn't even raise my head or turn over to take pain killers to ease it.

The best of everything is that I can now travel without any worries of having uncontrollable fever and shivering, which is a god send, as now it doesn't take good week to get over it.

Aeroplane travel was always the worst because of the cold inside the cabin, and I used to suffer severely especially on long haul flights.

Since the start of the treatment, not a winter has gone by when I haven't thanked heaven for medical science.

Thank you.

JK

The Impact of Canakinumab

Until I was referred to the National Amyloidosis Centre in May 2006, I had lived most of my life (I am 58) with chronic daily urticaria, intermittent inflamed eyes, almost daily aching joints and muscles, at times unbelievable fatigue, episodes of feeling extremely cold and in the ten years up to 2006 daily chronic oedema in my legs and particularly my ankles. I accepted this as normal for me and that I had just been unlucky in the genes I had inherited from my father who too suffered similar symptoms. I, like all the doctors and consultants I had sought advice from over the years looked at the symptoms in isolation and it was not until an appointment at the Urticaria Clinic at St Thomas's hospital in London did anyone ever suggest to me that the symptoms might be linked and that I could be suffering from Muckle Wells Syndrome. I was thus referred to the NAC for further investigation. My perseverance had been worth it - this was break through for me and my years of silently suffering.

To understand the impact Canakinumab has had on my life, I need to describe what it is like living without it.

Urticaria. Every part of my body except my trunk would be covered in large, raised red wheels which would start to appear in the second half of the day and get progressively worse until my body was covered by the evening. If I was lucky, the wheels would go down over night, only to start again the next day. Some days were worst than others and my legs and arms which were covered most would sometimes feel burning to touch and yet I could be shivering inside. Absolutely nothing alleviated the rash and it would determine what clothes I would wear. I was so embarrassed by the sight of my arms and legs; I would wear trousers or long sleeve tops to hide the rash. I never wore strappy dresses/tops in the summer and I virtually gave up playing tennis in the summer months as I wouldn't be 'seen dead' in shorts and it was too hot to play in track suit bottoms!

Inflamed Eyes. Once or twice a month, I would suffer with inflamed eyes. They would start to come up during the day and by the time I would go to bed on the first day, the pain when I lay down would be excruciating. Painkillers had minimal effect. The only way I could attempt to get any sleep was to press a tissue (to dry up the constant watering) against my eye with the palm of my hand. The pressure from my palm helped to alleviate the pain and I would lie there willing the night away. By morning I would have a very swollen eye which I could barely open and any light would increase the pain. The inflammation would subside over 24 – 48 hours, but leave me with cloudy vision for a further day.

Aching joints and muscles. I used to long for a day when I didn't have aching joints (hands, wrists, knees, ankles, hips). When really bad, I would be reduced to tears from the pain and just crawl upstairs and into bed at the end the day as at least in the morning, I might be slightly stiff when I got up, but invariably the pain would be gone. However, occasionally my hands and wrists would be very swollen and red and the inflammation would last up to 2 days or more so causing me difficulty holding and turning things.

Fatigue. There would be occasions at the end of the day when I was too tired to eat, let alone climb the stairs to bed. I have always played tennis, but found I was playing less often as I was coming home from work exhausted from sitting at a desk all day. What I could never understand was how I could feel drained of energy from just walking up the stairs when it was 8 o'clock in the morning and I had just had a night's sleep!

Episodes of feeling extremely cold. So cold, really cold inside, that not even layers of clothing and being wrapped in a duvet would alleviate. It would creep up on me during the day and by the time the evening came, I would be piling on the layers, the thermostat for the central heating would be cranked up and I would be miserable whilst everyone else in the

family roasted. Not even the thought of bed would comfort me for a warm bath had no effect and I would lie there, wrapped in numerous items of clothing, my body aching from the shaking of shivering, desperate to get to sleep, but sleep would allude me for hours.

Oedema. In the last ten years or so, I started to suffer with oedema with my legs always feeling swollen and I suffered really badly with fluid retention around my ankles. In fact, I could never really see my ankle bones, even first thing in the morning. I never dare take my shoes off during the day for fear of never getting them back on again and there were times when I missed out playing tennis because I couldn't get my trainers on, let alone flex my ankles. Trousers were the dress code.

I can be a very sceptical person and to be told that I would find out what it felt like to be symptom free if I tried Canakinumab, I didn't really believe, but within 24 hours of being given the first injection, I have never had any of the above symptoms again. To say that it has changed my life is an understatement – the impact has been immense. I am so happy to be symptom free, it is just fantastic!

This is how my life has changed:

- I no longer suffer with any aching joints or muscle pain – I am pain free
- I no longer suffer with oedema and have ankles!
- My urticaria has never re-occurred
- I have not had an inflamed eye since being on the drug
- I no longer suffer from extreme bouts of feeling cold
- I am more active as I do not suffer with fatigue
- I play tennis once a week, all year round
- I dog walk (briskly) almost every day
- I am not restricted in the clothes I wear
- I have gone down a shoe size
- I lost several pounds in weight (no fluid retention)
- I socialise more as I am free from pain, fatigue and the embarrassment of urticaria
- My family think it is wonderful and they are happy for me and so we all benefit
- I feel like a new person which has increased my confidence and positive attitude

I almost feel Canakinumab is too good to be true and my bubble will burst. To live a life symptom free is just second to none.

CB

Canakinumab Testimonial

In April 2013, I was diagnosed with the rare auto inflammatory syndrome, Cryopyrin-Associated Periodic Fever Syndrome (CAPS). This followed initial consultation with Professor Hawkins and Dr Lachmann in the Periodic Fever Syndrome Clinic at the Royal Free Hospital.

Over many years, my mother and I experienced frequent episodes of ill health, some very prolonged and debilitating and requiring hospitalisation. We shared the same recurring symptoms of high fever, nausea, abdominal pain, arthralgia, headache, red eyes and rashes. Both of us had been extensively investigated by various specialists including neurologists, gastroenterologists, rheumatologists and dermatologists. Although all confirmed that we seemed to have a very similar illness, the diagnosis remained elusive.

In childhood, I had a significant number of episodes of fever, arthralgia and abdominal pain, but it was not until I was a first year medical student that I had a major flare of symptoms presenting suddenly with a very high fever, severe headache, rash, abdominal pain and diarrhoea. Admitted to hospital, I was confirmed to have extremely high inflammatory markers but the cause of my symptoms could not be found. After several weeks of symptomatic treatment, my health gradually improved. However, throughout my studies I remained intermittently unwell with similar episodes.

More recently, I found life as a junior doctor challenging and stimulating but even more arduous than that of my colleagues: I was finding it difficult to keep up with the long hours of work and the enjoyable social activities that are an integral part of a junior doctor's life.

In January 2013, I had a severe flare of symptoms necessitating absence from work for several months. At this stage it became imperative that every avenue was explored in the hope of establishing a diagnosis. Detailed genetic testing confirmed that my mother and I carry a variant in NLRP3, the gene associated with CAPS, leading to the overproduction of the inflammatory cytokine, interleukin-1 beta.

Following detailed discussion with Professor Hawkins, Dr Lachmann and the research nurse team, I decided to begin treatment with the human monoclonal antibody Ilaris (canakinumab) which targets interleukin-1 beta and is approved for treatment of CAPS. I enrolled in the Ilaris Study which provides an international registry for patients treated with Ilaris; I have now received six months of treatment.

After years of poor health and struggling with the frequent flares of my illness, I was astounded to find that within two weeks of treatment I was dramatically better: I was free of arthralgia, headaches, vomiting and skin rashes and, remarkably, my fatigue and concentration were considerably improved. I began immediate discussions with the deanery to return to work as soon as possible and am now enjoying my work as a busy junior doctor and happily joining colleagues and friends for all the social activities on offer. With frequent flares of ill health in the past, studying for examinations was often a challenge. Since staring canakinumab I have been thrilled to pass MRCP Part One.

I had never heard of CAPS until I learnt that this rare genetic disorder was the cause of my illness. However, with the outstanding advice, support, care and encouragement I have received from Professor Hawkins, Dr Lachmann and the research nurses, Thirusha Lane and Rene Williams, my life has been transformed.

AVSC, November 2013.

I would like to start with a bit of background. I grew up in Lesotho..and also a small town called Ladybrand in south Africa. Not exactly a wealth of medical expertise. I remember from a very young age always being sick. Measles mumps colds flu..you name it I've probably had it. All the way to chickenpox at 18. I remember having pinkeye (conjunctivitis) at least twice a month where the sunlight would hurt my eye and cause headaches. I remember being fragile and always having sore muscles or bumps from school. I remember doing a lot of sport but getting injured a lot and pulling my Achilles tendons (sometimes even just waking up the next day and they would be as fat as a plum) I remember always being cold in winter because I would never put on any weight and being blue. My hands would turn into fat Shrek hands and would hurt to move. I remember getting a red rash all over my body..even in summer so I would wear long sleeves in the African sun to hide it...always looked weird to friends and class mates. I remember being in and out of doctors/hospitals..injection after injection trying to work out what I had..and a common answer would be arthritis.

Time passed and I learnt to manage what I had..obviously getting older and stronger I would be smarter in my clothes etc and not get as cold as often. I would still get rashes but learnt how to hide it well. I got used to the pain on my bones and got on with my life..it was part of my life and I wasn't going to let it bother me.
University was the norm..no real sports as id get injured but by then I was handling my " arthritis" well.

Then came the day in the UK I remember very well..I had decided I was tired of pink eye and sore hands. I went to the NHS and after a few months of being sent to eye specialists and arthritis specialists I finally went to a dermatologist who said I had Muckle-Wells syndrome. Fair enough..sounded interesting but little did I know my referral to the royal free would change my life completely.
After taking that first dose of canakinumab...I was no longer tired...no longer tired from the mental and physical fighting I didn't realise I was constantly doing to deal with the disease. As some of you know that weekend I went out all night dancing..didn't sleep..it was amazing.

Since 2009 I have not experienced any pink eye or sore joints or pulled Achilles or caps induced headaches... Happily I am a leopard that lost his spots too.
The main bonus of the drug..apart from not getting cold, sore, sick, tired..etc is that I have gained confidence...I am no longer shy...no longer body/image conscious.
I have gained over 12kgs since 2009. I like my body now... I have completed maybe 7 or 8 official 10km runs..I have stupidly completed tough mudder -a 21km obstacle course designed by the SAS. All these things I would never have dreamed of doing pre 2009.

I cant describe to you how my life has changed for the better since taking this drug..I am me... But a better me..a way better me. I cannot imagine life without it and actually the more the days pass the more I forget what life was like back then.

I cant express my gratitude enough for being able to take this drug and having a pain free and active lifestyle now.

Many thanks.
AS

Complete results for Subjects 1 to 7 in Chapter Four

SUBJECT 1 CRP dose 0.25 mg/kg															
			Screen	5 min	15 min	30 min	60 min	2 h	4 h	8 h	24 h	48 h	72 h	Day 6	Day 10
Ref Range	Unit														
<5	mg/L	C -reactive protein	2	7	.	6	.	.	5	5	3	2	3	6	2
< 10	mg/L	Serum amyloid A	<2.9	<2.9	.	<2.9	.	.	<2.9	<2.9	<2.9	<2.9	3.5	4.5	<2.9
135 - 145	mmol/L	Sodium	144	141	.	142	.	.	141	143	143	144	143	146	144
3.5 - 5.1	mmol/L	Potassium	4.3	Delayed	.	4.4	.	.	4.5	4	4.6	4.1	4.3	4.5	4.5
2.1 - 7.1	mmol/L	Urea	5.9	5.8	.	6	.	.	5.5	6.2	5.9	5.2	6.7	4.7	6.7
66 - 112	µmol/L	Creatinine	80	82	.	86	.	.	82	80	90	80	81	76	88
< 21	µmol/L	Total bilirubin	16	13	.	13	.	.	12	6	15	12	16	11	19
< 41	U/L	Alanine transaminase	18	51	.	50	.	.	50	48	45	38	33	22	19
< 37	U/L	Aspartate transaminase	27	65	.	64	.	.	61	57	44	33	28	21	22
< 129	U/L	Alkaline phosphatase	59	58	.	58	.	.	60	64	64	60	59	58	63
35 - 50	g/L	Albumin	46	45	.	45	.	.	46	46	47	46	46	46	46
< 0.030	µg/L	Cardiac troponin T	0.003	0.003	.	0.003	.	.	0.003	0.003	.	0.003	.	0.003	.
	pmol/L	NT pro-BNP	3	3	.	3	.	.	3	3	.	7	.	5	.
11.5 - 17.0	g/dL	Hemoglobin	14.1	14.5	.	14.7	.	.	14.7	14.9	15.1	14.2	14.3	14.3	14
3.5 - 11	10^9/L	White cells	6.98	6.41	.	6.35	.	.	6.88	7.63	6.72	6.66	8.75	7.08	7.44
140 - 400	10^9/L	Platelets	228	213	.	221	.	.	223	230	220	208	221	211	249
4.32 - 5.6	10^{12}/L	Red cells	4.91	4.97	.	5	.	.	4.96	5.01	5.08	4.85	4.83	4.92	4.74
0.39 - 0.52	L/L	Hematocrit	0.415	0.42	.	0.423	.	.	0.415	0.423	0.436	0.413	0.406	0.411	0.404
80 - 98	fL	MCV	84.5	84.5	.	84.6	.	.	83.7	84.4	85.8	85.2	84.1	83.5	85.2
27 - 33	pg	MCH	28.7	29.2	.	29.4	.	.	29.6	29.7	29.7	29.3	29.6	29.1	29.5
31 - 37	g/dL	MCHC	34	34.5	.	34.8	.	.	35.4	35.2	34.6	34.4	35.2	34.8	34.7
1.7 - 8.0	10^9/L	Neutrophils	4.57	3.85	.	3.68	.	.	4.09	4.69	4.13	3.94	5.84	4.28	4.79
1.0 - 3.5	10^9/L	Lymphocytes	1.56	1.72	.	1.88	.	.	2.07	2.01	1.83	1.81	1.91	1.84	1.86
0.1 - 1.0	10^9/L	Monocytes	0.73	0.68	.	0.67	.	.	0.58	0.74	0.68	0.77	0.86	0.71	0.64
0.0 - 0.46	10^9/L	Eosinophils	0.08	0.12	.	0.1	.	.	0.11	0.16	0.06	0.1	0.12	0.22	0.12
0.00 - 0.20	10^9/L	Basophils	0.04	0.04	.	0.02	.	.	0.03	0.03	0.02	0.03	0.03	0.03	0.03
45-175	IU/dL	vWF antigen	96	87	.	81	.	.	77	83	85	73	84	86	97
<130	ng/mL	Fibrinogen D-dimer	48	60	.	43	.	.	51	42	34	19	49	233	77
69 - 229	pmol/L	Prothrombin F1+2	113	111	.	116	.	.	102	103	104	121	303	142	108
1.4 - 8.4	ng/mL	PAI-1 antigen	1.8	4.6	.	5.3	.	.	0.9	2	2	2.2	1.7	0.9	0.4
	mm Hg	BP Systolic	118	116	104	115	104	106	108	100	116	110	110	110	110
	mm HG	BP Diastolic	66	57	58	54	57	53	52	56	63	55	55	53	62
	b.p.m.	Heart rate	52	53	44	50	50	70	53	69	84	49	49	49	49
	°C	Temperature	36.7	36.5	36.4	36.2	36.4	36.2	36.5	36.6	36	36.1	36.1	36.1	36.2
		ECG	NAD	.	NAD	.	.	.	.	.	NAD	NAD	NAD	NAD	NAD
		Medications	None	None	None	None	None	None	None	None	None	None	None	None	None

SUBJECT 2 CRP dose 0.50 mg/kg																
			Screen	Rescreen	Baseline	15 min	30 min	60 min	2 h	4 h	8 h	24 h	48 h	72 h	Day 6	Day 10
Ref Range	Unit															
<5	mg/L	C-reactive protein	<1	1	1	.	12	.	.	11	9	3	2	1	<1	<1
< 10	mg/L	Serum amyloid A	<2.9	<2.9	<2.9	.	<2.9	.	.	<2.9	<2.9	<2.9	<2.9	<2.9	<2.9	<2.9
135 - 145	mmol/L	Sodium	143	142	144	.	.	.	.	.	.	142	139	142	146	141
3.5 - 5.1	mmol/L	Potassium	4.1	4	4.2	.	.	.	.	.	.	4.1	3.9	4.4	4.4	4.1
2.1 - 7.1	mmol/L	Urea	5	4.4	5.5	.	.	.	.	.	.	4.9	3.9	4.6	4.3	4
66 - 112	µmol/L	Creatinine	82	86	94	.	.	.	.	.	.	94	83	80	88	84
< 21	µmol/L	Total bilirubin	19	12	7	.	.	.	.	.	.	15	14	12	15	15
< 41	U/L	Alanine transaminase	19	22	12	.	.	.	.	.	.	21	20	19	19	16
< 37	U/L	Aspartate transaminase	25	31	30	.	.	.	.	.	.	27	26	25	26	21
< 129	U/L	Alkaline phosphatase	50	61	49	.	.	.	.	.	.	49	47	52	58	44
35 - 50	g/L	Albumin	49	48	50	.	.	.	.	.	.	47	44	46	50	47
	mmol/L	Glucose	4.9	5.5	.	.	.	.	.	.	.	.	.	4.9	.	.
< 0.030	µg/L	Cardiac Troponin T	0.003	0.003	.	.	.	.	.	.	.	.	0.003	0.003	0.003	.
	pmol/L	NT Pro-BNP	2	5	.	.	.	.	.	.	.	.	2	2	4	.
11.5 - 17.0	g/dL	Hemoglobin	14.8	14.2	15.4	.	15	.	.	15.9	15.1	14.5	14	14.2	15	14.7
3.5 - 11	10^9/L	White cells	5.92	7.45	5.35	.	5.48	.	.	7.25	5.72	5.47	3.84	6.02	6.41	4.23
140 - 400	10^9/L	Platelets	182	184	165	.	157	.	.	193	182	175	153	189	195	173
4.32 - 5.6	10^{12}/L	Red cells	4.57	4.34	4.6	.	4.55	.	.	4.79	4.47	4.42	4.33	4.34	4.49	4.37
0.39 - 0.52	L/L	Hematocrit	0.424	0.409	0.43	.	0.423	.	.	0.441	0.413	0.411	0.4	0.402	0.414	0.408
80 - 98	fL	MCV	92.8	94.2	93.5	.	93	.	.	92.1	92.4	93	92.4	92.6	92.2	93.4
27 - 33	pg	MCH	32.4	32.7	33.5	.	33	.	.	33.2	33.8	32.8	32.3	32.7	33.4	33.6
31 - 37	g/dL	MCHC	34.9	34.7	35.8	.	35.5	.	.	36.1	36.6	35.3	35	35.3	36.2	36
1.7 - 8.0	10^9/L	Neutrophils	3.97	5.42	3.23	.	3.78	.	.	5.31	3.8	3.73	2.21	3.95	4.84	2.71
1.0 - 3.5	10^9/L	Lymphocytes	1.48	1.51	1.46	.	1.12	.	.	1.36	1.3	1.28	1.2	1.63	1.19	1.17
0.1 - 1.0	10^9/L	Monocytes	0.43	0.44	0.59	.	0.52	.	.	0.53	0.56	0.43	0.36	0.36	0.36	0.3
0.0 - 0.46	10^9/L	Eosinophils	0.02	0.06	0.05	.	0.04	.	.	0.02	0.02	0.01	0.04	0.05	0	0.03
0.00 - 0.20	10^9/L	Basophils	0.02	0.02	0.02	.	0.02	.	.	0.03	0.04	0.02	0.03	0.03	0.02	0.02
0 -15	mm 1st h	Sedimentation rate	2	2	2	.	2	.	.	3	4	2	2	3	4	6
45-175	IU/dL	VWF antigen	69	80	51	.	57	.	.	64	56	61	60	58	57	58
<130	ng/mL	Fibrinogen D-dimer	33	64	50	.	47	.	.	29	33	11	8	9	38	0
69 - 229	pmol/L	Prothrombin F1+2	161	180	135	.	141	.	.	462	325	123	127	117	130	126
1.4 - 8.4	ng/mL	PAI-1 antigen	2.2	1.7	9.2	.	8.9	.	.	15.3	3.4	2.1	3.9	1.8	6.6	3.4
	mm Hg	BP Systolic	126	.	125	126	126	121	121	121	118	112	116	113	124	127
	mm HG	BP Diastolic	126	.	74	77	77	78	64	61	67	72	65	63	65	72
	b.p.m.	Heart rate	57	.	60	59	59	50	81	81	65	55	57	66	65	71
	°C	Temperature	37.1	.	36.3	36.3	36.3	36.2	36.3	36.3	36.5	36.6	36.2	36.3	36.6	36.5
		ECG	NAD	.	.	NAD	.	.	.	.	.	NAD	NAD	NAD	NAD	NAD
		Medications	None	.	None	None	None	None	None	None	None	None	None	None	None	None

SUBJECT 3 CRP dose 1.00 mg/kg																
			Screen	Rescreen	Baseline	15min	30min	60min	2hr	4hr	8hr	24hr	48hr	72hr	D6	D10
Ref Range	Unit															
<5	mg/L	C-reactive protein	<1	<1	1	.	20	.	.	18	15	8	4	3	1	<1
< 10	mg/L	Serum amyloid A	<2.9	<2.9	<2.9	.	<2.9	.	.	<2.9	<2.9	<2.9	<2.9	<2.9	<2.9	<2.9
135 - 145	mmol/L	Sodium	144	141	142	.	.	.	.	.	.	143	139	140	141	140
3.5 - 5.1	mmol/L	Potassium	4.4	4.8	4.7	.	.	.	.	.	.	4.5	4.3	4.3	4.4	4.4
2.1 - 7.1	mmol/L	Urea	8.8	7.1	7.1	.	.	.	.	.	.	7.5	6	6.8	7.7	6.3
66 - 112	µmol/L	Creatinine	97	115	95	.	.	.	.	.	.	86	92	93	105	101
< 21	µmol/L	Total bilirubin	9	19	9	.	.	.	.	.	.	10	15	10	22	18
< 41	U/L	Alanine transaminase	13	16	11	.	.	.	.	.	.	12	14	14	13	13
< 37	U/L	Aspartate transaminase	28	30	22	.	.	.	.	.	.	19	22	24	28	28
< 129	U/L	Alkaline phosphatase	80	83	85	.	.	.	.	.	.	79	81	79	83	74
35 - 50	g/L	Albumin	49	49	47	.	.	.	.	.	.	46	49	48	48	46
	mmol/L	Glucose	3.9	.	.	.	.	.	.	.	.	.	.	.	.	.
< 0.030	µg/L	Cardiac Troponin T	0.003	.	.	.		.	.			.	.	.	.	.
	pmol/L	NT Pro-BNP	4	.	.	.	.	.	.	.	.	.	.	.	.	.
11.5 - 17.0	g/dL	Hemoglobin	14	14.4	14.2	.	14.1	.	.	14.9	14.1	13.9	14.3	14.1	13.7	Sample
3.5 - 11	10^9/L	White cells	5.93	4.07	4.35	.	3.99	.	.	4.16	5.31	4.04	4.1	4.79	4.08	clotted
140 - 400	10^9/L	Platelets	218	225	229	.	225	.	.	246	220	217	212	221	227	.
4.32 - 5.6	10^{12}/L	Red cells	4.78	4.85	4.71	.	4.69	.	.	4.82	4.63	4.53	4.71	4.72	4.56	.
0.39 - 0.52	L/L	Hematocrit	0.419	0.421	0.416	.	0.414	.	.	0.427	0.408	0.399	0.409	0.403	0.397	.
80 - 98	fL	MCV	87.7	86.8	88.3	.	88.3	.	.	88.6	88.1	88.1	86.8	85.4	87.1	.
27 - 33	pg	MCH	29.3	29.7	30.1	.	30.1	.	.	30.9	30.5	30.7	30.4	29.9	30	.
31 - 37	g/dL	MCHC	33.4	34.2	34.1	.	34.1	.	.	34.9	34.6	34.8	35	35	34.5	.
1.7 - 8.0	10^9/L	Neutrophils	3.06	1.96	1.84	.	1.71	.	.	1.95	2.71	2.21	2.24	2.36	2.25	.
1.0 - 3.5	10^9/L	Lymphocytes	1.94	1.45	1.73	.	1.6	.	.	1.55	1.77	1.22	1.26	1.67	1.26	.
0.1 - 1.0	10^9/L	Monocytes	0.54	0.3	0.42	.	0.36	.	.	0.3	0.42	0.31	0.3	0.37	0.3	.
0.0 - 0.46	10^9/L	Eosinophils	0.36	0.33	0.33	.	0.31	.	.	0.34	0.38	0.29	0.27	0.35	0.24	.
0.00 - 0.20	10^9/L	Basophils	0.03	0.03	0.02	.	0.01	.	.	0.02	0.03	0.01	0.03	0.04	0.03	.
0 -15	mm 1st h	Sedimentation rate	6	6	6	.	6	.	.	6	6	5	7	8	5	6
45-175	IU/dL	VWF antigen	108	111	91	.	86	.	.	89	84	102	114	108	117	126
<130	ng/mL	Fibrinogen D-dimer	30	19	4	.	0	.	.	8	28	30	21	9	26	17
69 - 229	pmol/L	Prothrombin F1+2	133	136	286	.	111	.	.	111	76	152	117	125	96	123
1.4 - 8.4	ng/mL	PAI-1 antigen	11.3	1	16.2	.	9.8	.	.	4.8	0.5	2.1	1.3	3.4	1.9	3.2
	mm Hg	BP Systolic	110	.	115	113	112	133	117	125	116	118	135	116	118	106
	mm HG	BP Diastolic	59	.	58	59	70	70	55	61	57	62	62	58	63	60
	b.p.m.	Heart rate	78	.	66	72	66	61	71	71	71	81	83	70	78	65
	°C	Temperature	36	.	36.1	36.4	36.3	36.4	36.1	36.1	36.6	36.9	36.1	36.3	36.5	36.2
		ECG	NAD	.	.	NAD	.	.	.	.	.	NAD	NAD	NAD	NAD	NAD
		Medications	None	.	None	None	None	None	None	None	None	None	None	None	None	None

SUBJECT 4 CRP dose 1.25 mg/kg															
			Screen	Baseline	15min	30min	60min	2hr	4hr	8hr	24hr	48hr	72hr	D6	D10
Ref Range	Unit														
<5	mg/L	C-reactive protein	<1	<1	.	21	.	.	17	16	8	5	2	<1	<1
< 10	mg/L	Serum amyloid A	<3.2	<3.2	.	<3.2	.	.	<3.2	<3.2	<3.2	<3.2	<3.2	<3.2	<3.2
135 - 145	mmol/L	Sodium	139	142	.	.	.	.	.	.	143	141	142	141	142
3.5 - 5.1	mmol/L	Potassium	3.8	3.9	.	.	.	.	.	.	3.9	3.9	4	4	4
2.1 - 7.1	mmol/L	Urea	5.8	4.4	.	.	.	.	.	.	6.2	5.5	5.5	5.9	4.2
66 - 112	µmol/L	Creatinine	84	82	.	.	.	.	.	.	77	81	70	80	80
< 21	µmol/L	Total bilirubin	10	10	.	.	.	.	.	.	8	7	8	13	10
< 41	U/L	Alanine transaminase	16	18	.	.	.	.	.	.	18	20	17	21	16
< 37	U/L	Aspartate transaminase	23	24	.	.	.	.	.	.	22	27	20	42	22
< 129	U/L	Alkaline phosphatase	75	77	.	.	.	.	.	.	70	79	65	74	67
35 - 50	g/L	Albumin	48	49	.	.	.	.	.	.	46	53	45	50	46
	mmol/L	Glucose	4.5	.	.	.	.	.	.	.	.	.	.	.	.
< 0.030	µg/L	Cardiac Troponin T	0.003	.	.	.	.	.	.	.	.	.	.	.	.
	pmol/L	NT Pro-BNP	6	.	.	.	.	.	.	.	.	.	.	.	.
11.5 - 17.0	g/dL	Hemoglobin	15	14.9	.	14.2	.	.	14.1	14	14	Sample	13.1	13.2	13.1
3.5 - 11	10^9/L	White cells	4.59	4.32	.	3.92	.	.	4.52	4.82	4.41	clotted	4.06	4.26	3.91
140 - 400	10^9/L	Platelets	250	243	.	224	.	.	239	223	244	.	225	233	265
4.32 - 5.6	10^{12}/L	Red cells	4.88	4.81	.	4.66	.	.	4.59	4.55	4.63	.	4.29	4.36	4.33
0.39 - 0.52	L/L	Hematocrit	0.423	0.413	.	0.403	.	.	0.394	0.394	0.401	.	0.372	0.372	0.379
80 - 98	fL	MCV	86.7	85.9	.	86.5	.	.	85.8	86.6	86.6	.	86.7	85.3	87.5
27 - 33	pg	MCH	30.7	31	.	30.5	.	.	30.7	30.8	30.2	.	30.5	30.3	30.3
31 - 37	g/dL	MCHC	35.5	36.1	.	35.2	.	.	35.8	35.5	34.9	.	35.2	35.5	34.6
1.7 - 8.0	10^9/L	Neutrophils	2.74	2.41	.	2.3	.	.	2.57	2.55	2.37	.	2.1	2.2	2.21
1.0 - 3.5	10^9/L	Lymphocytes	1.29	1.33	.	1.09	.	.	1.39	1.64	1.37	.	1.24	1.5	1.12
0.1 - 1.0	10^9/L	Monocytes	0.4	0.36	.	0.37	.	.	0.35	0.43	0.42	.	0.5	0.48	0.38
0.0 - 0.46	10^9/L	Eosinophils	0.15	0.21	.	0.15	.	.	0.2	0.2	0.24	.	0.21	0.17	0.19
0.00 - 0.20	10^9/L	Basophils	0.01	0.01	.	0.01	.	.	0.01	0	0.01	.	0.01	0.01	0.01
0 -15	mm 1st h	Sedimentation rate	5	4	.	3	.	.	3	3	5	5	6	4	3
45-175	IU/dL	VWF antigen	82	82	.	72	.	.	70	74	79	79	82	83	85
<130	ng/mL	Fibrinogen D-dimer	29	28	.	26	.	.	13	6	24	511	32	19	32
69 - 229	pmol/L	Prothrombin F1+2	152	172	.	151	.	.	132	122	201	1884	205	89	165
1.4 - 8.4	ng/mL	PAI-1 antigen	6.9	14.7	.	12.7	.	.	3	1.5	3.6	17.9	2.5	1.9	1.7
	mm Hg	BP Systolic	119	120	121	112	104	108	122	102	122	113	117	120	126
	mm HG	BP Diastolic	59	63	56	52	56	54	57	61	61	57	58	68	58
	b.p.m.	Heart rate	65	80	77	73	66	59	81	61	98	73	73	72	66
	°C	Temperature	37.2	36.7	37.1	37.1	37.1	36.7	36.7	37.1	36.9	37	37	36.3	36.8
		ECG	NAD	.	NAD	.	.	.	.	.	NAD	NAD	NAD	NAD	NAD
		Medications	None	None	None	None	None	None	None	None	None	None	None	None	None

SUBJECT 5 CRP dose 1.25 mg/kg															
			Screen	Baseline	15min	30min	60min	2hr	4hr	8hr	24hr	48hr	72hr	D6	D10
Ref Range	Unit														
<5	mg/L	**C-reactive protein**	<1	3	.	19	.	.	18	14	8	5	3	<1	<1
< 10	mg/L	**Serum amyloid A**	<3.2	4.5	.	4.4	.	.	4.2	<3.2	<3.2	<3.2	<3.2	<3.2	<3.2
135 - 145	mmol/L	**Sodium**	141	141	.	.	.	.	.	.	143	.	141	142	142
3.5 - 5.1	mmol/L	**Potassium**	3.9	4.1	.	.	.	.	.	.	4.2	.	4.1	4.2	4.5
2.1 - 7.1	mmol/L	**Urea**	4.6	5.1	.	.	.	.	.	.	5.1	.	3.7	5.3	4.9
66 - 112	µmol/L	**Creatinine**	81	76	.	.	.	.	.	.	72	.	65	75	69
< 21	µmol/L	**Total bilirubin**	**27**	13	.	.	.	.	.	.	15	.	10	13	15
< 41	U/L	**Alanine transaminase**	9	14	.	.	.	.	.	.	15	.	14	15	15
< 37	U/L	**Aspartate transaminase**	20	21	.	.	.	.	.	.	22	.	18	21	21
< 129	U/L	**Alkaline phosphatase**	62	62	.	.	.	.	.	.	61	.	60	62	60
35 - 50	g/L	**Albumin**	48	47	.	.	.	.	.	.	47	.	44	47	48
< 0.030	µg/L	**Cardiac Troponin T**	0.013	.	.	.	.	.	.	.	.	.	.	.	.
	pmol/L	**NT Pro-BNP**	3	.	.	.	.	.	.	.	.	.	.	.	.
11.5 - 17.0	g/dL	**Hemoglobin**	13.9	13.3	.	13.4	.	.	13.9	13.4	13.2	13.2	12.8	12.7	13.4
3.5 - 11	10^9/L	**White cells**	4.37	5.56	.	5.15	.	.	5.25	5.27	4.55	4.38	4.82	5.11	4.5
140 - 400	10^9/L	**Platelets**	212	213	.	207	.	.	212	217	208	215	215	201	207
4.32 - 5.6	10^{12}/L	**Red cells**	4.85	4.52	.	4.59	.	.	4.77	4.57	4.66	4.62	4.52	4.45	4.62
0.39 - 0.52	L/L	**Hematocrit**	0.42	0.39	.	0.4	.	.	0.412	0.389	0.399	0.401	0.384	0.382	0.398
80 - 98	fL	**MCV**	86.6	86.3	.	87.1	.	.	86.4	85.1	85.6	86.8	85	85.8	86.1
27 - 33	pg	**MCH**	28.7	29.4	.	29.2	.	.	29.1	29.3	28.3	28.6	28.3	28.5	29
31 - 37	g/dL	**MCHC**	33.1	34.1	.	33.5	.	.	33.7	34.4	33.1	32.9	33.3	33.2	33.7
1.7 - 8.0	10^9/L	**Neutrophils**	1.8	2.47	.	2.64	.	.	2.34	2.63	1.94	2.12	2.28	2.39	1.99
1.0 - 3.5	10^9/L	**Lymphocytes**	2.02	2.42	.	1.96	.	.	2.41	2.16	2.11	1.73	1.98	2.02	1.99
0.1 - 1.0	10^9/L	**Monocytes**	0.39	0.47	.	0.42	.	.	0.36	0.35	0.34	0.37	0.36	0.52	0.34
0.0 - 0.46	10^9/L	**Eosinophils**	0.14	0.17	.	0.12	.	.	0.13	0.1	0.15	0.14	0.18	0.16	0.16
0.00 - 0.20	10^9/L	**Basophils**	0.02	0.02	.	0.01	.	.	0.01	0.03	0.01	0.02	0.02	0.02	0.02
0 -15	mm 1st h	**Sedimentation rate**	2	6	.	6	.	.	6	10	6	7	4	4	8
45-175	IU/dL	**VWF antigen**	88	86	.	88	.	.	90	87	91	95	88	86	99
<130	ng/mL	**Fibrinogen D-dimer**	20	45	.	63	.	.	34	23	29	28	29	15	4
69 - 229	pmol/L	**Prothrombin F1+2**	84	83	.	72	.	.	58	64	101	72	72	65	62
1.4 - 8.4	ng/mL	**PAI-1 antigen**	4.2	0.4	.	2.3	.	.	2.1	1.6	2.3	1.3	1.2	1.4	0.6
	mm Hg	**BP Systolic**	120	118	109	109	108	118	117	112	114	122	117	121	123
	mm HG	**BP Diastolic**	69	72	62	63	68	63	67	58	61	71	65	68	75
	b.p.m.	**Heart rate**	64	64	59	60	58	59	64	62	70	76	63	70	74
	°C	**Temperature**	36.5	36.2	36.2	36.7	36.3	36.7	36.1	36.3	36.3	36.8	36.5	36.7	36.5
		ECG	NAD	.	NAD	.	.	.	.	.	NAD	NAD	NAD	NAD	NAD
		Medications	None	None	None	None	None	None	None	None	None	None	None	None	None

SUBJECT 6 CRP dose 2.00 mg/kg															
			Screen	Baseline	15min	30min	60min	2hr	4hr	8hr	24hr	48hr	72hr	D6	D10
Ref Range	Unit														
<5	mg/L	**C-reactive protein**	2	2	.	36	.	.	26	22	12	7	6	3	4
< 10	mg/L	**Serum amyloid A**	7.5	8.2	.	8.1	.	.	7.3	8	7.5	6.4	8.6	7.6	12.1
135 - 145	mmol/L	**Sodium**	144	145	.	.	.	.	.	.	145	142	140	142	.
3.5 - 5.1	mmol/L	**Potassium**	4.3	4.4	.	.	.	.	.	.	4.4	4.4	4.3	4.2	.
2.1 - 7.1	mmol/L	**Urea**	3.4	4.6	.	.	.	.	.	.	5.1	4.3	4.5	4.5	.
66 - 112	µmol/L	**Creatinine**	77	79	.	.	.	.	.	.	87	77	82	84	.
< 21	µmol/L	**Total bilirubin**	11	11	.	.	.	.	.	.	10	13	14	8	.
< 41	U/L	**Alanine transaminase**	10	12	.	.	.	.	.	.	10	11	12	15	.
< 37	U/L	**Aspartate transaminase**	15	17	.	.	.	.	.	.	18	17	23	22	.
< 129	U/L	**Alkaline phosphatase**	72	70	.	.	.	.	.	.	67	68	74	69	.
35 - 50	g/L	**Albumin**	48	49	.	.	.	.	.	.	46	47	50	48	.
	mmol/L	**Glucose**	4.3	.	.	.	.	.	.	.	.	.	.	.	.
< 0.030	µg/L	**Cardiac Troponin T**	0.009	.	.	.	.	.	.	.	.	.	.	.	.
	pmol/L	**NT Pro-BNP**	Insufficient	.	.	.	.	.	.	.	.	.	.	.	.
11.5 - 17.0	g/dL	**Hemoglobin**	13.5	13.6	.	13.6	.	.	13.1	12.8	12.9	12.8	12.6	12.6	12
3.5 - 11	10^9/L	**White cells**	6.07	5.25	.	5.87	.	.	6.16	6.91	5.57	4.25	10.52	4.71	4.94
140 - 400	10^9/L	**Platelets**	158	146	.	151	.	.	148	140	144	140	151	141	132
4.32 - 5.6	10^{12}/L	**Red cells**	5.11	5.1	.	5.06	.	.	4.75	4.8	4.74	4.85	4.72	4.71	1.47
0.39 - 0.52	L/L	**Hematocrit**	0.409	0.408	.	0.405	.	.	0.371	0.39	0.389	0.384	0.372	0.374	0.361
80 - 98	fL	**MCV**	80	80	.	80	.	.	78.1	81.3	82.1	79.2	78.8	79.4	80.8
27 - 33	pg	**MCH**	26.4	26.7	.	26.9	.	.	27.6	26.7	27.2	26.4	26.7	26.8	26.8
31 - 37	g/dL	**MCHC**	33	33.3	.	33.6	.	.	35.3	32.8	33.2	33.3	33.9	33.7	33.2
1.7 - 8.0	10^9/L	**Neutrophils**	4.34	3.62	.	3.63	.	.	4.27	4.96	3.72	2.65	9.03	3.08	3.15
1.0 - 3.5	10^9/L	**Lymphocytes**	1.07	1.09	.	1.54	.	.	1.18	1.4	1.23	1.1	0.85	1.08	1.13
0.1 - 1.0	10^9/L	**Monocytes**	0.5	0.38	.	0.54	.	.	0.55	0.42	0.46	0.37	0.6	0.43	0.51
0.0 - 0.46	10^9/L	**Eosinophils**	0.15	0.15	.	0.16	.	.	0.15	0.12	0.15	0.12	0.03	0.12	0.14
0.00 - 0.20	10^9/L	**Basophils**	0.01	0.01	.	0	.	.	0.01	0.01	0.01	0.01	0.01	0.01	0.01
0 -15	mm 1st h	**Sedimentation rate**	12	14	.	15	.	.	14	10	12	15	14	15	16
45-175	IU/dL	**VWF antigen**	75	75	.	71	.	.	66	64	61	69	128	69	77
<130	ng/mL	**Fibrinogen D-dimer**	377	355	.	374	.	.	361	333	317	365	344	371	334
69 - 229	pmol/L	**Prothrombin F1+2**	78	85	.	93	.	.	86	79	90	86	92	87	87
1.4 - 8.4	ng/mL	**PAI-1 antigen**	2.7	3.9	.	4	.	.	8.7	1.3	2.5	2.4	2.5	5	2.8
	mm Hg	**BP Systolic**	128	118	121	113	124	125	117	117	131	117	132	141	117
	mm HG	**BP Diastolic**	81	71	68	61	74	69	67	61	64	70	59	62	76
	b.p.m.	**Heart rate**	50	70	63	50	45	65	51	70	65	77	84	78	61
	°C	**Temperature**	36.2	36.6	36.6	36.8	36.6	36.8	36.7	36.8	36.8	36.5	36.8	36.2	36.4
		ECG	NAD	.	NAD	.	.	.	.	.	NAD	NAD	NAD	NAD	NAD
		Medications	None	None	None	None	None	None	None	None	None	None	None	None	None

SUBJECT 7 CRP dose 2.00 mg/kg															
			Screen	Baseline	15min	30min	60min	2hr	4hr	8hr	24hr	48hr	72hr	D6	D10
Ref Range	Unit														
<5	mg/L	**C-reactive protein**	<1	<1	.	44	.	.	38	31	14	7	4	1	<1
< 10	mg/L	**Serum amyloid A**	<3.2	3.2	.	3.5	.	.	4	3.4	<3.2	<3.2	<3.2	<3.1	3.4
135 - 145	mmol/L	**Sodium**	141	142	.	.	.	.	.	.	140	136	140	140	143
3.5 - 5.1	mmol/L	**Potassium**	4	Delayed	.	.	.	.	.	.	4.4	4.1	Delayed	Delayed	3.8
2.1 - 7.1	mmol/L	**Urea**	5.7	5.2	.	.	.	.	.	.	5	5.8	6.1	8	5.3
66 - 112	µmol/L	**Creatinine**	90	93	.	.	.	.	.	.	100	103	96	103	96
< 21	µmol/L	**Total bilirubin**	11	8	.	.	.	.	.	.	8	7	8	8	14
< 41	U/L	**Alanine transaminase**	17	19	.	.	.	.	.	.	18	17	19	21	19
< 37	U/L	**Aspartate transaminase**	16	19	.	.	.	.	.	.	19	17	18	16	20
< 129	U/L	**Alkaline phosphatase**	69	65	.	.	.	.	.	.	68	63	62	58	64
35 - 50	g/L	**Albumin**	44	43	.	.	.	.	.	.	44	42	43	42	46
< 0.030	µg/L	**Cardiac Troponin T**	<0.003	.	.	.	.	.	.	.	.	.	.	.	.
	pmol/L	**NT Pro-BNP**	2	.	.	.	.	.	.	.	.	.	.	.	.
11.5 - 17.0	g/dL	**Hemoglobin**	14.5	14.6	.	14.7	.	.	15.7	14.8	14.5	13.2	13.4	13.6	13..3
3.5 - 11	10^9/L	**White cells**	4.49	4.14	.	4.49	.	.	4.92	5.21	4.54	4.58	4.94	4.91	6.3
140 - 400	10^9/L	**Platelets**	198	212	.	217	.	.	220	227	220	211	220	201	216
4.32 - 5.6	10^{12}/L	**Red cells**	4.93	5.06	.	5.02	.	.	5.32	5.1	4.88	4.59	4.65	4.51	4.55
0.39 - 0.52	L/L	**Hematocrit**	0.415	0.43	.	0.425	.	.	0.455	0.435	0.42	0.387	0.396	0.377	0.385
80 - 98	fL	**MCV**	84.2	85	.	84.7	.	.	85.5	85.3	86.1	84.3	85.2	83.6	84.6
27 - 33	pg	**MCH**	29.4	28.9	.	29.3	.	.	29.5	29	29.6	28.8	28.8	30.2	29.2
31 - 37	g/dL	**MCHC**	34.9	34	.	34.6	.	.	34.5	34	34.5	34.1	33.8	36.1	34.5
1.7 - 8.0	10^9/L	**Neutrophils**	2.58	2.37	.	2.5	.	.	2.57	2.71	2.64	2.65	2.87	2.83	4.06
1.0 - 3.5	10^9/L	**Lymphocytes**	1.3	1.21	.	1.33	.	.	1.82	1.75	1.35	1.36	1.44	1.42	1.64
0.1 - 1.0	10^9/L	**Monocytes**	0.44	0.37	.	0.47	.	.	0.36	0.55	0.39	0.42	0.48	0.43	0.43
0.0 - 0.46	10^9/L	**Eosinophils**	0.15	0.17	.	0.17	.	.	0.15	0.18	0.15	0.14	0.15	0.22	0.15
0.00 - 0.20	10^9/L	**Basophils**	0.02	0.02	.	0.02	.	.	0.02	0.01	0.01	0.01	0.01	0.01	0.02
0 -15	mm 1st h	**Sedimentation rate**	3	3	.	4	.	.	3	4	5	5	4	4	4
45-175	IU/dL	**VWF antigen**	91	88	.	94	.	.	90	88	123	127	114	103	102
<130	ng/mL	**Fibrinogen D-dimer**	72	75	.	96	.	.	115	71	98	49	36	84	59
69 - 229	pmol/L	**Prothrombin F1+2**	104	108	.	108	.	.	96	120	101	107	101	109	132
1.4 - 8.4	ng/mL	**PAI-1 antigen**	1.6	4.2	.	1.7	.	.	0.5	0.4	3.5	1.6	1.7	3.4	1.9
	mm Hg	**BP Systolic**	120	108	112	110	116	110	112	116	111	115	109	119	126
	mm HG	**BP Diastolic**	70	74	75	72	88	69	62	57	74	75	59	74	78
	b.p.m.	**Heart rate**	80	71	60	62	54	63	61	55	79	79	82	78	71
	°C	**Temperature**	36.8	36.3	36.4	36.5	36.7	36.8	36.5	36.3	36.4	36.1	36.8	36.1	36.7
		ECG	NAD	.	NAD	.	.	.	.	.	NAD	NAD	NAD	NAD	NAD
		Medications	None	None	None	None	None	None	None	None	None	None	None	None	None